Regulation of Aging in Yeast by Glycation Inhibitors

Thesis Submitted to AcSIR
for the Award of the Degree of

Doctor of Philosophy

In

BIOLOGICAL SCIENCES

By

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10BB11J26118

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2018

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CERTIFICATE

This is to certify that the work incorporated in this Ph.D. thesis entitled **"Regulation of Aging in Yeast by Glycation Inhibitors"** submitted by **Ms. Rubina Sikandar Kazi** to the Academy of Scientific and Innovative Research (AcSIR) in fulfilment of the requirement for the award of the degree of **Doctor of Philosophy**, embodies original research work under my guidance. I further certify that this work has not been submitted to any other University or institution in part or full for the award of any degree or diploma. Research material obtained from other sources has been duly acknowledged in the thesis. Any text, illustration, table etc., used in the thesis from other sources have been duly cited and acknowledged.

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Dedicated to my beloved parents

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Declaration by Research Scholar

I hereby declare that I am the sole author of this thesis entitled **"Regulation of Aging in Yeast by Glycation inhibitors"** submitted by me for the degree of Doctor of Philosophy to Academy of Scientific and Innovative Research (AcSIR) and is a record of the research performed by myself with exception to few experiments. The work was performed at CSIR-National Chemical Laboratory (Pune, India).

This work is original and has not been submitted previously for any higher degree and was supervised by Dr. Mahesh Kulkarni.

Rubina Sikandar Kazi **Date: April 23, 2018**

Acknowledgement

I am extremely grateful to my advisor Dr. Mahesh Kulkarni. He has taught me, both consciously and unconsciously, how good experimental biology is done. I appreciate all his contributions of time, ideas, and funding to make my Ph.D. experience productive and stimulating. The joy and enthusiasm he has for his research was contagious and motivational for me, even during tough times in the Ph.D. pursuit. I am also thankful to him and his family for the excellent suggestions they have provided to maintain the balance between family and professional life. His intuition and expertise in scientific writings are the great learning experiences and I am very pleased to have learned many of such things from him. He is an inspiration.

I would like to thank Director, CSIR-NCL and Head of Department Biochemical Sciences, Dr. Ashok Giri and former head Dr. Vidya Gupta for providing the infrastructure and laboratory facilities. The members of my Doctoral Advisory Committee (DAC) have contributed immensely to my Ph.D. They have been a source of good advice for me. I would like to acknowledge my DAC members Dr. Shashidhar, and Dr. Thulasiram for sharing their valuable feedback and deep insights. I am extremely grateful to Dr. Ashok Giri, for his guidance and all the useful discussions especially during the difficult stages. I am also grateful to Dr. Santhakumari for her unending understanding and support. I gratefully acknowledge UGC for providing me financial assistance.

My friends and colleagues who have made this journey so memorable must also be mentioned. I spent major time with Proteomics and MBL group. The Ph.D. years with you all were great and influential years of my life. I would like to thank my senior friends Dr.Ashok Chougule, Dr. Hemangi Bhosale, Dr. Arvind Korwar, Dr. Sachin, Santosh and Dr.Yogesh Kolekar. Special thanks to Dr. Suresh KK and Dr.Sandeep Golegaonkar who taught me proteomics and mass spectrometry techniques. I am grateful to all my labmates Kedar, Jaggi, Shrikant, Swami, Gouri, Shakuntala, Rajeshwari, Prachi, Sharada, Arvind, Babasaheb, Yugendra, Rashmi and all other labmates from Proteomics. I would like to thank my senior friends Sneha and Shweta for their support. It was very convenient for me to work at MBL. I had great intellectual interactions here. My honest thanks goes to my friends Avinash, Shiva,

Krithika, Rinci, Sonia,Vishwa, Jyoti, Nivedita,Chaya, Arathi at MBL. It is pleasure to thank my friends from PMB group Sheon, Amey, Priyanka, Tejas, Rahul and Dr. Rakesh Joshi. I have greatly benefited from discussions with Ashish from PMB group. I would like to thank my batchmates Ruchira, Ashish, Sayali, Deepanvita and Rupa for all the information, course work, CSIR-800 and AcSIR document preparation.

My honest thank goes to Reema for her unconditional support and encouragement during tough times of Ph.D. and personal life. Heartiest thanks to my friend Sagar. Special mention goes to my best friend Arati, importance of her presence during my Ph.D. was worth more than I can express on paper.

I feel incredibly grateful to have the love and support of my family through my Ph.D. program, and also long before that. I owe my existence to the people who have shaped my identity over the years, especially my Mammy and Abba. They stood behind me and motivated me every-time, to achieve my dreams by providing the quality education. I am fortunate to have loving, caring and supporting siblings like Reshma, Riyaz, Rukaiyya and their families. I am also grateful to my teachers, my mentors, my classmates and friends. During my difficult time I could continue my education, because of huge support, highest motivation and financial assistance from RTO Officer Rajendra Madane and my beloved Mrs. Sunandatai Pawar. They receive my deepest gratitude and love.

The presence of my mother in-law here at NCL from last few years was a great relief for me. My sincere gratitude is reserved for her for caring, loving, supporting, encouraging me. I am fortunate to have supporting sister in law and her family. My husband Asif, whose faithful support during my Ph.D. is highly appreciated. My husband has been a true and great supporter and has unconditionally loved me during my good and bad times. We both are fortunate to have our baby boy Arsh. I love you all dearly!

Finally, I would like to thank many people who have helped and befriended me along the way. The list of names is so long that, were I attempt to write it, I would inevitably

leave someone out and suffer a pang of regret upon realizing my mistake. Therefore, I hope that those whom I would like to thank know that they have my gratitude.

Above all, I owe it all to Almighty God for granting me the wisdom, health, and strength to undertake this research task and enabling me to its completion.

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Definitions/Abbreviations

AC	Adenylate cyclase
ADE3	Purine biosynthetic protein adenosine 3
ADE4	Purine biosynthetic protein adenosine 4
AGEs	Advanced glycation End Products
AKT	Protein kinase B
AMG	Aminoguanidine
AMP	Adenine Monophosphate
AMPK	5'AMP activated protein kinase
ANOVA	Analysis of Variance
ARGP	Argpyrimidine
ATG	AuTophaGy related genes
ATG1	AuTophaGy related 1
ATG7	AuTophaGy related 7
ATP	Adenosine Triphosphate
BINGO	Biological Network Gene ontology
ACN	Acetonitrile
cDNA	Complementary DNA
CEL	Carboxyethyl lysine
CFU	Colony forming units
CHAPS	(3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate)
CLS	Chronological lifespan
CML	Carboxymethyl lysine
CR	Calorie Restriction
CT	Cycle threshold
DAF-16	Transcription factor
DAVID	Database for Annotation, Visualization and Integrated Discovery
DHE	Dihydroethidium
DIA	Data Independent Acquisition
DNA	Deoxy Nucleic Acid
DNP	Dinitrophenol
DR	Dietary Restriction
DTT	Dithiothreitol
ERCs	Extrachromosomal ribosomal DNA circles
FA	Formic acid
FDR	False Discovery Rate
FL	Fructosyl-lysine
FOXO	Fork head box protein O
FWHM	Full Width Half Maximum
GFP	Green Fluorescent Protein
Gis1	Transcription factor
GMP	Guanine Monophosphate
H2DCFDA	2',7'- dichlorodihydrofluorescein diacetate
HDMS	High Definition Mass spectrometer

HIF-1	Hypoxia inducible factor1
HRP	Horse Radish Peroxidase
HSCs	Hematopoietic stem cells
HYD	Hydralazine
IAA	Indole-3-acetic acid
IDA	Information Dependant Acquisition
IGF-1	Insulin like growth factor 1
IIS	Insulin and Insulin like growth factor signaling
IMP	Inosine Monophosphate
iTRAQ	Isobaric Tags for relative and absolute quantitation
LC-MS^E	Liquid Chromatography-Mass spectrometry
LDL	Low Density lipopolysaccharide
LSG	Longevity related genes
MALDI	Matrix assisted laser desorption ionization
MET	Metformin
MG-H1	Hydroimidazolones
MGO	Methylglyoxal
mRNA	Messenger RNA
Msn2/4	stress responsive transcriptional activator 2/4
mtDNA	Mitochondrial DNA
mTOR	mammalian TOR
NAB	N-aryl benzimidazole
NAD^+	Nicotinamide Adenine Dinucleotide
NF-kB	Nuclear factor kappa-light chain enhancer of activated B cells
NR	Non- calorie Restriction condition
OD	Optical Density
PBS	Phosphate buffer saline
PCA	Principal component analysis
PKA	Protein Kinase A
PLGS	Protein lynx global server
PVDF	polyvinylidine fluoride
Ras2	GTP-binding protein
rDNA	ribosomal DNA
Rim15	Serine/Threonine Protein Kinase Rim15
RLS	Replicative lifespan
ROS	Reactive Oxygen Species
RT-PCR	Real-time Polymerase chain reaction
S6K	Ribosomal protein S6 Kinase
SC	Synthetic Complete medium
Sch9	Serine/Threonine Protein Kinase
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEER	Surveillance Epidemiology and End Results
SEM	Standard Error of the mean
SILAC	Stable isotope labeling with amino acids in cell culture
Sir2	Silent Information regulator 2
SIRT	sirtuins

SNF1	Sucrose non fermenting 1 protein kinase
SRM	Selected Reaction monitoring
SWATH-MS	Sequential Window Acquisition of all Theoretical masses-Mass Spectrometry
TCA	Tricarboxylic acid
TDH3	Glyceraldehyde -3-phosphate dehydrogenase 3
TFA	Trifluroacetic acid
TOR	Target of Rapamycin kinase
TPI1	Triosephosphate isomerase1
UPLC	Ultra Performance liquid chromatography
VPS30	Vacuolar Protein sorting-associated protein 30
WHO	World's Health Organization
YPD	Yeast Extract Peptone Dextrose medium

Preface

Aging is an irreversible degenerative process characterized by a general decline in cellular metabolic activity accompanied with progressive deterioration of cellular components resulting in enhanced mortality. Although, average life expectancy has increased, it is associated with different aging related diseases such as cardiovascular diseases, cancer, diabetes and so on. Hence, there is demand of active research on aging process and aging associated diseases to make added years of older people healthy. Short lived organisms, such as yeast are excellent model for aging research, as longevity-defining genes and signaling pathways discovered in yeast have been shown to define cellular and organismal longevity in eukaryotes across phyla. Yeast displays two distinct lifespan, namely, replicative lifespan (RLS) and chronological lifespan (CLS), which serve as models for proliferating (mitotic) and non-proliferating (post-mitotic) tissues in higher eukaryotes, respectively. RLS is defined as the number of daughter cells produced by a mother cell before cell division ceases; whereas CLS is the duration of cell survival in the stationary phase.

Nutrients play important role in chronological aging of yeast. Calorie restriction is associated with lifespan extension in yeast and other higher organism including C elegans, drosophila, and mouse. While, non calorie restriction in terms of glucose (2%) leads to activation of various signaling pathways such as TOR/Sch9 and RAS/AC/PKA, which stimulate cellular senescence in yeast. In addition to these factors, non calorie restriction in terms of glucose can promote formation of advanced glycation reaction products (AGEs). AGEs are formed as a result of series of non-enzymatic reactions between protein and reducing sugars. AGEs are known to affect functions of many intracellular proteins and also AGE modification of proteins results in protease resistance, which affect the protein homeostasis. Glycated proteins and aggregates are responsible for several age associated diseases like Alzheimer's disease, Parkinson's disease, familial amyloidotic polyneuropathy, amyloidotic lateral sclerosis and diabetic complications.

Thus, targeting AGE formation could be a rational approach to extend the lifespan and delay aging. Therefore, thesis deals with the development of yeast as a model system to study glycation induced aging and targeting AGEs by glycation inhibitors.

For that, we have studied the effect of glycation and glycation inhibitors on yeast chronological lifespan. Further, we explored the effect of presence of glycation inhibitors on AGE modification and on global proteome through proteomics approach. Lastly, we have investigated the effect of glycation inhibitors on aging responsive genes using microarray and real time PCR.

Chapter 1

Introduction

Chapter 1 Introduction

1.1 Aging

Aging is a complex process associated with accumulation of damage, loss of function and increased vulnerability to disease, leading ultimately to death. Aging is widely characterized by loss or decrease in mental and physical fitness. Broadly, time dependent accumulation of cellular damage is considered as cause of aging [1]. Phenotype of human aging can be illustrated as accumulation of multiple types of damage within single tissue with age, which has great variability among individuals [2, 3].

1.1.1 Hallmarks of aging

Lopez-Otin *et al*, have identified nine tentative cellular and molecular hallmarks of aging. Genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication are the nine candidate hallmarks which contribute to the aging process [4] (Figure 1.1).

1.1.1.1 Genetic instability

Accumulation of DNA damage was observed in numerous premature aging diseases such as Werner syndrome, Bloom syndrome, etc [5]. Stability and integrity of DNA get affected by exogenous factors like physical, chemical and biological agents along with endogenous factors such as reactive oxygen species (ROS), replication errors, etc [6]. In addition to these, defects in mitochondrial DNA and nuclear lamina also contribute to genome instability resulting in premature aging syndromes [7, 8].

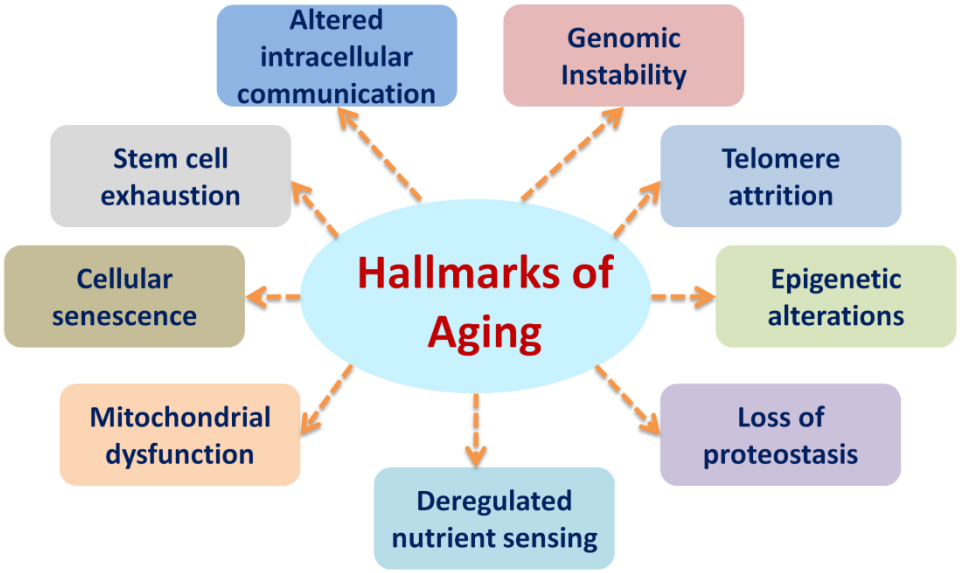


Figure 1.1 Hallmarks of aging.
Figure adapted and modified from [4]

1.1.1.2 Telomere attrition

DNA damage affects genome randomly but telomeres, terminal ends of DNA molecules, are more susceptible to age dependant deterioration [9]. Mammalian somatic cells do not express telomerase, a DNA polymerase which replicate the telomere region. In view of this, telomere shortening is observed in humans [10]. Meta-analysis in humans suggested strong relationship between telomere length and risk of mortality [11].

1.1.1.3 Epigenetic alterations

Epigenetic changes which affect cells and tissues involve alterations in DNA methylation patterns, posttranslational modification of histone, and chromatin remodeling [12]. Inhibiting histone demethylases extends lifespan in worms through insulin/IGF-1 signaling pathway [13]. In mammals, members of sirtuin family (SIRT1, SIRT3 and SIRT6), NAD-dependant histone deacetylase, have been reported to contribute to healthy aging by regulating genome stability, NF-κB signaling and glucose homeostasis [14-17]. Resveratrol, anti-aging molecule, showed upregulation of SIRT1 activity.

1.1.1.4 Loss of proteostasis

Proteostasis involves correct folding of proteins by the family of heat shock proteins and degradation of misfolded or unfolded proteins by the proteasome or lysosome [18-20]. Impaired proteostasis is associated with aging and aging associated diseases [21]. Several reports in model organisms suggested enhanced proteostasis by chaperones [22-24]. Two proteolytic systems, important for protein quality control, viz., 1) autophagy- lysosomal system, 2) ubiquitin- proteasome system were showed decline in activity with aging [25, 26]. Rapamycin and spermidine, both promote longevity in yeast, worms, flies via induction of autophagy [27]. Likewise, Enhanced proteasomal activity extends replicative lifespan in yeast [28].

1.1.1.5 Deregulated nutrient sensing

Different nutrient sensing pathways are active in model organisms which regulate aging process. Inhibition of the insulin- and insulin like growth factor 1(IIS) pathway extends lifespan in worms, flies and mice. Dietary restriction inhibits IIS pathway which leads to activation of transcription factor FOXO mediating increased lifespan [29-31]. Other nutrient sensing systems are mTOR which senses high amino acid concentration, AMPK which recognizes low energy states by detecting high levels of AMP and sirtuins which senses low energy states from high NAD^+ levels [32]. Downregulation of TORC1 extends lifespan in yeast, worms and flies [33]. In contrast to IIS and mTOR, activation of AMPK and sirtuins extends longevity. AMPK signals about nutrient scarcity and it is reported to inhibit mTORC1 [34]. Dietary restriction favors healthy aging by inhibiting IIS pathway and mTOR and activating AMPK and sirtuins (Figure 1.2).

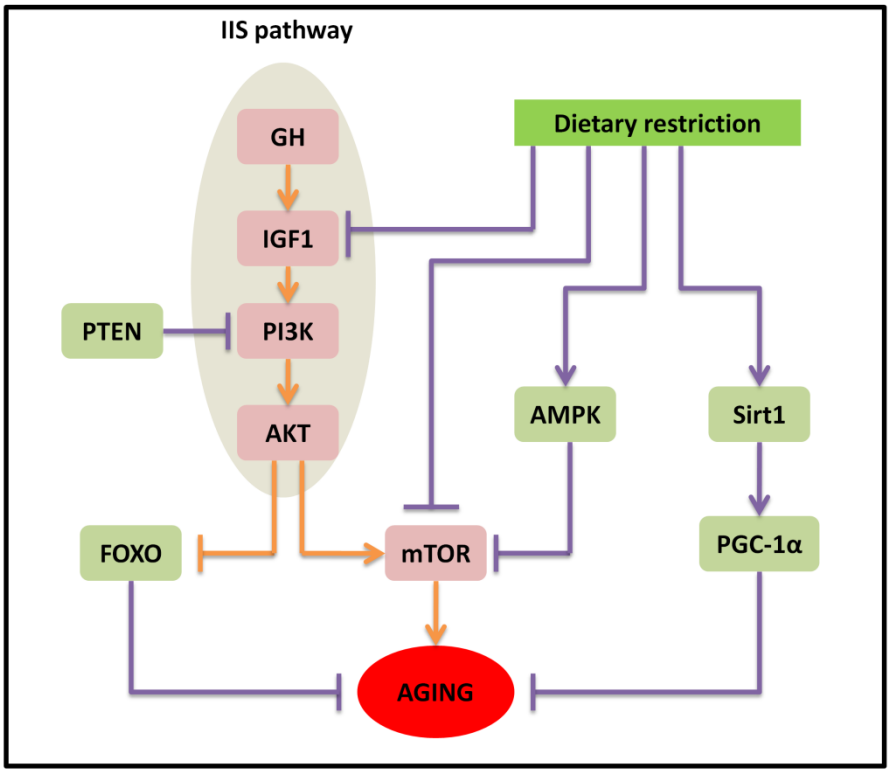


Figure 1.2 Nutrient sensing pathways leading to aging
Dietary restriction inhibits IIS pathway leading to extension of lifespan in worms, flies and mice. Figure adapted and modified from [4]

1.1.1.6 Mitochondrial dysfunction

Decline in mitochondrial function was observed with aging which implicated in reduced ATP generation and increased production of ROS [35, 36]. Multiple reports suggest the role of ROS in aging, however there are few contradictory observations reported in last 5 years. Extension of longevity was observed in yeast and worms with increased ROS [37, 38]. Likewise, few evidences proved role of ROS in survival in response to stress condition [39]. In this sense, a new concept that is been raised is upto a certain threshold ROS helps in survival under cellular stress and damage but beyond that it aggravates age associated damage [40]. Independent of ROS, reduced mitochondrial biogenesis can also contribute to aging [41]. SIRT1 enhances mitochondrial biogenesis through removal of damaged mitochondria by autophagy [42]. Defect in mitochondrial bioenergetics caused by oxidation of mitochondrial proteins, accumulation of mutations in mtDNA, and defective mitophagy may aggravate aging process [43].

1.1.1.7 Cellular Senescence

Cellular senescence is nothing but stable arrest of cell cycle [44]. It is beneficial compensatory response to prevent propagation of damaged cells. However, it requires efficient clearance of damaged cells and replenishment of cell number. This cell replacement system becomes inefficient with age causing accumulation of damaged cells which accelerates aging process [45].

1.1.1.8 Stem cell exhaustion

Regenerative potential of tissues decline with age, as observed in case of haematopoiesis which lead to anemia and myeloid malignancies [46]. Cell cycle activity of hematopoietic stem cells (HSCs) decreases with age in mice [47]. Stem cell decline serves as a consequence of aging- associated damage [48].

1.1.1.9 Altered intracellular communication

Intracellular communications can be endocrine, neuroendocrine or neuronal which get affected during aging [49, 50]. Inflammation is the prominent aging associated alteration in intracellular communication. Damaged tissue and dysfunctional immune system to clear pathogens and damaged cells result into aging associated inflammation [51]. Over-activation of NF-κB pathway was observed during aging whereas inhibition of NF-κB signalling alleviates age associated response in mouse model [52, 53].

1.1.2 Aging associated diseases

Aging is characterised by decline in cellular function with advancing age [3]. Loss of cellular functions during aging leads to onset of different diseases which are termed as aging-associated diseases. Age associated diseases can be divided into two types: 1) those diseases that progress with age such as neurodegenerative diseases and cardiovascular diseases; 2) those disease that occur at particular age e.g. Multiple sclerosis. Aging - associated diseases are more prevalent in older age and result from chronic degenerative processes [54]. So, it is of prime concern to study the aging process and aetiology of aging associated diseases to improve quality of life. Aging

associated diseases are cardiovascular diseases, cancer, arthritis, dementia, cataract, osteoporosis, diabetes, hypertension, neurodegenerative diseases, etc.

1.1.2.1 Cardiovascular diseases

Age is important determinant of cardiovascular health. By 2030, 40% of deaths in the age group of 65 and older will be because of cardiovascular diseases and its treatment cost will triple [55]. Cardiovascular system plays vital role in the health of an organism by providing oxygenated blood to all tissues in the body [56]. Aging affects function of heart and arterial system leading to increase in occurrence of cardiovascular diseases like atherosclerosis, hypertension, myocardial infarction, and stroke [57].

1.1.2.2 Cancer

According to the US National Cancer Institute's Surveillance Epidemiology and End Results (SEER) Database, 43% of men and 38% of women will develop an invasive cancer over a lifetime. Among these, 23% of men and 19 % of women will die from cancer. Around 50% of cancers occur in individuals older than 70 [58, 59]. Genomic instability observed during aging is one of the hallmarks of cancer [58].

1.1.2.3 Arthritis, Cataract and Osteoporosis

Rheumatoid arthritis is a chronic inflammatory disease caused by synovial inflammation leading to cartilage damage and destruction of the joints. It is an autoimmune disease caused by degeneration of immune system with age [60, 61].

Cataract is a visual opacity in lens which leads to visual loss. With aging, lens proteins undergo modification, its transparency falls down leading to opacity in lens [62].

Osteoporosis is characterized by low bone mass and increased bone fragility. Susceptibility to inflammation and degenerated immune system, characteristic of aging, are associated with osteoporosis [63].

1.1.2.4 Diabetes

Diabetes is a metabolic disorder. The prevalence of diabetes mellitus is increasing. In 2000, 2.8% of the world-wide population was diabetic and it will increase up to 4.4% by 2030. Almost half of the diabetic population is over 60 years of age. Number of older diabetic persons is expected to double by the year 2030 in developing countries. The largest number of diabetics lives in India, China and the United States [64]. In US, 8 in 10 adults aged 65 or more have some form of dysglycemic condition. Older diabetics show both impaired insulin secretion as well as insulin resistance [65].

1.1.2.5 Hypertension

Hypertension contributes to 4.7% of total disease population world-wide; in which majority of population is over 60 years of age. According to National Health and Nutrition Health Survey 2015, 70% of older adults have hypertension. Hypertension increases numerous other health risks [66].

1.1.2.6 Neurodegenerative diseases

Alzheimer's disease, Parkinson's disease, amyotrophic, Lateral sclerosis, and so on come under neurodegenerative diseases. They show symptoms from progressive dysfunction of motor control to mood disorders and cognitive deficits. These diseases not only have impact on individuals but also on family and society. These diseases cannot be cured. Alzheimer's disease alone affects around one-third to half of old age people [67].

1.1.3 Need of aging research

Over the past decades, global life expectancy has improved a lot. WHO's statistics shows that a world-wide average life expectancy was about 70 years in 2014 which is 6 years longer than in 1990 and around 80 years in the developed countries [68]. The number of people aged 65 or older is projected to grow from 524 million in 2010 to 1.5 billion in 2050; this increase is almost double and majority is from developing countries [69]. Improvement in sanitation, medical advances, rising living standards and a decline in child mortality are the factors contributing to increased life expectancy.

Increase in average life expectancy reflects positive human development. However, many aging-associated diseases such as cardiovascular disease, cancer, diabetes, and neurodegenerative diseases which affect older people impose greatest burden on global health. This affects economic and societal growth of a nation [69]. So, socio-economic and health related challenges of aging societies to a nation are 1) Increasing health care cost and financial stress 2) Neurodegenerative diseases demand additional cost for intensive care 3) a declining active workforce 4) Increasing number of pensioners 5) Decline in quality of life [70]. Aging is a complicated physiological process and the research progress in aging is at its preliminary stage [71]. All these factors demand active research on aging process and aging-associated disease to make added years of older people healthy.

1.1.4 Aging research in different model organisms

Human aging process cannot be studied in human because of its longer lifespan. To study human aging major model organisms used are 1) Unicellular yeast *Saccharomyces cerevisiae* 2) The roundworm *Caenorhabditis elegans* 3) the fruit fly *Drosophila melanogaster* 4) rodents such as mice (*Mus musculus*).

Like other biological processes, aging process is also subject to regulation by different signalling pathways and transcriptional factors [72]. These signalling pathways are evolutionarily conserved from yeast to mammals (Figure 1.3) [73].

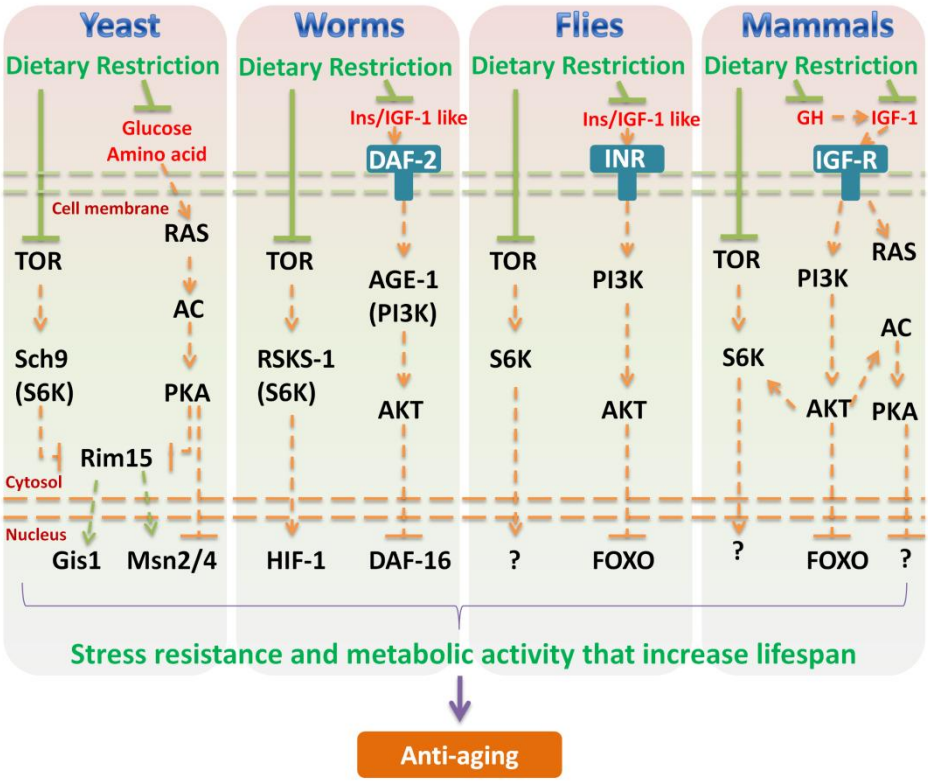


Figure1.3 Aging pathways are evolutionarily conserved from yeast to mammals Dietary restriction inhibits TOR/Sch9 and RAS/AC/PKA pathway and extends lifespan in yeast. TOR/Sch9 (S6K) which promotes aging appears to be conserved. RAS/AC/PKA pathway along with TOR/Sch9 pathway accelerates aging in yeast and mammals. Whereas, IIS pathway plays important role in aging of worms and flies. Figure adapted and modified from [73].

Dietary restriction without malnutrition is reported to extend lifespan of various organisms such as yeast, worms, flies and mice through signalling pathways that regulate aging [74]. Dietary restriction inhibits different signal transduction pathways. Inhibition of TOR and S6K pathway leading to longevity appears to be conserved in yeast, worms, flies, and mice. However, the AC-PKA pathway promotes aging by inactivating transcription factors GIS1 and MSN2/4 in yeast. Whereas, insulin/Insulin like growth factor accelerates aging by inactivating transcription DAF-16 and HIF-1 in worms, and FOXO in flies and mice. Inhibition of these nutrient-sensing signalling pathways extends lifespan by increasing stress resistance, maintaining proteostasis and activation of autophagy [73].

1.2 Aging in yeast

Yeast, *Saccharomyces cerevisiae*, is widely used model organism to study different genes and pathways relevant to human aging and diseases. *Saccharomyces cerevisiae* is unicellular eukaryotic budding yeast. It divides by asymmetric budding wherein daughter cell produced is smaller than mother cell. It stops dividing after limited number of divisions usually 20-25 and enter into stationary phase [75].

1.2.1 *Saccharomyces cerevisiae* - A model organism for aging

Fundamental research on understanding of aging process and interventions to slow down aging has been emerging from past decades. *Saccharomyces cerevisiae* has made immense contribution to this research [76]. It has contributed in identification of more human aging genes than any other model organisms [75]. There are many factors allowing use of *S. Cerevisiae* as model organism for aging research, one of the important factors is that longevity defining genes and signalling pathway discovered in yeast defines organismal longevity throughout eukaryotic phyla [77]. Ease in experimentation because of short lifespan provides rapidity in the quantification of longevity than other complex organisms. It allows rapid progress in revealing molecular mechanisms of organismal aging and identification of factors that modify its longevity [76]. It proliferates in both haploid and diploid forms and its manipulation is easy compared to other model organisms. It has small and relatively less complex genome, about 1% that of mammalian genome [78]. Fully sequenced genome provides an array of functional genomics tools such as collection of yeast deletion mutants, genome-wide over-expression libraries, and GFP-tagged yeast strains. Yeast shares a significant fraction of functional pathways with humans which control key aspects of eukaryotic cell biology like cell cycle, metabolism, protein folding, and so on. Many yeast genes have orthologs in the human genome including disease-causing genes. Out of 2271 known disease-associated genes, 526 have orthologs in yeast genome. For example, study of yeast set1 protein, ortholog of human mixed lineage leukemia gene, led to identification of highly conserved regulatory complex namely COMPASS (Complex of proteins associated with set1) in human leukemia. In addition, humanized yeast model which express human disease gene, is also in use to study numerous age-associated diseases like cancer, and

neurodegenerative diseases. N-aryl benzimidazole (NAB), compound which protects cells from α-synuclein toxicity, was discovered in humanized yeast model of Parkinson's disease [79]. As aging pathways are evolutionarily conserved (Figure 1.4), molecular mechanisms of aging defined in yeast can be easily compared with more complex multicellular organisms [77]. With all this, *S.cerevisiae* will continue to serve as model organism to study human aging and diseases.

1.2.2 Replicative and chronological models of yeast aging

Yeast, *S.cerevisiae* has two phases in its lifespan; replicative and stationary. Replicative phase lifespan acts as model for replicative aging, whereas stationary phase lifespan acts as model for chronological aging in yeast. Replicative lifespan is termed as the number of times a mother cell divides before cell cycle arrest. Chronological lifespan is the length of time for which a cell is viable in stationary phase (Figure 1.4) [76].

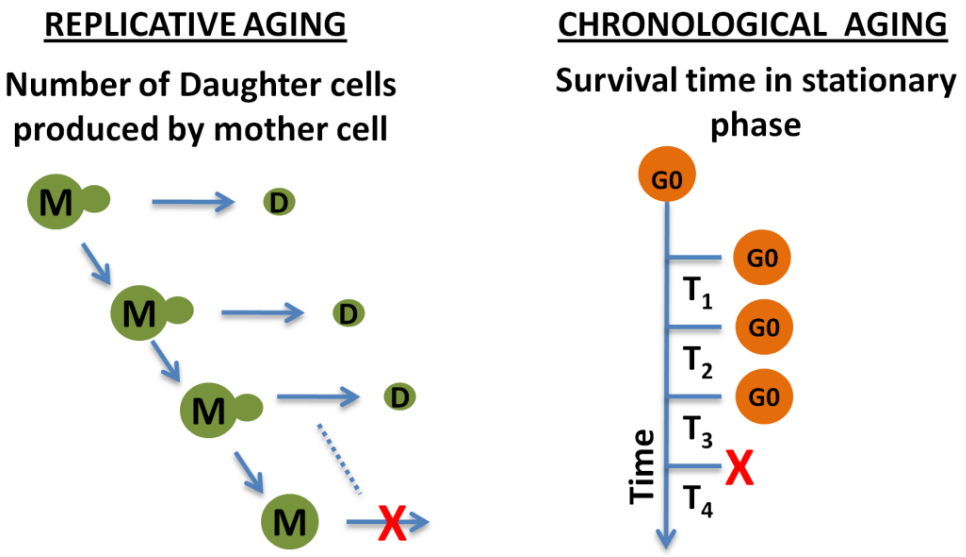


Figure 1.4 Two commonly used aging assays in yeast.
Replicative aging is measured in terms of generations as number of daughter cells produced by a mother cell. Chronological aging can be studied in terms of viability of cell over time after cell cycle arrest. Figure adapted and modified from [77].

1.2.2.1 Replicative lifespan (RLS)

Replicative lifespan in *S.cerevisiae* used to study aging of proliferating, mitotically active cells in multicellular eukaryotic organisms. *S.cerevisiae* divides by budding, asymmetric cell division, where daughter cell detaches from mother cell once mature [80]. RLS analysis is performed using a standard tetrad dissection microscope equipped with a micromanipulator. In this method, daughter cells are manually separated using micromanipulator to count number of cell divisions a single mother cell can undergo at given laboratory condition. Recently, new high throughput methods have been introduced which involves selective killing of daughter cells or microfluidic flow chambers. Although, micromanipulator method remains gold-standard for RLS study [75].

Aging is characterized by different kinds of damage, so nature of damage contributing to aging and aging-associated diseases play important role in its research. In replicative aging model, as cell division is asymmetric, damage inherited by mother cell must be asymmetric. Age-related damage gets accumulated in dividing mother cell with time. Young mother cell retains most of the age related damage than aging mother cell (Figure 1.5). Aged mother cell transfers sufficient damage to daughter cell which becomes prematurely aged. One of the widely studied molecular damage during RLS, which is specific to mother cell, is accumulation of extrachromosomal rDNA circles (ERCs) (Figure 1.5). Homologous recombination within rDNA leads to formation of self-replicating circular DNA molecules, termed as ERCs. ERCs are formed in mother cell nucleus and inherited asymmetrically to daughter cell [76, 77]. Sir2, NAD^+- dependant histone deacetylase, inhibit homologous recombination resulting in inhibition of ERCs formation. Thus, it is reported that overexpression of Sir2 increases RLS whereas deletion of Sir2 decreases RLS [76].

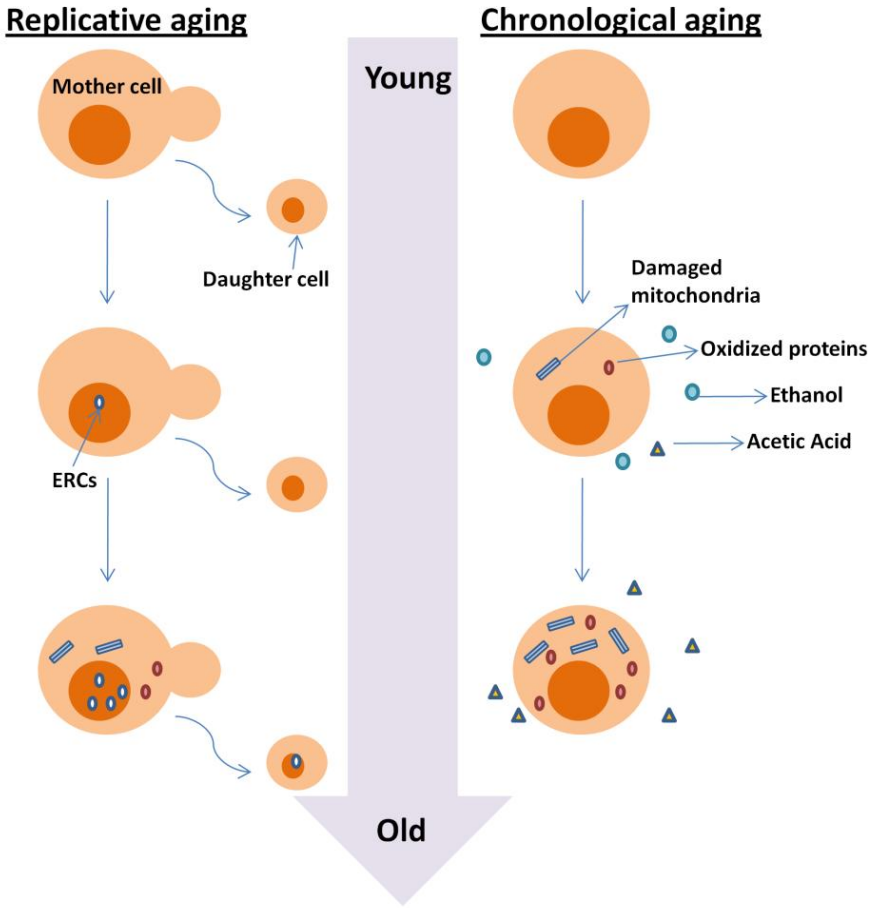


Figure1.5 Damage accumulation during yeast aging, replicative and chronological aging.
In RLS, Damage is asymmetrically inherited from mother cell to daughter cell. In CLS, damage accumulates in non-dividing cell. In chronologically aging cell, ethanol accumulates in the medium which further gets converted into acetic acid and induces cell death. In addition, damaged mitochondria and oxidized proteins contribute to senescence. In replicative aging, dysfunctional mitochondria and oxidized proteins along with ERCs stimulate senescence. Figure adapted and modified from [76].

1.2.2.2 Chronological lifespan (CLS)

Chronological lifespan measures survival of non-dividing cells. CLS of yeast can be serving as model for non-dividing, post mitotic cells aging in multicellular organisms. It is a simple model to study organismal aging [81]. CLS under laboratory conditions can be assessed using clonogenic assay. Clonogenic assay measures percent viability of yeast cells in liquid medium with 2% glucose at different time points following entry into stationary phase. Percent viability can be calculated by monitoring ability of a cell to form colony on the surface of solid medium [76, 82].

In contrast to RLS, in yeast CLS, age-related damage accumulates in non-dividing cell over time (Figure 1.5). Standard CLS assay involves growing yeast cells in synthetically defined liquid medium with 2% glucose as carbon source. Yeast cell utilise glucose through fermentation and produce ethanol in the extracellular environment. Once glucose gets exhausted, cells start metabolizing ethanol through mitochondrial respiration [76]. Several studies suggest that reactive oxygen species (ROS) formed during electron transport chain lead to oxidative damage to proteins, lipids and DNA within mitochondria. Oxidative damage accelerates process of chronological aging. In addition to ROS, reduced oxidative stress resistance during CLS worsen the problem. Oxidative stress in the form of acetic acid is found to be primary limiting factor for CLS. Acetic acid gets accumulated in extracellular environment when cells utilise ethanol through mitochondrial respiration (Figure 1.6). Acetic acid acidifies growth medium and thereby shortens yeast CLS [76, 77].

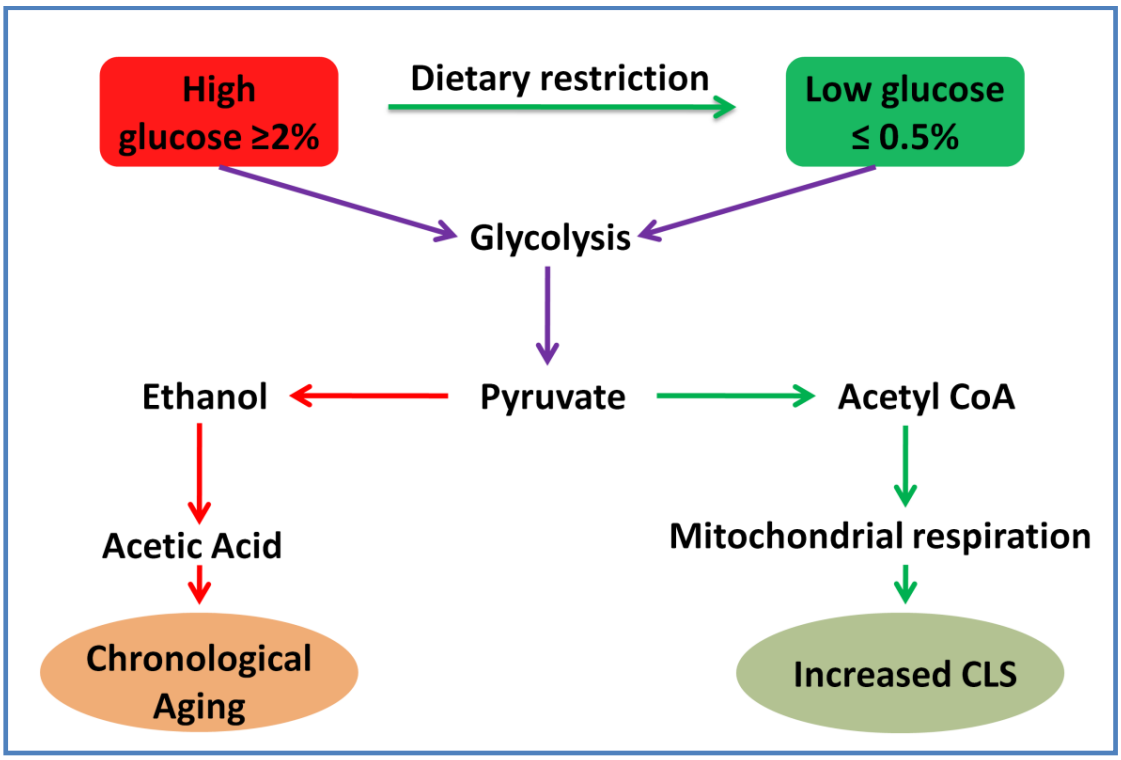


Figure 1.6 The metabolism of carbon sources in the chronological aging of S. cerevisiae.
In 2% glucose (non-caloric restriction), the yeast cells metabolize glucose into ethanol. Once glucose is depleted, ethanol is then metabolized through mitochondrial respiration, with acetic acid as the final product. In contrast to 2% glucose, in 0.5% glucose (caloric restriction), glucose is metabolized through mitochondrial respiration rather than through fermentation, thus extending chronological lifespan and increasing oxidative stress resistance [76].

Arlia-Ciammo *et al*. has proposed the concept of "biomolecular networks" which define the longevity of chronologically aging yeast. These networks operate at several distinct checkpoints during the CLS of *S. cerevisiae*. A set of protein regulators regulate the entry and progression though these checkpoints. The CLS checkpoints are defined according to growth phases of yeast (Figure 1.7).

The checkpoints occur in the logarithmic (early), diauxic, post-diauxic, and stationary (late) growth phases [82]. Cells inoculated in 2% glucose medium progress into logarithmic phase. When 50% of glucose is utilized glycogen synthesis begins. The diauxic shift phase is a phase in which glucose has been utilized completely; cells reduce their growth rate and then enter into next phase that is the post-diauxic phase. In this phase, yeast cells undergo slow respiratory growth using the ethanol and acetate as carbon sources [77, 83].

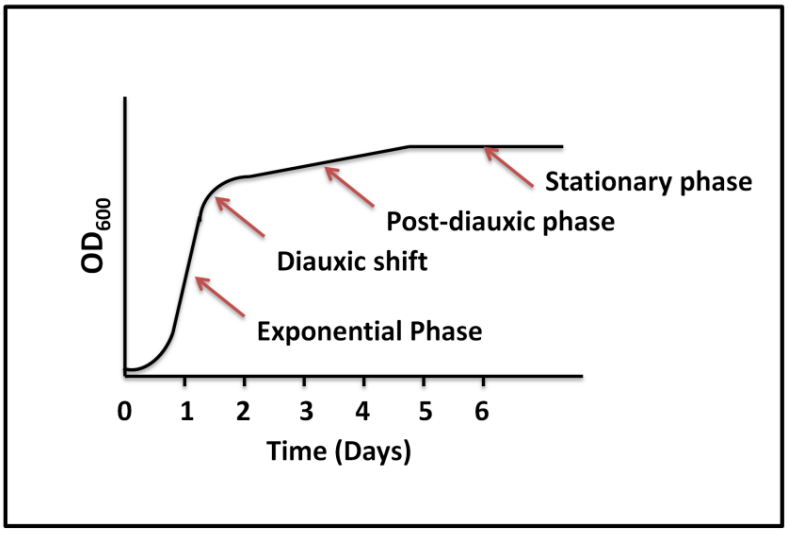


Figure 1.7 Growth curve for yeast cultured in 2% glucose medium.
Figure adapted and modified from [77]

Another important aspect to study chronological aging of yeast is quiescence and non-quiescence. Yeast CLS is monitored using cells grown in 2%glucose medium and progressing through stationary phase. Because of starvation in stationary phase, cells exit cell cycle in the G1 phase and enter into non-dividing phase of cell cycle G0 which termed as cellular quiescence. The yeast aging at quiescent state is a valuable model to understand aging of post-mitotic mammalian cells; as in this state cells

undergo complex changes in their metabolism and physiology. Quiescence state is clearly nutrient specific. Once nutrients are available, quiescent cells re-enter into cell cycle to proliferate. Under starvation conditions, not all cells enter into quiescent state; some cells enter a different state to become non-quiescent cells. Non-quiescent cells lose their ability to re-enter into cell cycle and die eventually [83].

1.2.3 Molecular mechanism of chronological aging in yeast

The major genes and signalling pathways which regulate yeast CLS are observed to be conserved from yeast to mammals. There are two main pro-chronological aging pathways operating in *S.cerevisiae* one is TOR/Sch9 (S6K) and second one is Ras/Adenylate cyclase/PKA pathway. Both of these pathway sense nutrient availability and its utilization [75]. Similarly, SNF1 pathway and ATG (autophagy) pathway are two anti-chronological aging pathways and Protein kinase Rim15 acts as anti-aging protein (Figure 1.8). Transcription factors downstream to these pathways play important role in regulation of longevity-defining cellular processes, including stress responses, protein synthesis in the cytosol and mitochondria, maintenance of nuclear and mitochondrial genomes, autophagy, mitochondrial respiration, peroxisome biogenesis, gluconeogenesis, lipid metabolism, glyoxylate cycle, glycogen synthesis and degradation, and the synthesis of amino acids and fatty acids [77].

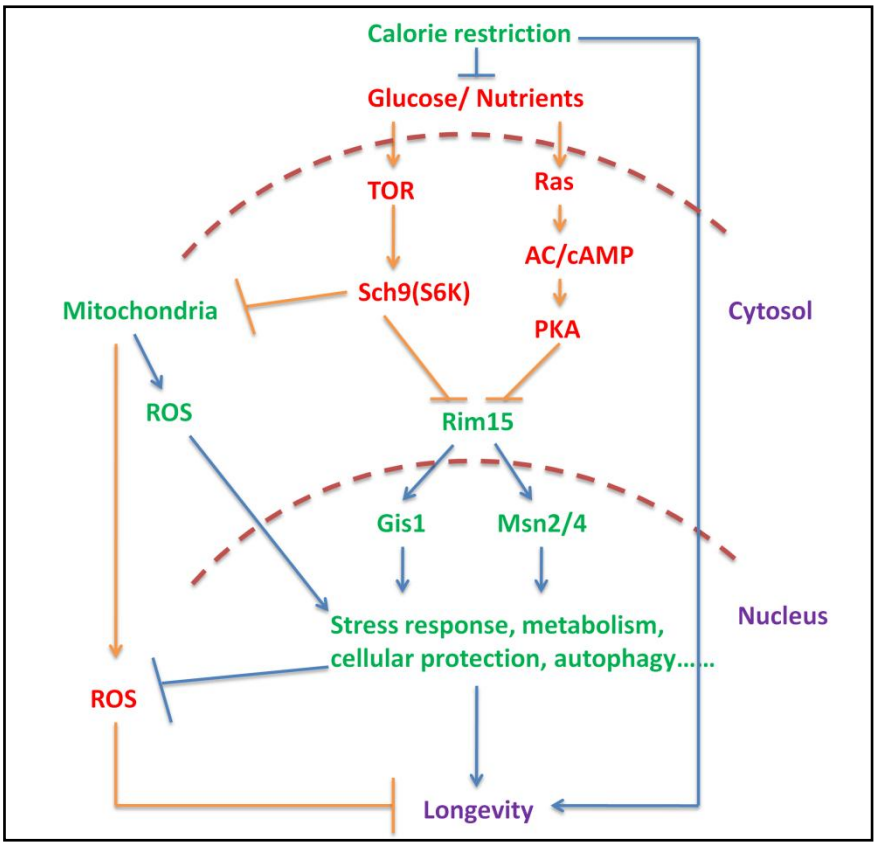


Figure 1.8 Regulatory pathways of yeast chronological lifespan.
TOR/Sch9 and Ras/AC/PKA are two major pro-aging pathways converging at protein kinase Rim15. Calorie restriction mediated longevity is through down-regulation of Tor/Sch9 and Ras/AC/PKA pathway and subsequent activation of Rim15 further activate stress responsive transcription factors Msn2/4 and Gis1. Figure adapted and modified from [75].

TOR (Target of Rapamycin) is an evolutionary conserved master regulator protein of longevity in all eukaryotic organisms. In higher eukaryotes, only one TOR iso-form is present which functions in two multiprotein complexes, TORC1 and TORC2. In yeast *S. cerevisiae* two TOR kinases are present, Tor1 and Tor2, which function in complex TORC1 and TORC2 [81]. The TORC2 complex regulates cell polarity and the actin cytoskeleton. The TORC1 regulates cell growth, the transition from the logarithmic growth to the diauxic and the stationary growth phases, and the progression through the stationary phase. Sch9 functions downstream to TORC1. Sch9, structurally and functionally related to ribosomal protein S6 kinase (S6K), is one of the key regulators of cell cycle initiation and ribosome assembly. Inhibition of TORC1 by rapamycin or by nitrogen starvation dephosphorylates and inactivates Sch9 [84]. Inhibition of Tor1 and Sch9 activates the nuclear translocation of anti-aging protein kinase Rim15 and stress-responsive transcriptional factor Gis1, thereby reprogramming gene expression

and extending yeast CLS [73, 84]. Similarly, inhibition of Sch9 or TORC1 decreases ethanol and acetic acid concentration in growth medium as well as increases the efflux of glycerol from the yeast cell which also contributes to CLS extension.

Like TOR/Sch9 pathway, Ras/AC/PKA pathway limits yeast CLS. The first chronologically long lived mutant identified was ras2Δ. RAS2 codes for highly conserved G-protein known to activate PKA via adenylate cyclase (Cyr1). Inhibition of RAS/AC/PKA pathway activates Rim15 which in turn activate stress responsive transcription factors Msn2/4 [81]. Ras/PKA and Tor/Sch9 converge on transcription factors which mediate expression of stress-responsive genes [75]. TORC1 and PKA inhibit anti-aging SNF1 pathway and autophagy. PKA inhibits stress response and some metabolic pathways in yeast cells entering the diauxic growth phase and then to the stationary phases of growth. Decrease in PKA activity is reported to extend yeast CLS by enhancing stress response [77, 84].

SNF1 (Sucrose non-fermenting, protein 1), an anti-aging signaling pathway, is known to regulate transcription of almost 400 genes. Yeast SNF1 is an ortholog of the mammalian AMP-activated protein kinase (AMPK) which controls transcription of numerous genes [77, 83, 84]. SNF1 has also been shown to play key role in chromatin modification, autophagy and activation of some metabolic pathways. The SNF1 pathway is inhibited when glucose is available to yeast cells. Similarly, autophagy is also a beneficial process regulated by ATG signaling pathway [84]. Autophagy is the process of degradation of cytoplasmic substrates in the vacuole of yeast cell. It is important for survival during nutrient deprivation. It is also a regulator of organellar homeostasis, specifically of mitochondria. Dysfunctional mitochondria which lead to increase in oxidative stress are removed by autophagy. Indeed, it is not a destructive process but rather a cytoprotective process leading to extension of CLS of yeast. TORC1 inhibit autophagy causing chronological aging in yeast [25, 77].

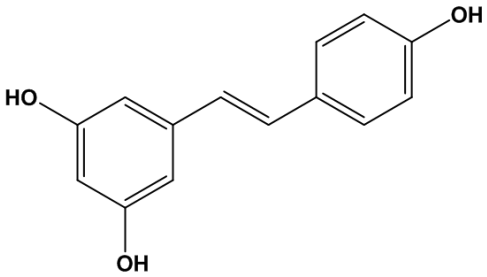
Caloric restriction (CR), reduction in nutrients availability without malnutrition, is the only non genetic intervention known to extend lifespan in all species [81].CR reported to extends yeast CLS by inhibiting Tor1/Sch9 and Ras/PKA pathway. It results into activation of Rim15 which further activate stress responsive transcription factor.

Under CR condition, ethanol and acetic acid concentration is reduced contributing to CLS extension [76].

1.2.4 Anti-aging drugs from studies on yeast

1.2.4.1 Resveratrol

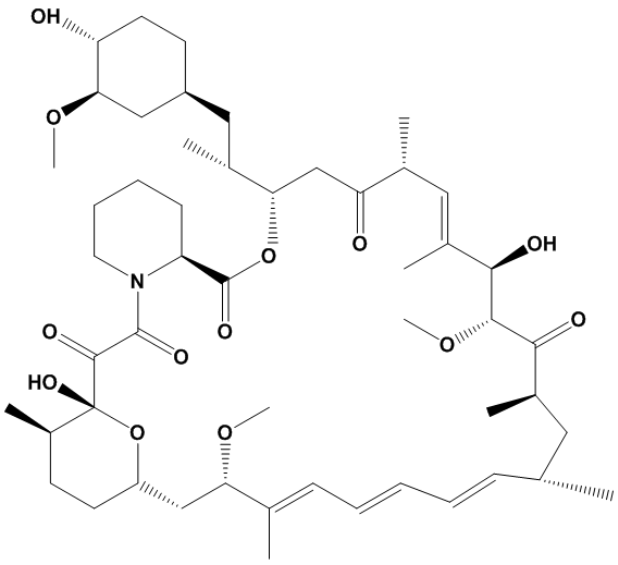
Resveratrol is a polyphenolic compound found in red wine. Howitz, KT. *et al*. for first time reported that resveratrol extends yeast RLS by activating Sir2. It extends lifespan in nematodes, flies and a short-lived fish. Resveratrol is currently being studied for safety and efficacy against type 2 diabetes in human clinical trials [76].



Resveratrol

1.2.4.2 Rapamycin

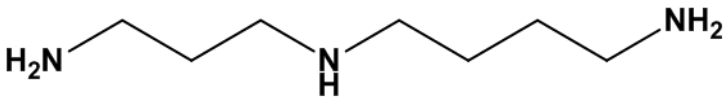
Rapamycin is macrolide antibiotic that inhibits TOR activity. Powers, RW. *et al*. in 2006 reported that rapamycin extends yeast CLS. Further, it is reported to extends yeast RLS and lifespan in nematodes and flies. Clinically, it is used to treat certain forms of cancers and as an immunosuppressant [76].



Rapamycin

1.2.4.3 Spermidine

Spermidine is naturally occurring polyamine which modulates different cellular processes such as DNA stability, apoptosis and translation. Spermidine was reported to extends yeast CLS and lifespan in nematodes and flies in 2009 by Eisenberg, T. *et al.* It promotes longevity through induction of autophagy [76].



Spermidine

1.3 Glycation and Aging

1.3.1 Glycation

Glycation is a spontaneous, non-enzymatic reaction of reducing sugars with free amino group of proteins resulting in the formation of a reversible Schiff's base. Further, it undergoes rearrangements to form amadori product. Oxidation of amadori product generate dicarbonyl compounds which further form cross-linking fluorescent as well as non-fluorescent adducts termed as advanced glycation end products (AGEs) [85, 86]. AGEs are stable and irreversible end products of glycation reaction. AGEs further react with free amino groups of proteins leading loss of protein function [85]. Not only glucose but glucose derived dicarbonyls such as glyoxal, methylglyoxal, glucosones and 3-deoxyglucosone are the major precursors of AGEs [86]. Also, glycolytic intermediates such as dihydroxyacetone phosphate and glyceraldehyde 3-phosphate can form AGEs [87]. Fructosyl-lysine (FL), Carboxymethyl lysine (CML), Carboxyethyl lysine (CEL) and pentosidine are the predominant AGE modifications [86]. In addition to direct glycation, glucose reduces molecular oxygen yielding free radicals by process of glyco-oxidation. A large amount of reactive oxygen species (ROS) is formed during process of glycation which leads to oxidative stress. Oxidative stress in turn increases glycation [87, 88].

1.3.2 Role of glycation in aging and aging-associated diseases

Glycation plays critical role in aging and aging associated diseases as it affects all fundamental cellular processes. Glycation affects proteins and enzymes involved in different cellular processes such as glucose metabolism, bioenergetics, cell repair and stress response. Plasma proteins have been seen to be glycated predominantly [89]. Glycation inhibits specific function of proteins via cross-linkage, aggregation and precipitation. Glycated, misfolded proteins accumulate in the system and become protease resistance. Ultimately, it results into decline in protein homeostasis [90].

AGEs like pentosidine and CML, are considered as biomarkers of aging and their levels increase five times from infancy to old age [91]. AGEs accumulate slowly in the body with age. It is reported that insulin resistance increases with age because of AGEs and oxidative stress which interfere with insulin receptor functioning and signal transduction [92]. Damaged mitochondria and accumulation of modified proteins are the major sources of stress associated with aging. AGEs like CML have been observed in mitochondria of aged animals. Hyperglycemia induces overproduction of mitochondrial ROS which increases production of AGEs via methylglyoxal (MGO) formation. MGO causes mitochondria damage and affects respiratory rate [93].

AGEs have been detected in β-amyloid plaques in patients with Alzheimer's disease and Parkinson's disease [94, 95]. Rheumatoid arthritis patients showed increased levels of pentosidine in plasma which may evoke inflammatory response contributing to aging [96]. AGEs such as CML and pentosidine accumulate in long lived tissue proteins which implicated into aging. Glycated collagen plays a critical role in vascular complications during aging [86]. Aging is associated with defective vascular relaxation and hypertension which is caused by collagen bound AGEs in vascular walls. LDL, plays crucial role in atherogenesis, was also found to be AGE modified [87, 97]. Accumulation of AGEs in eye lens was observed during aging induce cataract formation [98]. Cancer is associated with higher intracellular carbonyl stress and AGEs formation. AGEs modified proteins were detected in breast cancer tissue [86, 99].

1.3.3 Glycation inhibitors

Involvement of AGEs in aging and aging-associated diseases makes it promising therapeutic target. Indeed, reducing AGEs by using glycation inhibitors may be the rational approach to extend lifespan and delay aging. Aminoguanidine (AMG) was the first glycation inhibitor reported to scavenge dicarbonyl compounds [100]. Aspirin prevents glycation by acetylating proteins [101]. Carnosine reacts with reducing sugar thereby inhibiting reaction [102]. Nucleophillic compound, Hydralazine removes glucose moiety bound to protein [103]. Metformin, the first line medication for the treatment of type-2 diabetes, is also reported to trap reactive carbonyl intermediates formed during glycation reaction [104]. Pyridoxamine inhibits conversion of Amadori intermediates [105]. Metal chelators, AGE cross-link breakers, antioxidants and RAGE blockers are known to inhibit glycation and AGE formation at one or other stage [86].

With all this background, following objectives were designed for thesis,

1. To develop yeast as a model system to study glycation induced aging
2. To study the effect of glycation inhibitors on yeast chronological lifespan at proteomic level
3. To study the effect of glycation inhibitors on aging responsive genes and pathway

Chapter 2

Development of yeast as a Model system to study glycation induced aging

Partial contents of this chapter are published as a research article in Journal of Proteomics, 2017, vol-156, 104-112.

Chapter 2 Development of Yeast as a model system to study glycation induced aging

2.1 Background

The single-celled budding yeast, *Saccharomyces cerevisiae* is one of the most efficiently used model organisms in aging research [106]. This is because the aging related genes and signaling pathways discovered in yeast have been shown to define aging process in eukaryotes across phyla [75]. Also, *S. cerevisiae* has short lifespan in comparison with other, more complex organisms which provides ease in experimentation [76]. This gives accelerated progress in discovering molecular mechanisms associated with process of aging and identifying factors responsible for longevity within the organism [76]. *S. cerevisiae* displays two distinct lifespan, namely, replicative lifespan (RLS) and chronological lifespan (CLS), which serve as models for proliferating (mitotic) and non-proliferating (post-mitotic) tissues in higher eukaryotes, respectively[76]. RLS is defined as the number of daughter cells produced by a mother cell before cell division ceases[107], whereas CLS is the duration of cell survival in the stationary phase[108].

Non calorie restriction in terms of glucose (2%) led to activation of nutrient signaling factors such as TOR/Sch9 and RAS/AC/PKA that contribute to reduced lifespan in yeast[109]. Calorie restriction, deletion of SCH9 and TOR extends CLS of yeast, accompanied by a shift in glucose metabolism from fermentation to respiration[110, 111]. In addition to these factors, non calorie restriction in terms of glucose can promote formation of intracellular advanced glycation end products (AGEs) [112, 113]. AGEs are formed as a result of series of non-enzymatic reaction between protein and reducing sugars[114]. AGE modification is known to affect function of several intracellular proteins like glyceraldehyde 3-phosphate dehydrogenase, bisphosphoglycerate mutase, and pancreatic glucokinase[115, 116], which is reviewed in great detail elsewhere [86]. AGE modification of proteins also results in protease resistance and accumulation of such proteins can cause proteotoxicity and affect the protein homeostasis[90]. Reactive carbonyl such as methylglyoxal formed as a result of glyco-oxidation, react with proteins and form aggregates[117]. Glycated proteins and aggregates are responsible for several age associated diseases like Alzheimer’s

disease[118, 119], Parkinson's disease[120], in familial amyloidotic polyneuropathy[121], amyloidotic lateral sclerosis[122] and diabetic complications[123]. Thus, targeting AGE formation could be a rational approach to extend the lifespan. Therefore, in this study, we have used yeast, *S.cerevisiae*, as a model system to study glycation induced aging. We show that non calorie restriction in terms of glucose shortens yeast CLS by increasing ROS and AGE modification of proteins. AGEs can be targeted using glycation inhibitors. Here, we are using 'Drug repositioning' strategy to develop anti-aging drugs, which has advantage of time and cost over classical drug discovery approach. In this study, we report that AMG, a known glycation inhibitor, reduces AGE modification of proteins and consequently extends the yeast CLS.

2.2 Materials and methods

2.2.1 Strains and Chemicals Used

S. cerevisiae strain BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) (life technologies, CA, USA) was used for all experiments. Cell stocks were maintained in YPD agar medium containing 0.5% yeast extract, 1% peptone, 2% agar, and 2% glucose. All experiments were performed in synthetic complete (SC) medium containing glucose (Himedia) as a carbon source and 0.67% yeast nitrogen base without amino acids (Sigma) supplemented with excess amino acids (Himedia). Cells grown in SC medium containing 0.5% glucose are referred as Calorie Restricted cells whereas cells grown in 2% glucose are referred as Non Calorie Restricted cells. 4% glucose is considered as high glucose treatment. Drug used was AMG (25mM) (Sigma cat#396494). All chemicals were procured from Sigma unless otherwise stated.

2.2.2 Chronological Lifespan

Starter culture was inoculated from plate and grown in SC medium containing 0.5% or 2% of glucose and then diluted into fresh SC medium with 0.5%, 2% and 4% concentration of glucose to obtain OD of ~0.1 at A_{600}(~1:100 dilution) with and without drug. These cultures were then incubated at 30°C with shaking at 200 rpm.

Cultures reached stationary phase at day 3 and this was considered as day 0 of CLS. Cellular viability was assessed at indicated days using quantitative plating experiment and by 10-fold serial dilutions spot test[109]. Quantitative plating experiment was done by counting colony-forming units (CFUs) assay [124]. Briefly, cell number was estimated by measuring the absorbance at 600 nm. The serially diluted cultures were plated onto 3 YPD agar plates at a cell density of 100 cells per plate. Plates were incubated at 30 °C for 2days and number of colonies was counted. Cell viability was recorded from 3rd day onwards at an interval of 2 to 3 days. The cell viability was determined by considering viability on the 3rd day as 100% [38, 125]. CLS assay was repeated for three times in independent experiments.

2.2.3 Quantification of Reactive Oxygen Species

Intracellular ROS levels were measured using Dihydroethidium(DHE) (Molecular Probes) [126]. Briefly, aliquots were taken at selected time points and 5 μM DHE was added and incubated for 10 min at 30 °C. Cells were then washed twice in PBS and analyzed by inverted fluorescence microscope [38].

2.2.4 Western blotting

Yeast protein extraction was performed by enzymatic disruption method. Briefly, 15 units of Zymolyase (GBiosciences) was added to 100mg of cell pellet and incubated at 37 °C for 1 h. Proteins were extracted from spheroblast formed in extraction buffer (8M Urea,2M Thiourea,1%DTT,4%CHAPS). Protein concentration was determined using the Bio-Rad Bradford assay kit. Proteins (40 μg protein per lane) were separated on 12% SDS-PAGE and transferred to polyvinylidine fluoride membranes using the Mini Trans-Blot system (Bio-Rad). Total proteins were stained with Ponceau S solution [0.5% (w/v) Ponceau S in 1% v/v glacial acetic acid] to confirm protein transfer. The membranes were incubated overnight at 4°C in blocking buffer containing 5% (w/v) skimmed milk in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2mM KH_2PO_4, pH 7.5). For AGE and CML detection, PVDF membranes were probed with anti-AGE antibody (1:1000) (Abcam, Cambridge, UK) and Anti-CML antibody (1:1000) (Abcam, Cambridge, UK)) respectively, for 3 h. After one wash with PBS-T (PBS containing 0.05% Tween 20) and two washes with PBS, the

membranes were incubated with secondary antibody conjugated with HRP (Bangalore Genei) at a dilution of 1:2000 for 1h at room temperature. After washing, Chemiluminescence detection was done using the WesternBright^{TM} Quantum Western Blotting detection Kit (Advansta) following the manufacturer's instructions. Levels of carbonylated proteins were measured using Oxiselect^{TM} protein carbonyl immunoblot kit (Cell Biolabs, San Diego, CA) following the manufacturer's instructions. Quantitative analysis was performed by Image studio Lite -LI CORE. Each immunoblot was repeated three times in independent experiments.

2.2.5 In-solution Trypsin Digestion

100µg of total protein mass per sample in 0.1% Rapigest (Waters) was used for digestion. Proteins were denatured by incubating at 80°C for 15min. Further, Proteins were reduced and alkylated by 100mM dithiothreitol for 15min at 60°C and 200mM Iodoacetamide for 30min at room temperature respectively. Trypsin (1:25) was added to it and incubated at 37°C for 18 h. Trypsin digestion was stopped by adding formic acid.

2.2.6 Desalting of peptide digest

Digested peptides were cleaned up or desalted by means of C18 chromatography using Zip tips (Millipore, Billerica, MA). Firstly, C18 Zip tips were activated using 100% MS grade ACN. Next step is column equilibration which is done using 0.1% TFA in MS grade water. Further, peptide digest was aspirated 3-4 times so that peptides will bind o C18 column. C18 Bound peptides were cleaned up by washing column 2-3 times with 0.1%TFA water. Lastly, peptides were eluted in 0.5%FA+50%ACN and concentrated using vacuum concentrator. Peptides were reconstituted in 3%ACN with 0.1%formic Acid.

2.2.7 Modification (PTM) Analysis by Label free LC-MS^{E}

Tryptic peptides were analyzed by nano LC-MS^{E} (MS at elevated energy) using a Nano Acquity UPLC system (Waters Corporation, Milford, MA) coupled to a SYNAPT-HDMS (Waters Corporation). The nano- LC separation was performed using a BEH-C18 (1.7 µm x 75 µm x 250 mm) column (Waters Corporation). The

binary solvent system used comprised 99.9% water and 0.1% formic acid (mobile phase A) and 99.9% acetonitrile and 0.1% formic acid (mobile phase B). Peptides were initially pre-concentrated and desalted online at a flow rate of 5 µl/min using a Symmetry C18 trapping column (180 µm x 2 cm) (Waters Corporation) with a 0.1% B. After each injection, peptides were eluted into the NanoLockSpray ion source at a flow rate of 250nL/min using a gradient of 3–40% B for 95 min. All mass spectrometric analysis was performed in a positive V-mode at a resolution of about 9000 full width half maximum (FWHM) in a mass range of 50-2000 m/z with a scan time of 0.75 s with alternating low (4 eV) and high (15 to 40 eV) collision energy. The instrument was calibrated with a MS/MS spectra of Glu-fibrinopeptide B (m/z 785.8426) (600 fmol/µl), and the lock mass correction was done every 30s by the same peptide.

The LC-MS^E data was analyzed by Protein Lynx Global Server 2.5.1 (PLGS; Waters Corporation) software. The ion accounting parameters used to search included precursor and product ion tolerance as automatic, minimum number of peptide matches (1); minimum number of product ion matches per peptide (3), minimum number of product ion matches per protein (5) and the one missed tryptic cleavage site. The false positive rate was 1%. Ion intensity threshold was set at 500 counts. A preliminary search was performed for protein identification using UniProt *Saccharomyces cerevisiae* database updated with Uniprot release 2011_5 at the end of December 2014 containing 7851 reviewed protein entries. LC-MS^E data were searched with a fixed carbamidomethyl modification for Cys residues. For identification of AGE modifications, a targeted search against subset of identified proteins was performed involving variable glycation modifications specific to lysine residues viz., carboxymethyllysine (CML) (+58.0055 Da) and carboxyethyllysine (CEL) (+72.0211 Da); Pyralline (+108.0211 Da). Those involving both lysine and arginine residues were Argpyrimidine (ARGP) (80.0262); Schiff's base/Amadori modification (+162.02 Da) and hydroimidazolones (MG-H1) (+54.0106 Da). Three replications were performed for each sample.

2.2.8 Gene Ontology Analysis using DAVID

S.cerevisiae uniprot accessions of identified proteins were uploaded to the bioinformatics tool DAVID[127] to look for functional annotation. Functional

clustering was performed with high stringency. Pathway enrichment was also determined using DAVID.

2.2.9 Statistical analysis

All experiments were performed either in triplicates. Statistical analysis was performed by Student's t test and two-way ANOVA. Data were expressed as mean ±SEM. A p value < 0.05 was considered as statistically significant.

2.3 Results

2.3.1 Increase in media glucose concentration shortens *S. cerevisiae* chronological lifespan

Yeast CLS can be measured by growing the cells to stationary phase in liquid media, whereupon they cease division but remain metabolically active [128]. Cells were removed periodically from stationary phase culture. Viable cells are the fraction of these cells capable of forming a colony when plated onto rich media. The percentage viability is measured by determining viability at a period compared with viability at day 3 (Considered as 100%). We observed that with increase in media glucose concentration from 0.5% to 2% and 4% cell viability decreases with time. Decrease in percent viability with 2%glucose is 25-30% whereas with 4%glucose it is 50-60% (Figure 2.1 A and B).

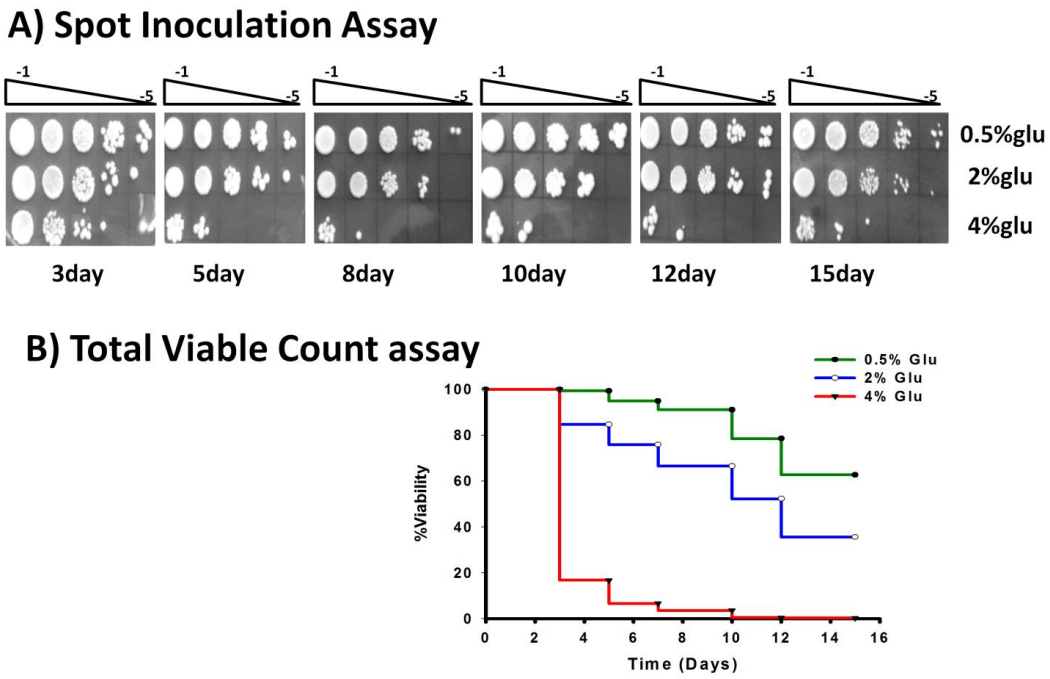


Figure 2.1 Increase in glucose concentration shortens yeast CLS.
A) 10-fold Serial dilution spot assay, where viability of yeast cells was observed on different days by inoculation of serially diluted yeast culture as a spot on YPD agar plate; and B) Quantitative CLS assay, where cell viability was measured as Colony Forming Unit (CFU) at 2-3 days interval beginning with the day when yeast culture was at stationary phase (day 3). The cell viability on different days was compared with viability on the day 3, which was considered as 100%. Values represent mean ±SEM from three biological replicates. Statistical significance was calculated by two-way ANOVA (Interaction P< 0.001, Time P< 0.001, treatment P< 0.001).

2.3.2 Increase in media glucose concentration increases ROS formation and glycation modification of proteins in *S. cerevisiae*

Increased oxidative stress is associated with death of the cells. Intracellular oxidative stress was studied using dihydroethidium (DHE) dye to confirm its effect on yeast CLS. DHE reacts with superoxide radicals and gives red fluorescence under fluorescence microscope. With increase in concentration of glucose, number of cells showing red fluorescence increases. High glucose increases intracellular ROS formation in yeast (figure 2.2 A). High glucose non-enzymatically reacts with proteins giving rise to formation of AGEs through glycation reaction. Protein glycation was studied by western blotting using anti-CML antibody. There was increase in AGE modification of proteins with increase in glucose concentration

(Figure 2.2 B). Protein carbonylation was studied to check overall protein modification because of oxidation reaction. Anti- DNP antibody was used to check protein carbonylation. Like AGE modifications, protein carbonylation was also increased with high glucose (figure 2.2 C).

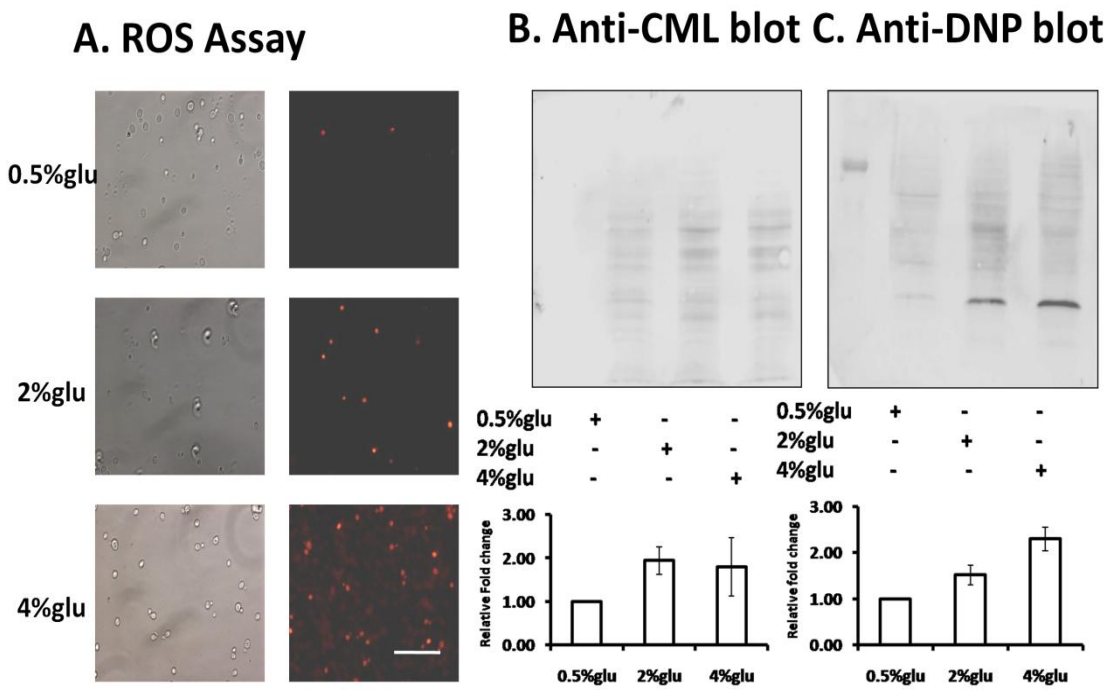


Figure 2.2 Increase in glucose concentration increases ROS formation and level of AGE modification of proteins.
A) ROS formation was studied using DHE dye and cells were observed under inverted fluorescence microscope. B) Western blot analysis was performed to detect AGE modification and carbonylation in proteins extracted on day 3 from cells in different conditions.

2.3.3 Aminoguanidine extends *S. cerevisiae* Chronological Lifespan

Non calorie restriction (2% glucose) is known to promote AGE modification of intracellular proteins affecting their function. Accumulation of AGE modified proteins can accelerate aging process. NR condition i.e. yeast grown on 2% glucose, is associated with reduced chronological lifespan, suggesting the possibility of involvement of AGEs. Therefore we have investigated the role of glycation in regulation of yeast CLS by growing the yeast cells either in 0.5% (CR), or 2 % (NR), or 2% (NR) with AMG, a glycation inhibitor. Yeast CLS was studied by measuring the survival of non dividing stationary phase cells using CFU counting and serial dilution spot assay. CLS of yeast was decreased in NR as compared to CR condition. AMG mimicked the CR condition as it increased yeast CLS by 45-50%. (Figure 2.3A and B).

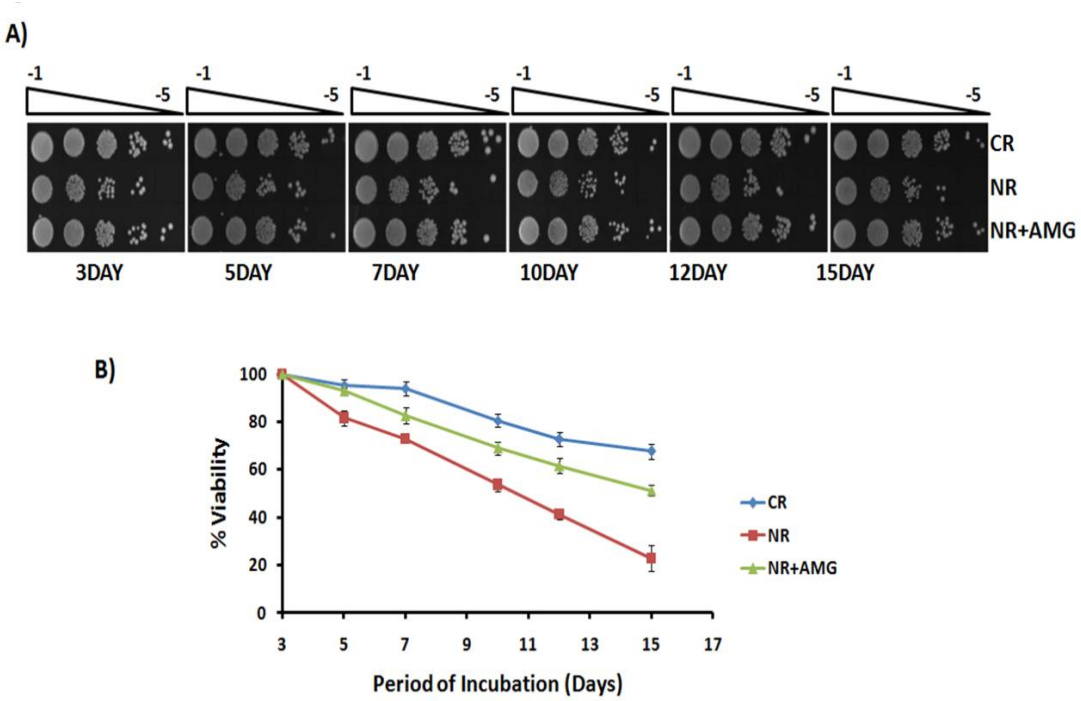


Figure 2.3 AMG extends yeast CLS in NR condition.
A) 10-fold Serial dilution spot assay, where viability of yeast cells was observed on different days by inoculation of serially diluted yeast culture as a spot on YPD agar plate; and B) Quantitative CLS assay, where cell viability was measured as Colony Forming Unit (CFU) at 2-3 days interval beginning with the day when yeast culture was at stationary phase (day 3). The cell viability on different days was compared with viability on the day 3, which was considered as 100%. Values represent mean ±SEM from three biological replicates. Statistical significance was calculated by two-way ANOVA (Interaction P< 0.001, Time P< 0.001, treatment P< 0.001).

2.3.4 Aminoguanidine decreases AGE modification of proteins in non-CR condition

Western blot analysis showed that AGE modification of proteins was more in NR than CR condition. Interestingly, AMG was able to reduce AGE modification of proteins in NR, which was detected by AGE specific antibodies, anti-AGE and anti-CML (Figure 2.4 A and B). Further, protein carbonylation was also decreased with AMG treatment, as analyzed by anti-DNP antibody (Figure 2.4C).

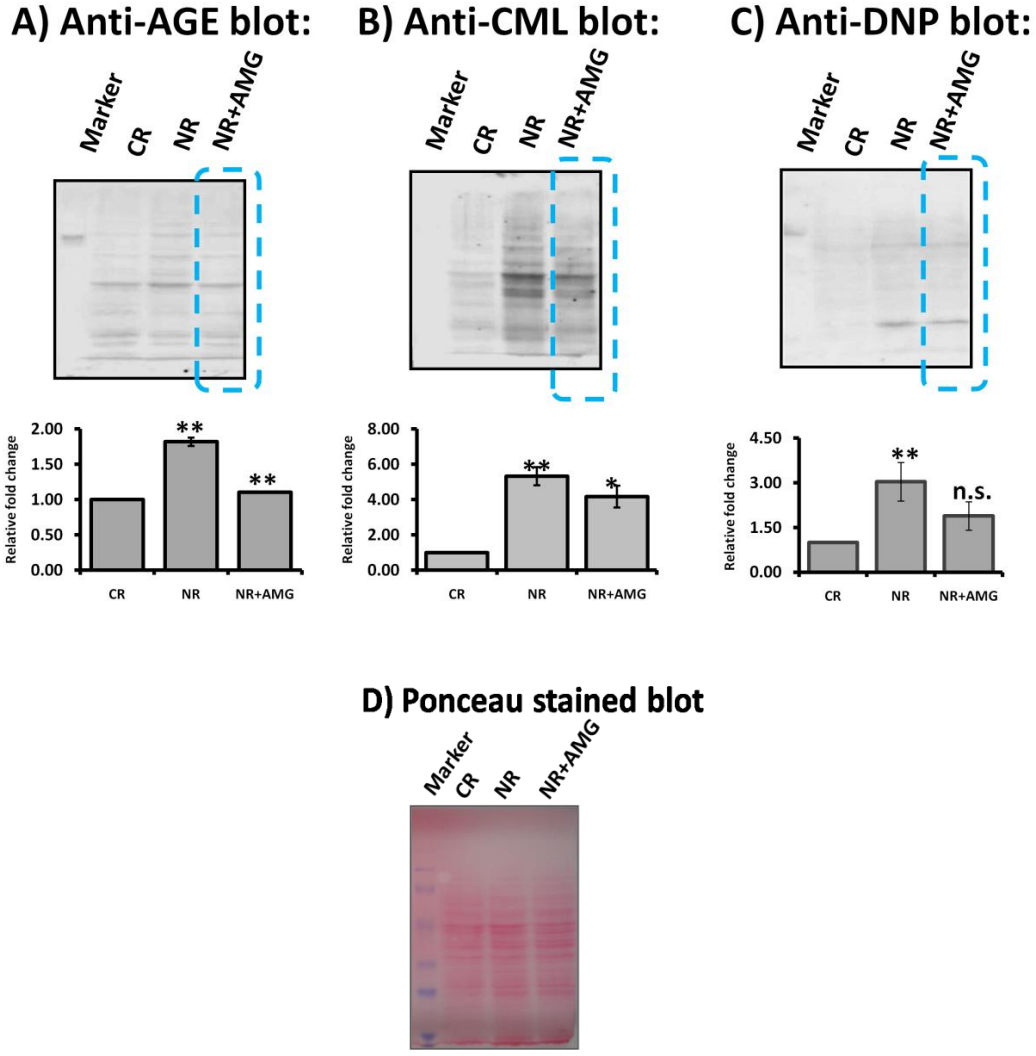


Figure 2.4 AMG reduces AGE modification of proteins.
Western blot analysis using antibodies against A) AGE B) CML and C) DNP to detect modifications in proteins extracted at 3day in different conditions. Extent of modifications was represented densitometrically in comparison with CR (considered as 1). D) Ponceau stained blot as a loading control.Three biological replicates for western blot were performed. Antibody signal values are mean ±SEM. Statistical significance (*P< 0.05 a, ** P<0.01 and n.s.- non significant) was calculated by student t-test.

AGE modification of proteins was also studied by label free LC-MS^E approach. A total of 39 and 10 proteins were found to be AGE modified in NR and CR condition respectively. The number of AGE modified proteins in NR decreased to 19 with the treatment of AMG (supplementary data S1). This trend was also reflected at the peptide level. Peptides were manually validated for presence of AGE modification (Figure 2.5) [129]. Some of the important proteins such as mitochondrial outer membrane protein porin 1 (por1) and ATP synthase subunit 2 (atp2) were found to be AGE modified. Although these proteins were down regulated in NR, showed increased modification as compared to CR and NR treated with AMG. Similarly up-regulated proteins such as Enolase 1 (eno1), ribosomal proteins and elongation factor 1 alpha (tef1) were showed increased number and extent of AGE modification in NR compared to CR and NR treated with AMG (Figure 2.6 A and supplementary data S1). AGE modification of proteins was not dependent on the protein abundance as both up-regulated and down regulated proteins in NR condition found to be AGE modified. Further pathway analysis by Database for Annotation, Visualization and Integrated Discovery (DAVID) of AGE modified proteins suggested that they are mainly involved in glycolysis and ribosomal proteins (Figure 2.6 B).

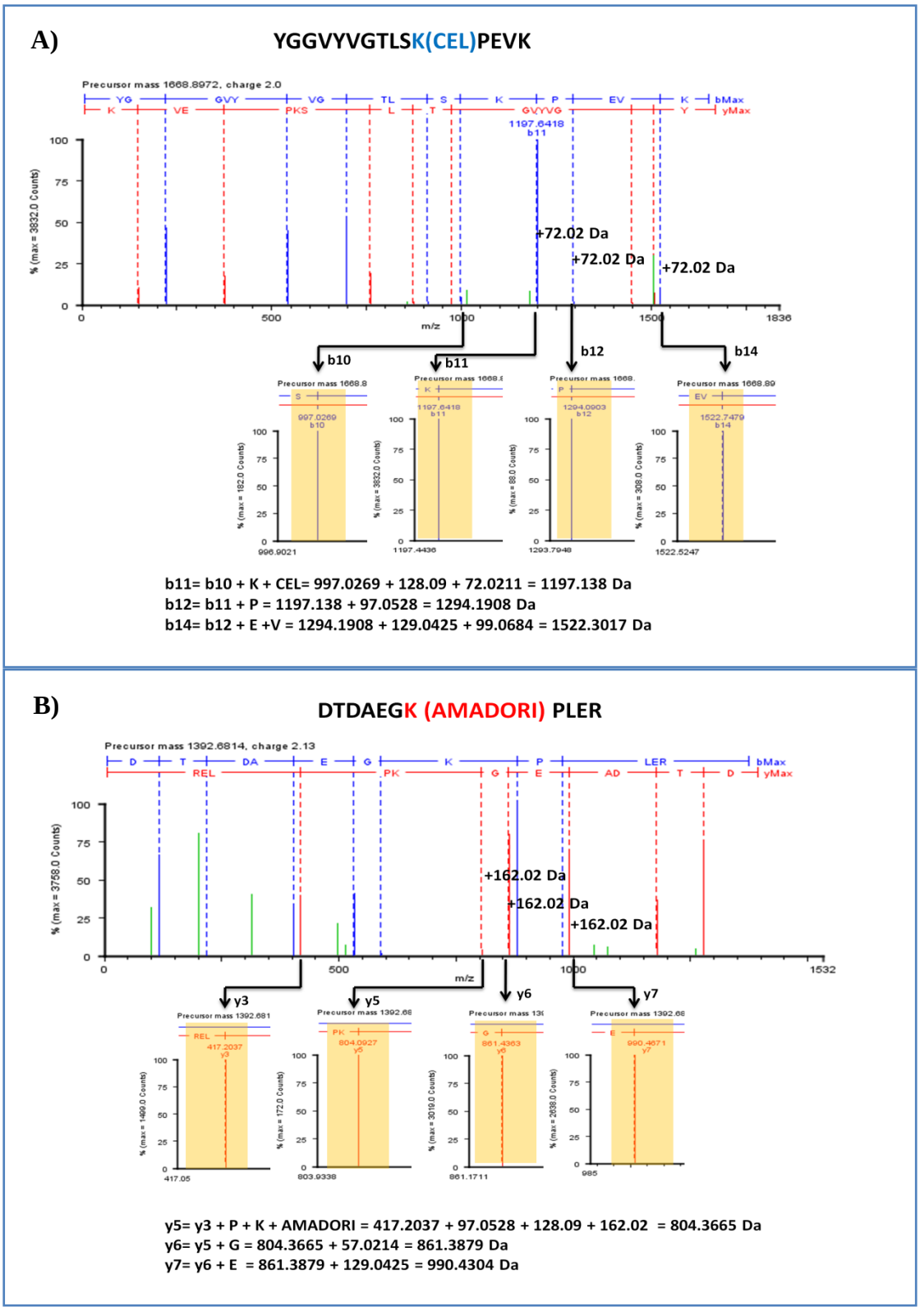


Figure 2.5 A representative MS/MS annotation of peptides
A representative MS/MS annotation of A) YGGVYVGTLSK*CEL PEVK, CEL modified peptide from PDC1 protein B) DTDAEGK*AMADORI PLER, AMADORI modified peptide from EF2 protein.

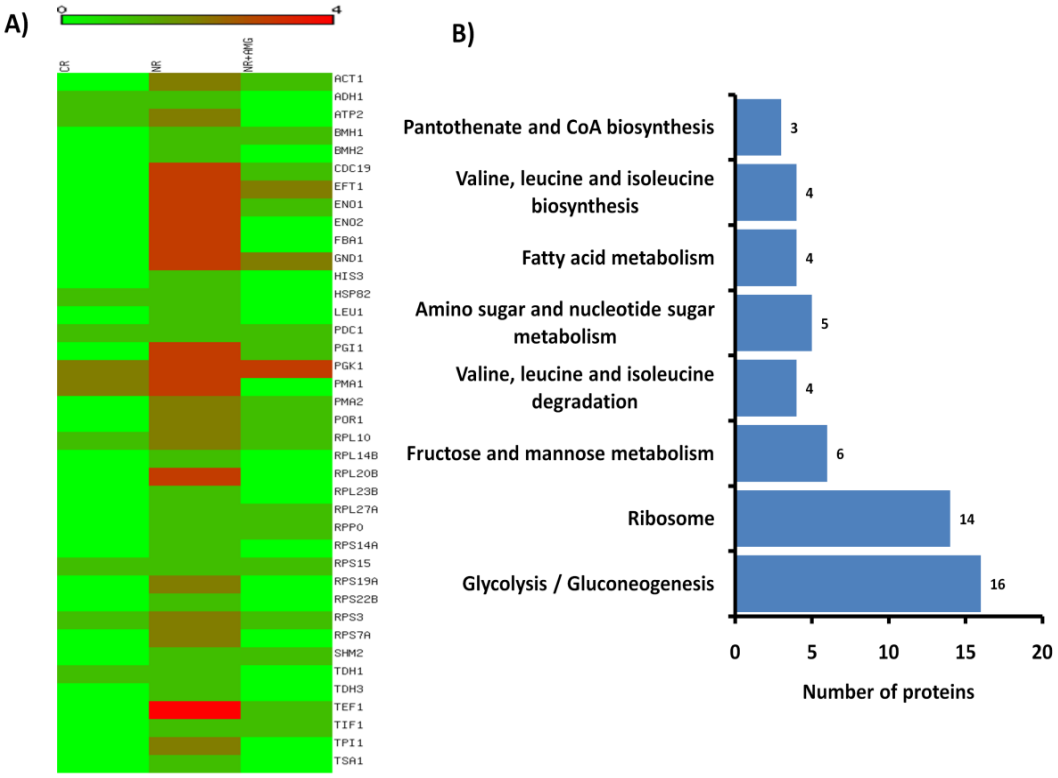


Figure 2.6 AMG reduce number of AGE modified proteins
A) AGE modified proteins were analyzed by LC-MSE. Heat map of manually inspected AGE modified peptides in different conditions. Three biological replicates for western blot and three technical replicates for LC-MSE were performed. B) Pathway analysis of modified proteins was done using DAVID (Categories represented in bar graph are with P< 0.05).

2.4 Discussion

Aging has been widely studied using different model systems including yeast. Different signalling pathways like TOR pathway, Ras-PKA pathway etc, are reported to be associated with aging in yeast [76]. Calorie restriction in terms of glucose is known to extends yeast CLS as compared to non calorie restriction or high glucose [110, 130, 131]. High glucose can lead to protein glycation. Protein glycation plays a vital role in the pathogenesis of age-related diseases, such as diabetes, atherosclerosis, end-stage renal disease, and neurodegenerative disease[85]. High glucose along with reactive carbonyl compounds like methylglyoxal and 3-deoxyglucosone[132] accelerate formation of intracellular AGEs[133]. AGE modification confers increased resistance to proteolysis, leading to their accumulation in the cell, which is known to accelerate aging process [90]. Thus, we have used yeast as a model system to study glycation induced aging. Our study has suggested that increase in glucose concentration shortens CLS of *S. cerevisiae* by increasing AGE modification of proteins and intracellular ROS. It can be expected that inhibition of glycation may prolong the lifespan as it restores protein structure and biological function which results into normal functioning of the cell[134]. Glycation inhibitors are known to reduce protein glycation and intracellular accumulation of AGEs. AMG[135] and pyridoxamine[136] are considered as potent carbonyl scavengers that prevent formation of AGEs. AMG showed a strong ability to react with dicarbonyl intermediates formed during glycation process. Some AGE inhibitors, such as pioglitazone, MET (anti-hyperglycemic drug) and pentoxifylline (used for treatment of diabetes- induced peripheral vascular diseases) were also reported to show moderate inhibitory effects at the early stage of glycation[137]. AMG[113], Benfotiamine[138], Carnosine[139], Tenilsetam[140] and a natural product, resveratrol[141] are reported to reduce intracellular AGEs[142]. In this study, we have showed that AMG extends CLS of *S. cerevisiae* in NR condition by decreasing AGE modification of many of the important cellular proteins such as alcohol dehydrogenase 1 (adh1), fructose-1,6-bisphosphate aldolase (alf), Enolase 1 and 2(eno1 and 2), ATP synthase b subunit (atpb) and pyruvate decarboxylase (pdc1). These proteins were also observed to be carbonylated in NR [143]. Glyceraldehyde 3 phosphate dehydrogenase 3 (g3p3), elongation factor 1-alpha (tef1) and mitochondrial outer membrane protein porin1 (porin1) were oxidatively damaged in aged yeast cells

[144]. Decreased AGE modifications of cellular proteins is associated with increased lifespan of *C.elegans*[145].

2.5 Conclusion

Our study showed that increase in media glucose concentration shortens the yeast CLS by increasing AGE modification of proteins and levels of intracellular ROS. Further, AMG, a glycation inhibitor, is able to extends yeast CLS by reducing AGE modification of proteins. As glycation plays role in yeast aging, glycation induced aging can be studied using yeast as a model system.

Chapter 3

Effect of Glycation inhibitors on yeast chronological lifespan at proteomic level

Contents of this chapter are published as a research article in Journal of Proteomics, 2017, vol-156, 104-112.

Chapter 3 Effect of glycation inhibitors on yeast chronological lifespan at proteomic level

3.1 Background

Glycation mainly affects structure and function of proteins. As protein is an ultimate functional unit of a cell, effect of glycation on CLS of *S.cerevisiae* can be studied through differential or quantitative proteomics. For proteomics analysis, two main LC-MS/MS strategies used so far are Shotgun proteomics and targeted proteomics [146]. Shotgun proteomics is used for discovering the maximal number of proteins from one or a few samples. But it has limited quantification capabilities on large sample sets because of irreproducible precursor ion selection [147]. In contrast, targeted proteomics can be used for the reproducible detection and accurate quantification of sets of specific proteins in many samples. However, it lacks the throughput to quantify large fractions of a proteome [148]. To alleviate limitations of either method, Data independent acquisition (DIA) strategy has been developed termed as SWATH-MS. SWATH-MS aims to complement traditional mass spectrometry-based proteomics techniques such as shotgun and SRM methods. It allows a complete and permanent recording of all fragment ions of the detectable peptide precursors present in a biological sample. It thus combines the advantages of shotgun (high throughput) with those of SRM (high reproducibility and consistency). SWATH-MS is done in two steps 1) DIA acquisition to generate spectral library and 2) SWATH acquisitions for quantification.

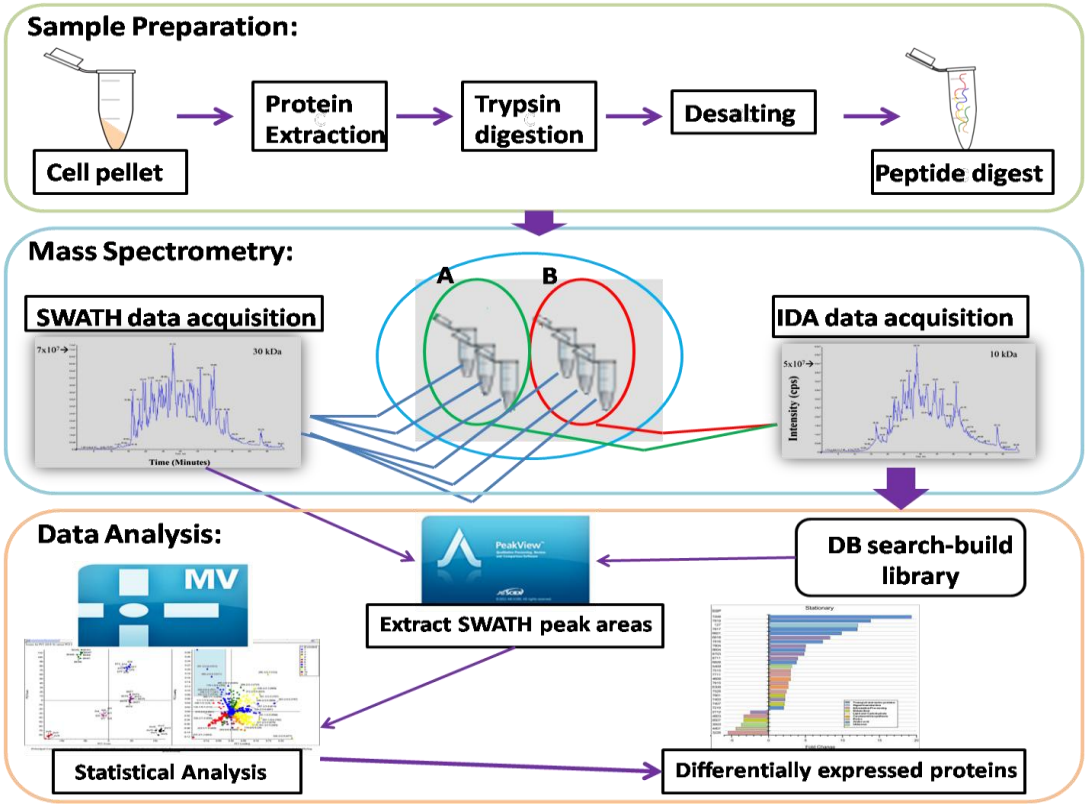


Figure 3.1 SWATH-MS workflow.

We have used SWATH-MS workflow (Figure 3.1) to study differential proteomics in presence of glycation inhibitors.

3.2 Materials and methods

3.2.1 Strains and Chemicals Used

S. cerevisiae strain BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) (life technologies, CA, USA) was used for all experiments. Cell stocks were maintained in YPD agar medium containing 0.5% yeast extract, 1% peptone, 2% agar, and 2% glucose. All experiments were performed in synthetic complete (SC) medium containing glucose (Himedia) as a carbon source and 0.67% yeast nitrogen base without amino acids (Sigma) supplemented with excess amino acids (Himedia). Cells grown in SC medium containing 0.5% glucose are referred as Calorie Restricted cells whereas cells grown in 2% glucose are referred as Non Calorie Restricted cells. Drugs used were AMG (25mM) (Sigma cat#396494), HYD (25mM) and MET (25mM). HYD and MET were generous gift from Dr. M. K. Gurjar, Director (R and D), Emcure, Pharmaceuticals Ltd. All chemicals were procured from Sigma unless otherwise stated.

3.2.2 Chronological Lifespan

Starter culture was inoculated from plate and grown in SC medium containing 0.5% or 2% of glucose and then diluted into fresh SC medium with the same concentration of glucose to obtain OD of ~0.1 at A_{600}(~1:100 dilution) with and without drug. These cultures were then incubated at 30°C with shaking at 200 rpm. Cultures reached stationary phase at day 3 and this was considered as day 0 of CLS. Cellular viability was assessed at indicated days using quantitative plating experiment and by 10-fold serial dilutions spot test[109]. Quantitative plating experiment was done by counting colony-forming units (CFUs) assay [124]. Briefly, cell number was estimated by measuring the absorbance at 600 nm. The serially diluted cultures were plated onto 3 YPD agar plates at a cell density of 100 cells per plate. Plates were incubated at 30 °C for 2days and number of colonies was counted. Cell viability was recorded from 3rd day onwards at an interval of 2 to 3 days. The cell viability was determined by considering viability on the 3rd day as 100% [38, 125]. CLS assay was repeated for three times in independent experiments.

3.2.3 Western blotting

Yeast protein extraction was performed by enzymatic disruption method. Briefly, 15 units of Zymolyase (GBiosciences) was added to 100mg of cell pellet and incubated at 37 °C for 1 h. Proteins were extracted from speroblast formed in extraction buffer (8M Urea,2M Thiourea,1%DTT,4%CHAPS). Protein concentration was determined using the Bio-Rad Bradford assay kit. Proteins (40 µg protein per lane) were separated on 12% SDS-PAGE and transferred to polyvinylidine fluoride membranes using the Mini Trans-Blot system (Bio-Rad). Total proteins were stained with Ponceau S solution [0.5% (w/v) Ponceau S in 1% v/v glacial acetic acid] to confirm protein transfer. The membranes were incubated overnight at 4°C in blocking buffer containing 5% (w/v) skimmed milk in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2mM KH_2PO_4, pH 7.5). For CML detection, PVDF membrane was probed with Anti-CML antibody (1:1000) (Abcam, Cambridge, UK)), for 3 h. After one wash with PBS-T (PBS containing 0.05% Tween 20) and two washes with PBS, the membrane was incubated with secondary antibody conjugated with HRP (Bangalore Genei) at a dilution of 1:2000 for 1h at room temperature. After washing, Chemiluminescence detection was done using the WesternBright^{TM} Quantum Western Blotting detection Kit (Advansta) following the manufacturer's instructions. Quantitative analysis was performed by Image studio Lite -LI CORE. Each immunoblot was repeated three times in independent experiments.

3.2.4 In-solution Trypsin Digestion

100µg of total protein mass per sample in 0.1% Rapigest (Waters) was used for digestion. Proteins were denatured by incubating at 80°C for 15min. Further, Proteins were reduced and alkylated by 100mM dithiothreitol for 15min at 60°C and 200mM Iodoacetamide for 30min at room temperature respectively. Trypsin (1:25) was added to it and incubated at 37°C for 18 h. Trypsin digestion was stopped by adding formic acid.

3.2.5 Desalting of peptide digest

Digested peptides were cleaned up or desalted by means of C18 chromatography using Zip tips (Millipore, Billerica, MA). Firstly, C18 Zip tips were activated using

100% MS grade ACN. Next step is column equilibration which is done using 0.1% TFA in MS grade water. Further, peptide digest was aspirated 3-4 times so that peptides will bind o C18 column. C18 Bound peptides were cleaned up by washing column 2-3 times with 0.1%TFA water. Lastly, peptides were eluted in 0.5%FA+50%ACN and concentrated using vacuum concentrator. Peptides were reconstituted in 3%ACN with 0.1%formic Acid.

3.2.6 Liquid Chromatography-Mass Spectrometry Analysis— SWATH MS Analysis

All samples were analyzed on Triple- TOF 5600 (AB Sciex; Concord, Canada) mass spectrometer coupled with Micro LC 200 (Eksigent; Dublin, CA) in high-sensitivity mode. To generate the SWATH spectral library equal amounts of peptide samples from each treatment were pooled together and analyzed via LC-MS/MS (Information dependent acquisition IDA). Accumulation time for MS was 0.25ms and for MS/MS was 0.01ms. Fragmentation was done using rolling collision energy.

SWATH MS datasets were acquired (in biological duplicates and technical duplicates) on Micro LC-Triple TOF 5600, briefly peptides were directly injected onto a Eskigent C18-RP HPLC column (100×0.3mm, 3µm, 120 Å) and then separated using a 95-min gradient of 3% to 35% mobile phase (Mobile phase A: 100% water with 0.1% (v/v) formic acid, mobile Phase B: 100% acetonitrile with 0.1% (v/v) formic acid) at a flow rate of 8 µL/min. In SWATH-MS mode, the instrument was specifically tuned to optimize the quadrupole settings for the selection of precursor ion selection windows 25 m/z wide. Using an isolation width of 26 m/z (containing 1 m/z for the window overlap), a set of 34 overlapping windows was constructed covering the precursor mass range of 400–1250 m/z. SWATH MS/MS spectra were collected from 100 to 2000 m/z. The collision energy was optimized for each window according to the calculation for a charge 2+ ion centered upon the window with a spread of 15eV. An accumulation time (dwell time) of 70 ms was used for all fragment-ion scans in high-sensitivity mode, and for each SWATH-MS cycle a survey scan in high-resolution mode was also acquired for 100 ms resulting in a duty cycle of 3.4 s [146, 149].

To get spectral library from IDA run, data was analyzed by Protein Pilot software 5.0 using UniProt *Saccharomyces cerevisiae* database updated with Uniprot release 2011_5 at the end of December 2014 containing 7851 reviewed protein entries. Generated spectral library was used as database for SWATH analysis with 20ppm mass error. SWATH runs were uploaded in swath processing window with 50 ppm error, 5min mass window and 99% confidence. SWATH file was exported to Marker View which gives quantitative analysis of proteins, peptides and ions in different samples.

3.2.7 Gene Ontology Analysis

DAVID: *S.cerevisiae* uniprot accessions of differentially expressed proteins were uploaded to the bioinformatics tool DAVID[127] to look for functional annotation. Functional clustering was performed with high stringency. Pathway enrichment was also determined using DAVID.

BINGO: Gene ontology clustering analysis for the deregulated proteins was performed using BINGO (Biological Network Gene Ontology) plug-in on Cytoscape 3.2, open source network visualization software. Significantly overrepresented functional categories from GOSlim_Yeast were determined by applying hypergeometric test and multiple test correction to obtain p- value ≤ 0.05 using Benjamin Hochberg FDR correction algorithm inbuilt in the BINGO software.

3.2.8 Quantification of Reactive Oxygen Species

Intracellular ROS levels were measured using 2', 7'-dichlorodihydrofluorescein diacetate (H2DCFDA) (Molecular Probes) [126]. Briefly, aliquots were taken at selected time points and 10 μM H2DCF-DA was added and incubated for 90 min at 30 °C. Cells were then washed twice in PBS and analyzed by Flow Cytometry [38] using BD Accuri C6 flow cytometer equipped with a 50 mW Argon laser. The emission of green fluorescence was monitored using FL1 channel. Data acquired from a minimum of 25,000 events per sample at medium flow rate were analyzed using BD Accuri C6 software. H2DCF-DA fluorescence is a direct output of FL1 channel without compensation.

3.2.9 Mitochondrial functional analysis by Antimycin A

Starter culture was grown in SC medium containing 0.5% or 2% of glucose. After 16 h, the culture was diluted into fresh SC medium with the same concentration of glucose to obtain OD of ~0.1 at A_{600} (1:100 dilution) with and without drug. These cultures were supplemented with 50 µM antimycin A (Sigma- Aldrich) [124] at stationary phase and then incubated at 30°C with shaking at 200 rpm. Cultures without antimycin A were supplemented with equal volume of drug vehicle (ethanol) as a control. After 3 days, these cultures were used to assess cellular viability by 10-fold serial dilution spot assay.

3.2.10 Statistical analysis

All experiments were performed either in triplicates or duplicates. Statistical analysis was performed by Student's t test and two way ANOVA. Data were expressed as mean ±SEM. A p value < 0.05 was considered as statistically significant. Number of matching peptides ≥ 2 and 1.5 fold change difference of protein expression (biological duplicates were acquired in duplicate).

3.3 Results

3.3.1 Aminoguanidine back regulated expression of differentially expressed proteins in non-CR condition

SWATH-MS based proteomic analysis was performed to understand the molecular mechanisms of AMG induced extension of yeast CLS in NR condition. All replicate acquisitions of different treatments are clustered together in PCA suggesting the reproducibility of replicate acquisitions (2 biological and 3 technical) (Figure 3.2). Spectral library had about 1500 proteins (Supplementary data S2 and S3). Using this spectral library, 59% of proteins were quantified with 4 peptides or more and 76% of proteins quantified with 2 peptides or more. SWATH analysis revealed expression of 207 up regulated proteins and 152 down regulated in NR with respect to CR (Figure 3.3A) (Appendix 1). These differentially expressed proteins were involved in various biological processes as shown in Figure 3.3 B. Briefly, most of the up regulated proteins are involved protein folding, ribosome biogenesis, stress response etc. While down regulated proteins are involved in cellular respiration, carbohydrate metabolism, generation of precursor metabolites and energy etc.

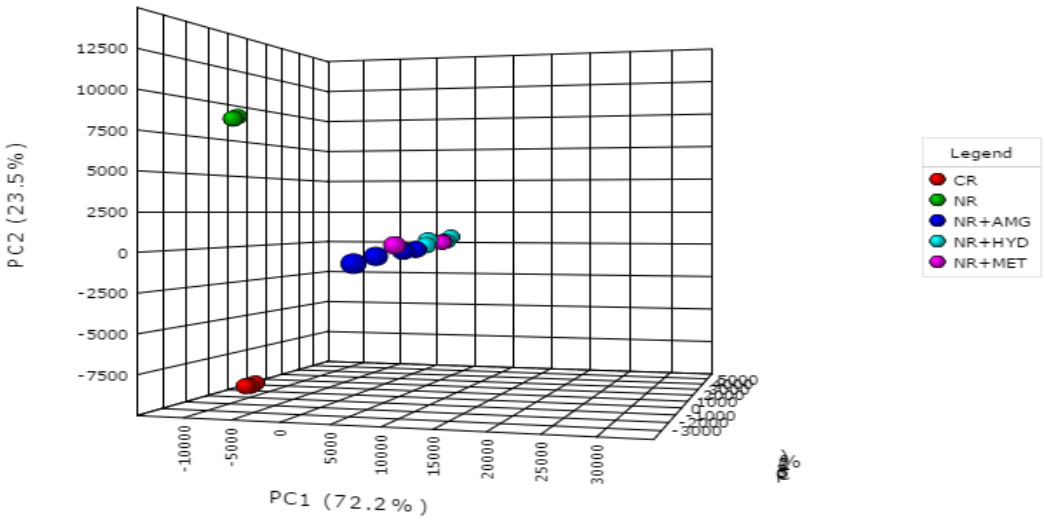


Figure 3.2 PCA plot
All replicate acquisitions of different treatments are clustered together suggesting reproducibility of replicate acquisitions.

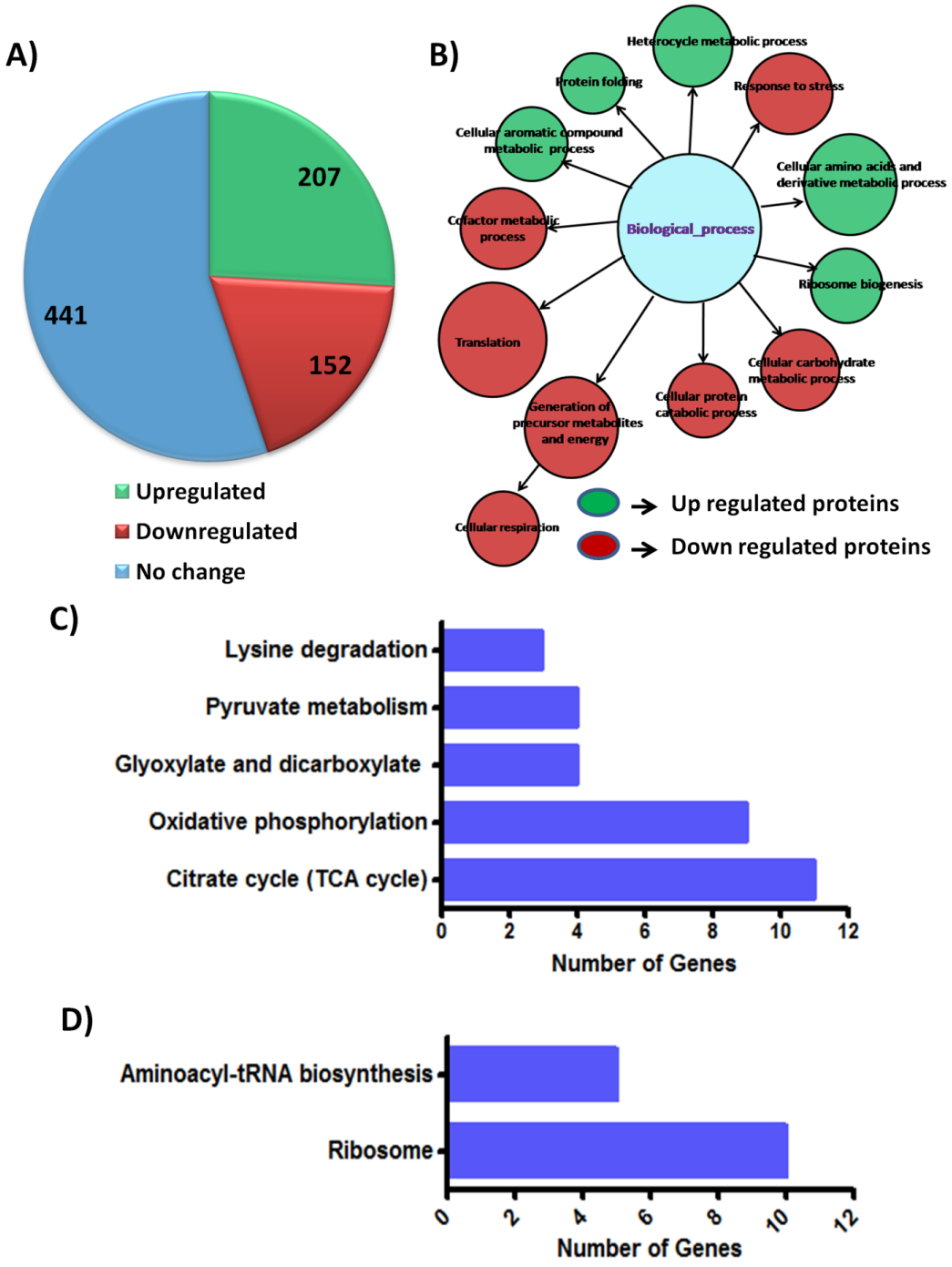


Figure 3.3 AMG back regulates the deregulated proteins in NR.
SWATH-MS based proteomics analysis was performed to understand the effect of AMG on yeast. A) Number of differentially expressed proteins in NR with respect to CR; B) Biological processes of differentially expressed proteins in NR with respect to CR analyzed by Cytoscape; C) Pathway analysis of AMG induced restoration of expression of down regulated proteins in NR; and D) Pathway analysis of AMG induced restoration of expression of up regulated proteins in NR (Pathways represented in bar graph are with $P< 0.05$). Two biological replicates and two technical replicates for SWATH-MS were performed. All proteins considered with $P< 0.05$.

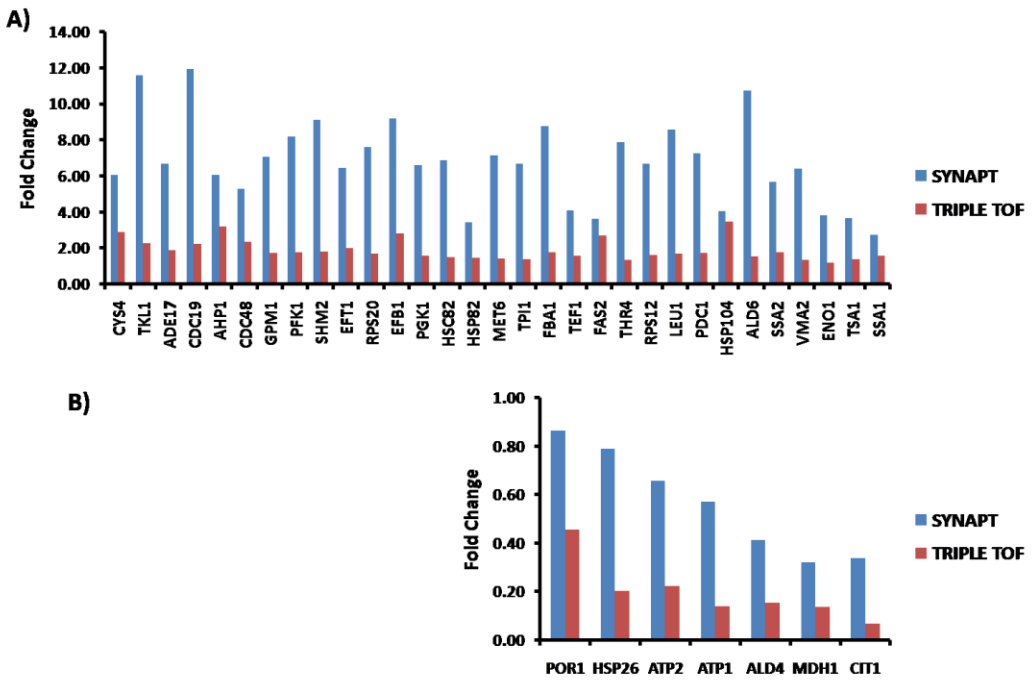


Figure 3.4 Corroboration of Triple TOF data with LC-MSE data
Proteins are showing same trend of differential expression in both the data.

The differential expression of proteins was also corroborated with LC-MS^E analysis, another label free quantitative mass spectrometric approach (Figure 3.4). AMG showed back regulation of many of the differentially expressed proteins in NR condition i.e. restored the level of expression similar to that observed in CR (Appendix 1). Pathway analysis of back regulated proteins by DAVID server revealed that AMG restored expression of 72 up-regulated proteins which are involved in protein synthesis (Figure 3.3D, Table 3.1). AMG restored the expression of 55 down regulated proteins which are involved in mitochondrial respiration, glyoxylate and dicarboxylate metabolism, pyruvate metabolism, lysine degradation (Figure 3.3C, Table 3.2). AMG back regulates expression of HXK1 and PGM2 which are very important not only in glycolysis but also in pentose phosphate pathway. AMG restored the expression of proteins involved in TCA cycle like CIT1, KGD1-2, MDH1, SDH1, IDH1-2 and FUM1, oxidative phosphorylation such as QCR2, ATP1, INH1, ATP5, TIM11, COX4, ATP7, SDH1, SDH2, ATP4 and ATP14. Whereas it reduced the expression of glycolytic proteins like PDC1, PFK1 and TPI1 suggesting decreased fermentation and increased respiration. AMG also decreased the expression of protein synthesis machinery which contained ribosomal subunits, amino acyl t-

RNA and translation factors. Presence of AMG increased the expression of proteins involved in glyoxylate pathway (e.g. CIT2, IDP1-2, ICL1, and MLS1) which suggest decreased methylglyoxal toxicity.

Table 3.1 List of upregulated proteins in NR showing back regulation on AMG treatment

Accession	Protein Description	Gene Name	2%GLU	2%AMG
			FOLD CHANGE	
P06169	Pyruvate decarboxylase isozyme 1	PDC1	1.49	0.95
P07264	3-isopropylmalate dehydratase	LEU1	1.55	1.45
P0CX44	60S ribosomal protein L1-B	RPL1B	1.55	0.98
P19097	Fatty acid synthase subunit alpha	FAS2	1.59	0.64
P02994	Elongation factor 1-alpha	TEF1	1.61	1.40
P29311	Protein BMH1	BMH1	1.62	1.43
P00942	Triosephosphate isomerase	TPI1	1.63	1.19
P05740	60S ribosomal protein L17-A	RPL17A	1.67	1.35
Q03148	Pyridoxine biosynthesis protein SNZ1	SNZ1	1.69	0.73
P02406	60S ribosomal protein L28	RPL28	1.69	1.63
P46655	Glutamate--tRNA ligase, cytoplasmic	GUS1	1.71	1.70
P32861	UTP--glucose-1-phosphate uridylyltransferase	UGP1	1.73	1.35
P39954	Adenosylhomocysteinase	SAH1	1.73	1.54
P02829	ATP-dependent molecular chaperone HSP82	HSP82	1.73	1.73
P15108	ATP-dependent molecular chaperone HSC82	HSC82	1.75	1.71
P38911	FK506-binding nuclear protein	FPR3	1.77	1.64
P21264	Phosphoribosylaminoimidazole carboxylase	ADE2	1.77	1.32
P05030	Plasma membrane ATPase 1	PMA1	1.82	1.64
Q01560	Nucleolar protein 3	NPL3	1.82	1.61
P10080	Single-stranded nucleic acid-binding protein	SBP1	1.83	1.82
P32836	GTP-binding nuclear protein GSP2/CNR2	GSP2	1.87	1.48
P14127	40S ribosomal protein S17-B	RPS17B	1.95	1.69
P38219	Obg-like ATPase 1	OLA1	1.96	1.91
P05739	60S ribosomal protein L6-B	RPL6B	2.01	1.75
P09436	Isoleucine--tRNA ligase, cytoplasmic	ILS1	2.03	1.61
P19358	S-adenosylmethionine synthase 2	SAM2	2.06	1.84
P10659	S-adenosylmethionine synthase 1	SAM1	2.06	1.85
Q02326	60S ribosomal protein L6-A	RPL6A	2.08	1.90
P27476	Nuclear localization sequence-binding protein	NSR1	2.12	1.94
Q06252	Uncharacterized protein YLR179C	YLR179C	2.13	1.94
P39015	Suppressor protein STM1	STM1	2.13	1.49
P32324	Elongation factor 2	EFT1	2.15	1.94
P0CX40	40S ribosomal protein S8-B	RPS8B	2.15	1.75
P37291	Serine hydroxymethyltransferase, cytosolic	SHM2	2.16	2.09
P16861	6-phosphofructokinase subunit alpha	PFK1	2.18	2.13
P04147	Polyadenylate-binding protein, cytoplasmic and nuclear	PAB1	2.18	1.94
Q3E757	60S ribosomal protein L11-B	RPL11B	2.18	1.76
P0CX54	60S ribosomal protein L12-B	RPL12B	2.19	1.69
P34223	UBX domain-containing protein 1	SHP1	2.19	1.57
P53334	Probable family 17 glucosidase SCW4	SCW4	2.20	2.10
P38999	Saccharopine dehydrogenase [NADP(+), L-glutamate-forming]	LYS9	2.24	1.83
P38711	40S ribosomal protein S27-B	RPS27B	2.26	2.14

Accession	Protein Description	Gene Name	2%GLU	2%AMG
			FOLD CHANGE	
P40825	Alanine--tRNA ligase, mitochondrial	ALA1	2.26	1.72
P40054	D-3-phosphoglycerate dehydrogenase 1	SER3	2.28	2.27
P0C2H7	60S ribosomal protein L27-B	RPL27B	2.37	2.05
P38013	Peroxiredoxin type-2	AHP1	2.37	2.02
P27616	Phosphoribosylaminoimidazole-succinocarboxamide synthase	ADE1	2.39	2.23
P42222	Enolase-related protein 3	ERR3	2.49	2.18
P0CX50	60S ribosomal protein L18-B	RPL18B	2.55	2.01
P15625	Phenylalanine--tRNA ligase alpha subunit	FRS2	2.57	2.04
P38061	60S ribosomal protein L32	RPL32	2.58	2.27
P38009	Bifunctional purine biosynthesis protein ADE17	ADE17	2.62	2.07
P54839	Hydroxymethylglutaryl-CoA synthase	ERG13	2.63	2.53
P0CX24	60S ribosomal protein L20-B	RPL20B	2.64	2.31
P38011	Guanine nucleotide-binding protein subunit beta-like protein	ASC1	2.77	2.56
P29952	Mannose-6-phosphate isomerase	PMI40	2.89	2.74
P87262	60S ribosomal protein L34-A	RPL34A	2.90	2.18
P38817	ADP-ribosylation factor-binding protein GGA2	GGA2	2.93	2.48
Q12485	Transposon Ty1-GR2 Gag polyprotein	TY1A-GR2	3.01	2.68
P39939	40S ribosomal protein S26-B	RPS26B	3.11	1.91
Q12470	Transposon Ty1-NL2 Gag polyprotein	TY1A-NL2	3.20	2.81
P32582	Cystathionine beta-synthase	CYS4	3.25	2.46
P34167	Eukaryotic translation initiation factor 4B	TIF3	3.25	2.49
P15180	Lysine--tRNA ligase, cytoplasmic	KRS1	3.38	3.03
P41807	V-type proton ATPase subunit H	VMA13	3.64	1.77
P16521	Elongation factor 3A	YEF3	4.34	3.64
P07263	Histidine--tRNA ligase, mitochondrial	HTS1	4.51	3.38
P0CX26	60S ribosomal protein L43-B	RPL43B	4.96	3.84
P39976	D-lactate dehydrogenase [cytochrome] 3	DLD3	5.85	3.55
P32503	Major capsid protein	gag	6.03	5.34
P07262	NADP-specific glutamate dehydrogenase 1	GDH1	6.13	4.14
P33399	La protein homolog	LHP1	6.86	4.69

Table 3.2 List of downregulated proteins in NR showing back regulation on AMG treatment

Accession	Protein Description	Gene Name	2%GLU	2%AMG
			FOLD CHANGE	
P29704	Squalene synthase	ERG9	1.74	1.56
P22137	Clathrin heavy chain	CHC1	1.76	1.59
P33416	Heat shock protein 78, mitochondrial	HSP78	1.99	1.81
P32352	C-8 sterol isomerase	ERG2	2.29	1.84
P04806	Hexokinase-1	HXK1	2.32	2.27
P07267	Saccharopepsin	PEP4	2.41	2.35
P36010	Nucleoside diphosphate kinase	YNK1	2.71	2.67
P14904	Vacuolar aminopeptidase 1	LAP4	2.77	2.30
Q00764	Alpha,alpha-trehalose-phosphate synthase [UDP-forming] 56 kDa subunit	TPS1	2.81	1.85
P04912	Histone H2A.2	HTA2	2.89	1.84
P23542	Aspartate aminotransferase, cytoplasmic	AAT2	3.11	2.99
P02992	Elongation factor Tu, mitochondrial	TUF1	4.28	1.66
P04840	Mitochondrial outer membrane protein porin 1	POR1	4.34	2.40
P12695	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	LAT1	4.69	3.10
Q3E841	Uncharacterized protein YNR034W-A	YNR034W-A	4.70	2.29

Accession	Protein Description	Gene Name	2%GLU	2%AMG
			FOLD CHANGE	
Q02486	ARS-binding factor 2, mitochondrial	ABF2	4.71	3.36
P38071	Enoyl-[acyl-carrier protein] reductase [NADPH, B-specific], mitochondrial	ETR1	4.91	3.13
P28834	Isocitrate dehydrogenase [NAD] subunit 1, mitochondrial	IDH1	4.94	4.94
P08417	Fumarate hydratase, mitochondrial	FUM1	4.98	4.45
P02309	Histone H4	HHF1	5.41	3.85
P00431	Cytochrome c peroxidase, mitochondrial	CCP1	5.58	3.23
P28241	Isocitrate dehydrogenase [NAD] subunit 2, mitochondrial	IDH2	6.03	5.30
Q04947	Reticulon-like protein 1	RTN1	6.32	3.00
P20967	2-oxoglutarate dehydrogenase, mitochondrial	KGD1	6.55	4.29
P07251	ATP synthase subunit alpha, mitochondrial	ATP1	6.68	6.55
P19262	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	KGD2	6.84	3.54
P46367	Potassium-activated aldehyde dehydrogenase, mitochondrial	ALD4	7.22	5.93
P07257	Cytochrome b-c1 complex subunit 2, mitochondrial	QCR2	7.35	6.26
P28240	Isocitrate lyase	ICL1	7.56	4.80
P05626	ATP synthase subunit 4, mitochondrial	ATP4	7.78	4.44
P30902	ATP synthase subunit d, mitochondrial	ATP7	7.83	5.30
P21801	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial	SDH2	7.85	6.39
Q12349	ATP synthase subunit H, mitochondrial	ATP14	7.99	6.98
P09457	ATP synthase subunit 5, mitochondrial	ATP5	8.68	3.91
P16547	Mitochondrial outer membrane protein OM45	OM45	9.43	7.94
P17505	Malate dehydrogenase, mitochondrial	MDH1	9.89	8.77
Q00711	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	SDH1	9.89	6.12
P32191	Glycerol-3-phosphate dehydrogenase, mitochondrial	GUT2	9.95	6.37
P81449	ATP synthase subunit e, mitochondrial	TIM11	10.18	7.56
P23641	Mitochondrial phosphate carrier protein	MIR1	10.35	6.39
P32796	Carnitine O-acetyltransferase, mitochondrial	CAT2	10.92	3.44
Q03104	Meiotic sister chromatid recombination protein 1	MSC1	11.03	4.08
P08679	Citrate synthase, peroxisomal	CIT2	11.81	9.78
P18239	ADP,ATP carrier protein 2	PET9	12.91	8.41
Q08179	Mitochondrial distribution and morphology protein 38	MDM38	13.84	13.39
P40185	Protein MMF1, mitochondrial	MMF1	13.92	11.88
P00890	Citrate synthase, mitochondrial	CIT1	14.31	5.46
P11632	Non-histone chromosomal protein 6A	NHP6A	15.11	14.19
P32316	Acetyl-CoA hydrolase	ACH1	15.29	6.09
P05749	60S ribosomal protein L22-A	RPL22A	18.06	2.70
Q12428	Probable 2-methylcitrate dehydratase	PDH1	19.97	6.28
A6ZRW6	Malate synthase 1, glyoxysomal	MLS1	20.74	7.61
P40466	Fork head protein homolog 1	FKH1	32.44	29.09
P33303	Succinate/fumarate mitochondrial transporter	SFC1	34.71	33.28
P01097	ATPase inhibitor, mitochondrial	INH1	98.71	84.15

3.3.2 Aminoguanidine induced back regulation of proteins is due to its glycation inhibition effect rather than its chemical effect

AMG induced back regulation of differentially expressed proteins could possibly due to its chemical effect or indirectly by glycation inhibition. To delineate this, proteomic analysis was performed in yeast cells treated with HYD, another potent glycation inhibitor but not an analogue of AMG, or MET, a structural analogue of AMG and a mild glycation inhibitor [150, 151] (Figure 3.5). Both HYD and MET were able to back regulate expression of deregulated proteins (Figure 3.6 A, B). However, HYD was more effective than MET in back regulating deregulated protein in NR (Supplementary data S4). DAVID analysis of back regulated proteins by all three molecules suggested that down regulated proteins were involved in mitochondrial respiration whereas up regulated were involved in protein synthesis (Figure 3.6 C, D, Supplementary data S4).

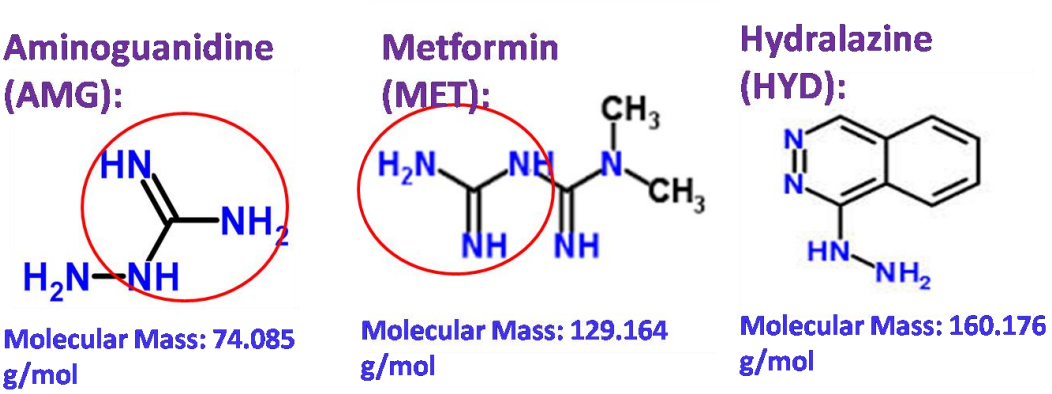


Figure 3.5 Structures of drug molecules used in study
Metformin is a structural analogue of Aminoguanidine and mild glycation inhibitor, whereas Hydralazine is a potent glycation inhibitor but not a structural analogue

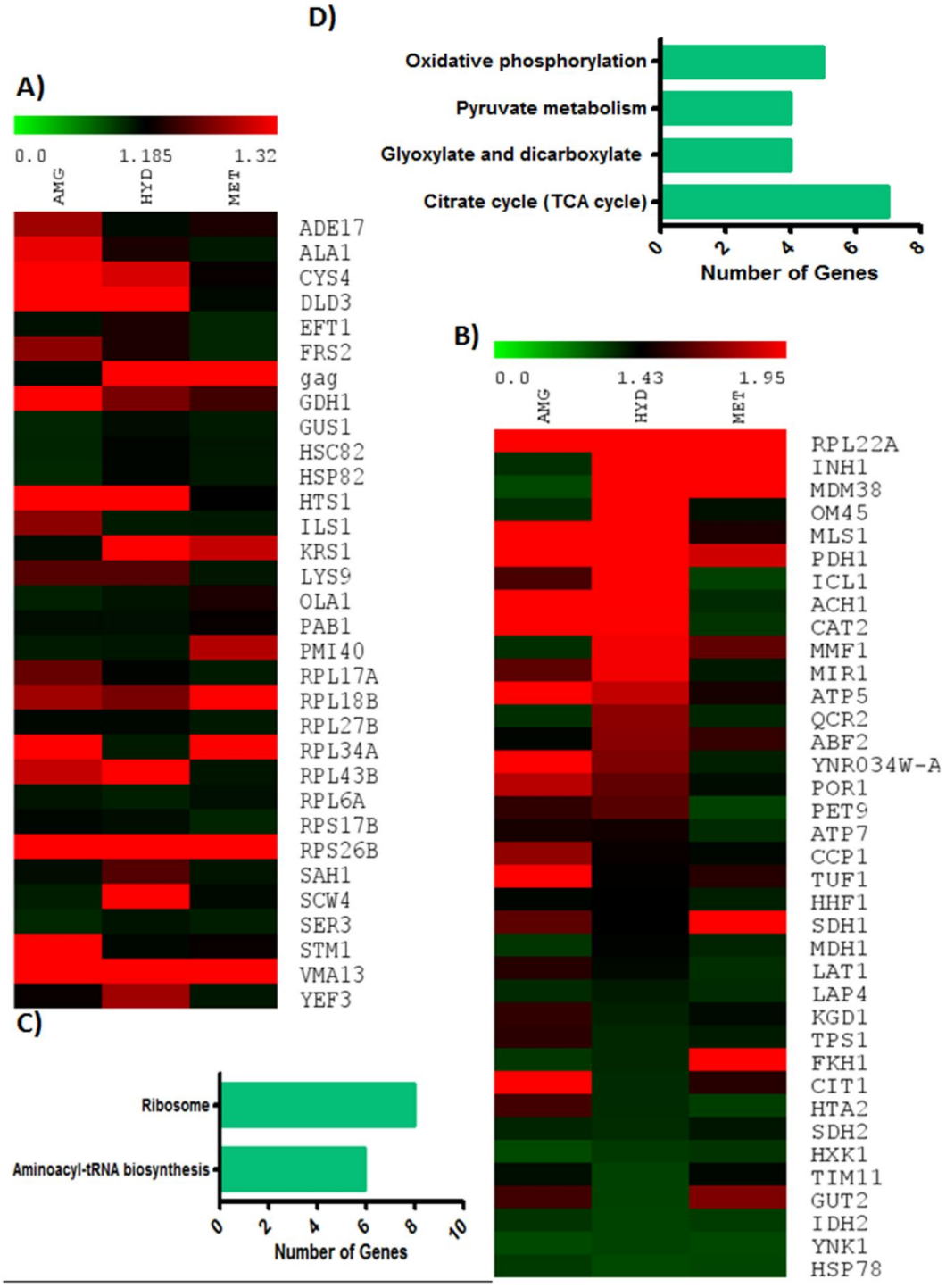


Figure 3.6 Glycation Inhibitors, AMG, MET and HYD back regulated expression of proteins mainly involved in mitochondrial respiration.
Expression Analysis was done by SWATH-MS. Heat map shows ratio of fold change of back regulated protein by AMG, MET and HYD in NR condition as shown in A) Up regulated proteins B) Down regulated proteins. Pathway Analysis of back regulated proteins by all three glycation inhibitors was performed using DAVID as shown in C) Up regulated proteins D) Down regulated proteins (Pathways represented in bar graph are with P< 0.05). Differentially expressed proteins in different conditions were compared with CR. Two biological replicates and two technical replicates for SWATH-MS were performed. All proteins considered with P< 0.05.

3.3.3 Glycation Inhibition by Aminoguanidine, Metformin and Hydralazine enhances mitochondrial respiration and CLS mimicking CR

AGE modification of proteins was analyzed by Western blotting using anti-CML antibody, HYD and MET showed reduced AGE modification along with AMG, albeit inhibition by MET was relatively milder (Figure 3.7A, B). AGEs are associated with elevated levels of ROS production. Therefore, we have evaluated the effect of these glycation inhibitors on the ROS production. Flow cytometry studies revealed that glycation inhibitors showed reduced levels of intracellular ROS similar to CR, as compared to NR (Figure 3.7 C). To confirm the role of mitochondrial respiration in lifespan extension by glycation inhibitors, culture media was supplemented with Antimycin A, a respiratory complex III inhibitor. AMG, MET and HYD were able to reduce the effect of Antimycin A, which inhibit electron transport chain and maintain the cell viability (Figure 3.7 D).

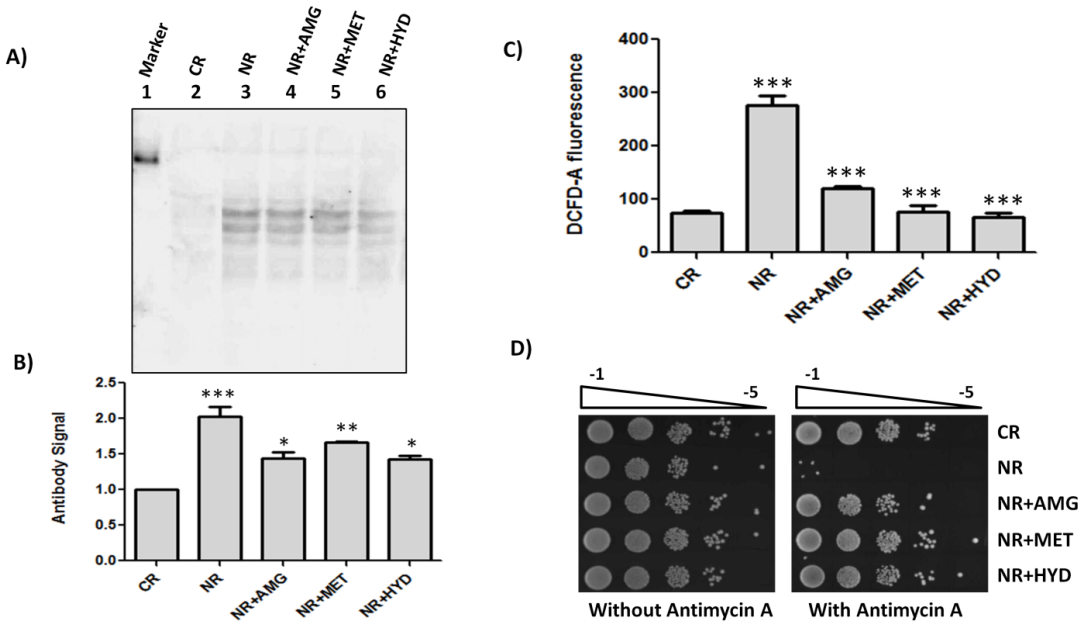


Figure 3.7 HYD and MET reduce AGE modification of proteins which affect ROS levels and mitochondrial respiration.

A) Western blot analysis was performed to detect AGE modifications in proteins extracted at day 3 in different conditions. B) Extent of modifications was represented densitometrically and the AGE modification of CR was considered as 1; C) ROS was quantified using 2', 7'-dichlorodihydrofluorescein diacetate (H2DCFDA) by flow Cytometry at day 3, fluorescence intensity represents ROS level; and D) 10-fold serial dilution spot assay was performed to check cell viability in different conditions with and without Antimycin A (50µM). Two biological replicates for western blot were performed. Three biological replicates for ROS assay and Antimycin A assay were performed. All values are mean ±SEM. Statistical significance (*P< 0.05, ** P<0.01 and ***P< 0.001) was calculated by student t-test.

The decrease in AGE modification and increase in mitochondrial respiration in terms of expression of mitochondrial proteins and ROS by glycation inhibitors was associated with increase in yeast CLS. All the three glycation inhibitors showed extension of CLS. AMG showed 45-50% increase, MET showed 20-25% increase and HYD showed 50-55% increase in yeast CLS (Figure 3.8 A, B).

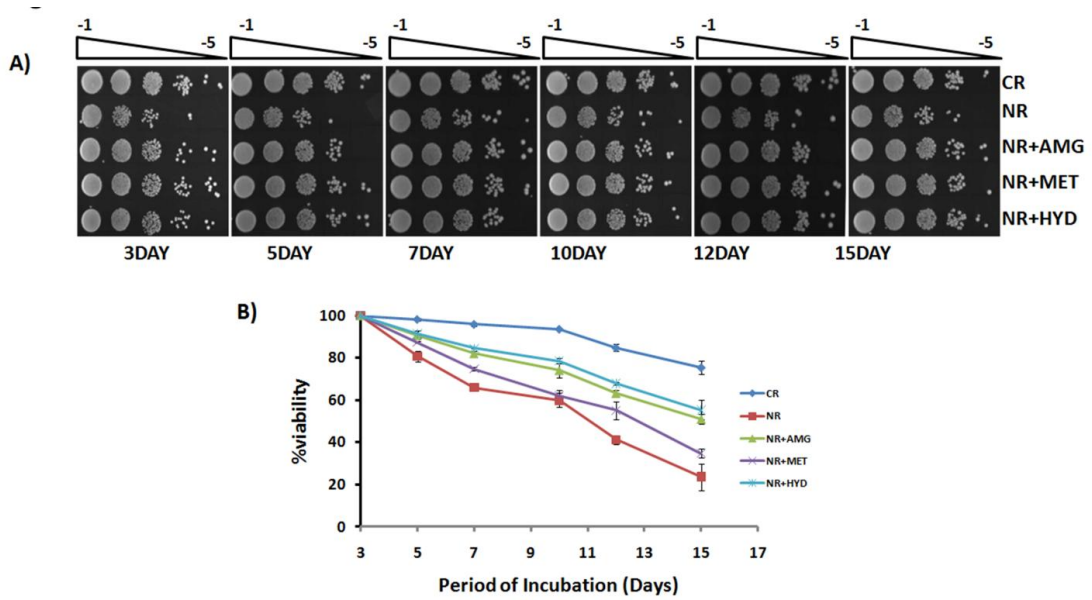


Figure 3.8 Glycation inhibitors extend yeast chronological lifespan.
Glycation inhibitors particularly AMG, MET and HYD extend yeast CLS in NR condition as observed by A) 10-fold serial dilution spot assay, where viability of yeast cells was observed on different days by inoculation of serially diluted yeast culture as a spot on YPD agar plate; and B) Quantitative CLS assay, where cell viability was measured as Colony Forming Unit (CFU) at 2-3 days interval beginning with the day when yeast culture was at stationary phase (day 3). The cell viability on different days was compared with viability on the day 3, which was considered as 100%. Values represent mean ±SEM of two biological replicates. Statistical significance was calculated by Two- way ANOVA (Interaction P< 0.001, Time P< 0.001, treatment P< 0.001).

3.4 Discussion

AMG extends CLS of *Saccharomyces cerevisiae* in NR condition by decreasing AGE modification of many cellular proteins. Apart from reducing AGE modification of proteins, AMG may enhance mitochondrial respiration, as in case of Metformin in type-2 diabetes [152]. Therefore, we performed SWATH-MS based differential proteomic analysis to understand the AMG induced extension of yeast CLS in NR condition. Non-caloric condition is known to induce transcriptional activation of many genes [153]. Glucose increases the expression of proteins involved in glycolysis, protein synthesis, de novo purine and amino acid biosynthesis [154], and decreases expression of proteins involved in TCA cycle, oxidative phosphorylation and gluconeogenesis [153, 155, 156]. Our proteomic analysis corroborates the previous studies in terms of glucose induced differential protein expression. Further, we compared our data with two previous studies, Giardina et al [155] and Kolkman et al [156] (Figure 3.9 and Supplementary data S5) using online tool Biovenn [157]. In the former study, using iTRAQ and MALDI approach 591 proteins were identified from yeast grown in glucose deficient medium and transferred to glucose rich medium for 2 h. While in the latter study, using SILAC approach 759 proteins were quantified in carbon and nitrogen limitation condition. With respect to our study, 205 proteins are common in all three studies, 60 proteins are common to former study, and 79 proteins are common to latter study. And there are 80 proteins unique to our study. Though the growth conditions in all three experiments are different, effect of glucose on overall proteome showed a similar pattern.

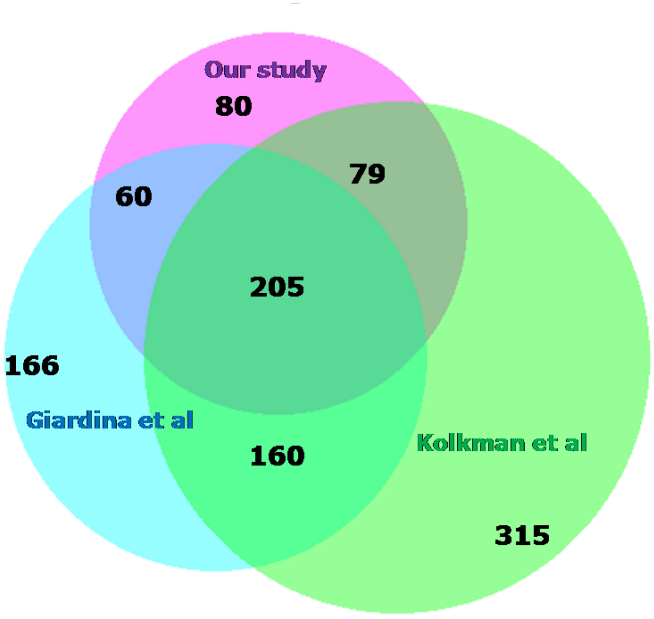


Figure 3.9 Comparison of differentially expressed proteins in our study with the previous study.
Differentially expressed proteins from our study were compared with previous studies by Giardiana et al and Kolkman et al using online tool Biovenn.

This analysis suggested that presence of glucose increases expression of proteins involved in glycolysis and decreases those involved in respiration. Glucose also increased the expression of proteins involved in protein synthesis [156]. Furthermore, we compared the proteomic profile with that of yeast grown in presence of glycation inhibitors. AMG treatment elicited changes at molecular level including back regulation of deregulated proteins in NR condition. Back regulated proteins are mainly involved in TCA cycle, oxidative phosphorylation and glyoxylate pathway. Activation of glyoxylate pathway may help in detoxification of methylglyoxal toxicity. AMG mimics CR in terms of shift in metabolism from fermentation to respiration. [131]. Furthermore, treatment of HYD, a potent glycation inhibitor and MET, structural analogue of AMG and a mild glycation inhibitor was able to reproduce AMG induced CLS. HYD was more effective than MET in reducing AGE modifications of proteins and back regulation of deregulated proteins. All three glycation inhibitors reduced ROS levels and rescued Antimycin A effect.

All these experiments suggested that AMG, HYD and MET induced CLS extension was perhaps through glycation inhibition. However, all these molecules comprise of

primary amine as one of the functional groups, as a result it is also possible these can scavenge glucose [158] and simulate CR condition, since the concentration used in this study was relatively higher. Nevertheless, these molecules also inhibited protein glycation as suggested by western blotting with anti-AGE antibodies, although the concentration required was relatively higher. A previous studies has also reported use of 100 mM AMG to reduce AGEs in the yeast [159]. Similarly another glycation inhibitor L-Carnosine at a concentration of 20mM enhanced reproductive potential of the yeast growing on medium containing glucose as a source of carbon [160]. It will be interesting to study glycation inhibition using molecules that lack ability to scavenge glucose or other reducing sugars.

3.5 Conclusion

In summary, our findings show that AMG, HYD, MET extends yeast CLS perhaps through glycation inhibition activity as well as scavenging of glucose. Treatment of glycation inhibitors, AMG, HYD, and MET, reduces AGE modification of cellular proteins, reduces ROS levels, back regulates deregulated proteins involved in mitochondrial respiration and possibly by scavenging glucose, which could facilitate shift of metabolism from fermentation to respiration and extends yeast CLS (Figure 3.10).

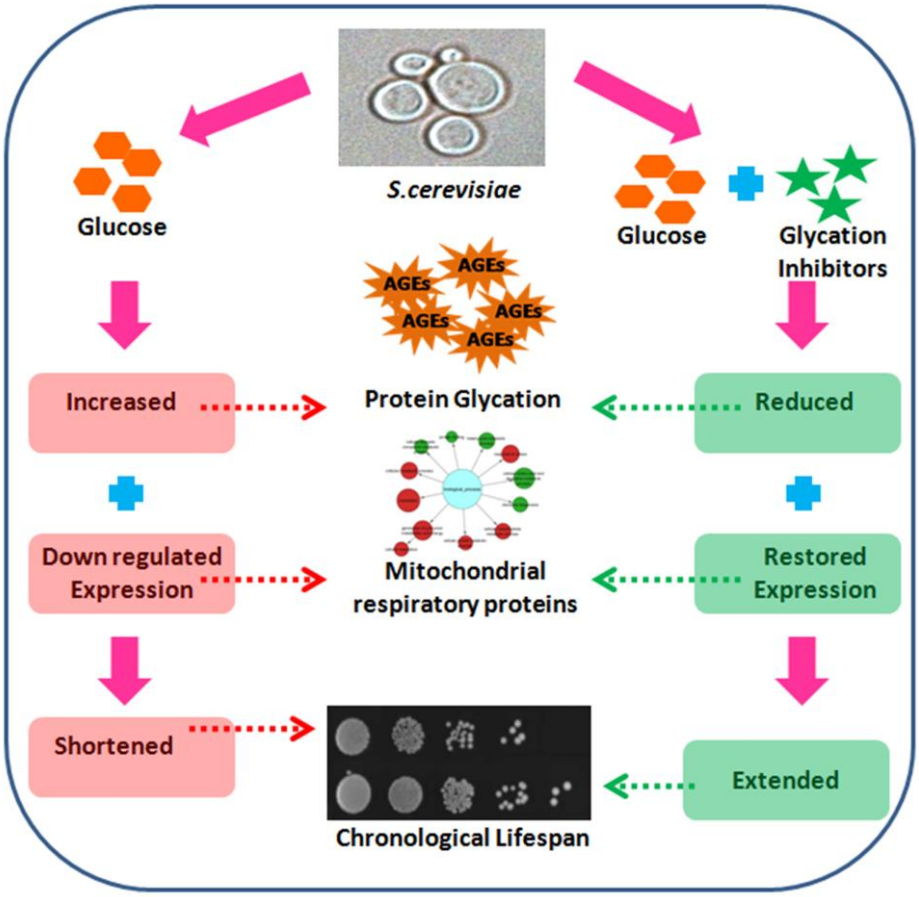


Figure 3.10 Summary
(Figure adapted from R. S. Kazi *et al*, Journal of proteomics, 2017)

Chapter 4

Effect of glycation inhibitors on genes involved in aging pathways

Chapter 4 Effect of glycation inhibitors on genes involved in aging pathway

4.1 Background

Aging is a multifaceted process caused by detrimental changes in many different cellular processes. Damage caused by increased ROS levels, dysfunctional mitochondria, and genomic instability seem to be common pro-aging factors [4, 161, 162]. Many pro- or anti aging cellular processes are the result of a core set of nutrient sensing and stress response pathways, conserved throughout eukaryotic phyla [163].

Dietary restriction (DR) or calorie restriction (CR) is one of the non genetic interventions which successfully extended lifespan in variety of model organisms in laboratory [29, 164]. Previous reports says that CR mediated longevity is evolutionary conserved from yeast to mammals [73, 165]. In yeast, longevity can be studied in terms of two lifespans; chronological lifespan (CLS) and replicative lifespan (RLS) [75]. CLS is survival time of a yeast cell in non-dividing state [166], whereas RLS is number of times a mother cell divides before senescence [107].

CR extends yeast CLS through inhibition of Ras/cAMP/PKA and TOR/Sch9 signaling [75, 128, 166-170]. Both of these pathways converge at protein kinase Rim15, which activate transcription factors such as Msn2/4 and Gis1 which regulate stress response [171-173]. A Transcriptome profile from different studies explains role of mitochondrial respiration in CR induced lifespan extension in yeast [110, 124, 174].

Transcriptome profiling helps in determining process of aging and how an intervention works. DNA microarray is one of the widely used methods for transcriptome study. It can measure mRNA levels of individual genes and helps to determine its expression at different condition [175]. Though mRNA levels cannot be directly correlated with protein levels, change in gene expression is immediate response of cell to any physiological change. Therefore, it helps to compute different cellular pathways working at different experimental conditions [176].

Apart from CR, few molecules were reported to extend yeast lifespan such as Resveratrol [177], Rapamycin [178] and Spermidine [27] through different signaling pathways. Resveratrol induce autophagy [179]. In addition to this, resveratrol is reported to inhibit AGEs also [141]. So, different glycation inhibitors may serve as anti-aging molecules. However, in previous chapter, we have already reported that three glycation inhibitors, AMG, MET and HYD, showed yeast CLS extension [180]. Indeed, there is need to compute its effect on aging pathways. In view of these, here we are studying transcriptome profile of yeast during CLS in presence of these three glycation inhibitors using microarray technique. Microarray data was validated using RT-PCR.

4.2 Materials and methods

4.2.1 Strains and Chemicals Used

S. cerevisiae strain BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) (life technologies, CA, USA) was used for all experiments. Cell stocks were maintained in YEPD agar medium containing 0.5% yeast extract, 1% peptone, 2% agar, and 2% glucose. All experiments were performed in synthetic complete (SC) medium containing glucose (Himedia) as a carbon source and 0.67% yeast nitrogen base without amino acids (Sigma) supplemented with excess amino acids (Himedia). Cells grown in SC medium containing 0.5% glucose are referred as Calorie Restricted cells whereas cells grown in 2% glucose are referred as Non Calorie Restricted cells. Drugs used were AMG (25mM) (Sigma cat#396494), HYD (25mM) and MET (25mM). HYD and MET were generous gift from Dr. M. K. Gurjar, Director (R and D), Emcure, Pharmaceutical. All chemicals were procured from Sigma unless otherwise stated.

4.2.2 Culture conditions

Starter culture was inoculated from plate and grown in SC medium containing 0.5% or 2% of glucose and then diluted into fresh SC medium with the same concentration of glucose to obtain OD of ~0.1 at A_{600}(~1:100 dilution) with and without drug. These cultures were then incubated at 30°C with shaking at 200 rpm. Cultures

reached stationary phase at day 3. At 3rd day cells were pelleted down and washed with PBS. Then, cells were resuspended in RNAlater solution (Thermo Fisher scientific) to stabilize RNA and kept at 4°C for 3-4 hrs. RNAlater solution was removed and cells were frozen till its use.

4.2.3 Microarray Analysis

Microarray was done by Genotype technology. In brief, total RNA for microarray analysis was harvested from triplicate frozen yeast pellet of CR, NR, NR+AMG, NR+MET and NR+HYD cultures at 3rd day using Qiagen mini kit with DNase treatment as per instructions. The gene expression profile of each sample was determined by using Affymatrix Yeast Genome Gene chip arrays. Pathway analysis of differentially expressed genes was done using DAVID software.

4.2.4 RNA extraction for RT-PCR

Total RNA from *S. cerevisiae* was extracted using RiboPure RNA Purification kit (ambion, cat.No.-AM1926) as per instructions at 3rd day of inoculation. Briefly pre-weighed yeast cell pellet was mixed with the lysis buffer along with 10% SDS and phenol:chloroform:IAA mixture followed addition of zirconia beads to allow complete lysis of the cells. It was vortexed thoroughly followed by centrifugation at 16,000 g at room temperature for 5 minutes. Aqueous layer containing RNA was collected into different tube. Binding buffer and molecular grade ethanol was added sequentially to the aqueous layer. The solution was passed through filter cartridge to allow RNA to bind to the column. Further, buffer washes were given to remove the impurities and RNA was eluted using pre-warmed elution buffer. RNA was measured by nanodrop spectrophotometer (Thermo scientific nanodrop 1000). Quality of RNA was checked on 1% agarose gel electrophoresis using gel red as an intercalating dye. To remove the DNA contamination, DNase I treatment was given. RNA was mixed with DNase I enzyme, buffer and incubated at 37^0C for 30 minutes. Reaction was stopped by addition of DNase inactivation agent. Samples were centrifuged to pellet down the inactivation agent and top aqueous layer containing RNA was taken forward for cDNA synthesis.

4.2.5 cDNA synthesis and Real-time PCR

Two micrograms of RNA was used for preparing cDNA using Applied Biosystems™ High Capacity cDNA Reverse Transcription kit (cat.no.-4368814). Quantitative real time PCR was performed using the Taqman gene expression master mix (cat.No.-4369016) and gene specific Taqman probes from Applied Biosystems™. Quantification was performed using 7900HT Fast Real-Time PCR System (Applied Biosystems, California, USA). All measurements were performed in biological and technical triplicates and conditions used were 95°C for 10min, denaturation at 95°C for 3 seconds followed by 30 seconds elongation at 60°C. TDH3 and TPI1 were used as the reference gene to calculate the relative expression of target genes. CT values were considered further for calculating the relative expression of genes which was normalised with the housekeeping gene. RQ manager software was used for CT values calculation.

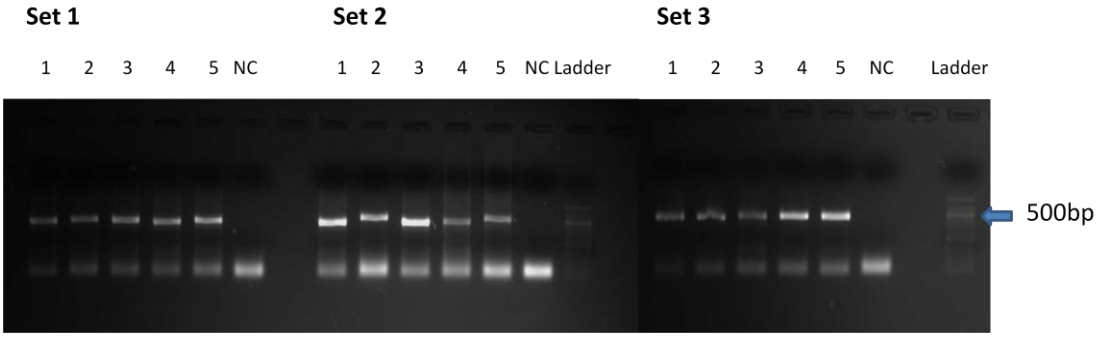


Figure 4.1 Agarose gel for cDNA synthesised

4.2.6 Gene Ontology Analysis

DAVID: *S.cerevisiae* gene names of differentially expressed genes were uploaded to the bioinformatics tool DAVID[127] to look for functional annotation. Functional clustering was performed with high stringency. Pathway enrichment was also determined using DAVID

4.2.7 Statistical analysis

All experiments were performed in triplicates. Statistical analysis was performed by one way ANOVA. Data were expressed as mean ±SEM. A p value < 0.05 was considered as statistically significant.

4.3 Results

4.3.1 Glycation inhibitors restore expression of differentially expressed genes in NR

Effect of glycation inhibitors on gene expression of *S.cerevisiae* during CLS was studied using microarray technique. Treatment of individual glycation inhibitors was given to yeast cells in NR condition. Microarray analysis was done on 3rd day of incubation, where cells are supposed to be in stationary phase. RNA extracted from three biological replicates was pooled together to average out variations [181] and used for microarray analysis. CR condition served as reference for other treatments. The analysis revealed that out of total 6225 genes in *S.cerevisiae* 2606 genes were upregulated, whereas 1738 genes were downregulated in NR condition (supplementary data S6). Treatment of glycation inhibitors restored the expression of 1716 upregulated genes (Figure 4.2A) and 1229 downregulated genes in NR (Figure 4.2B).

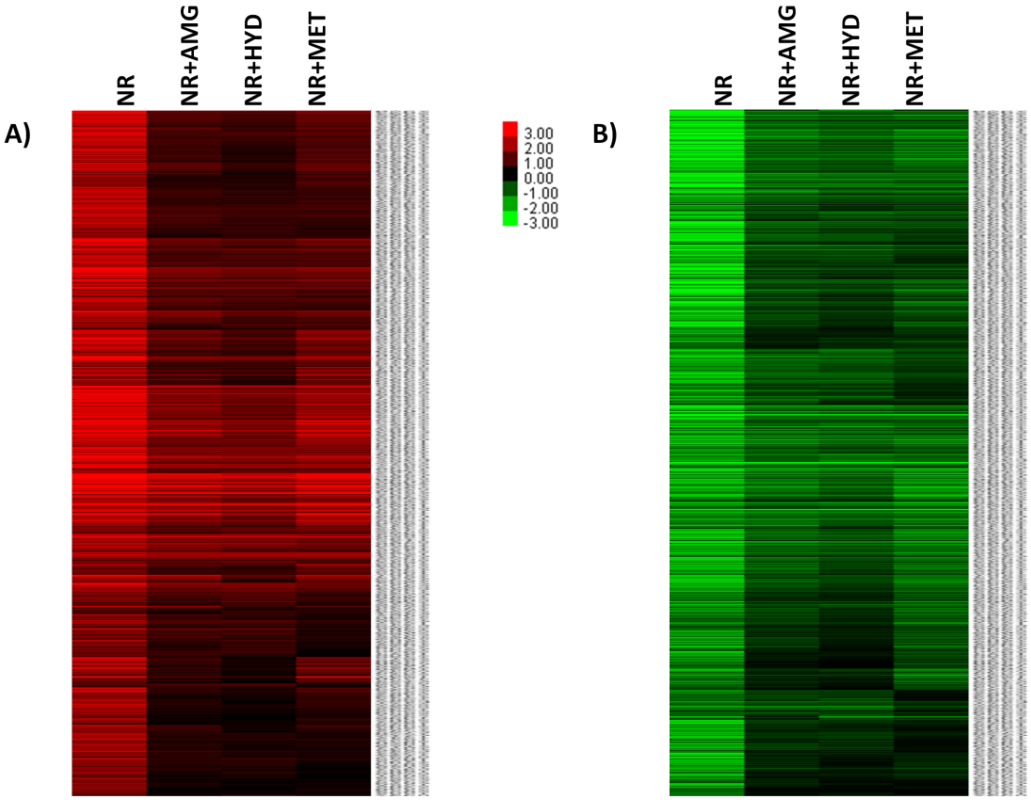


Figure 4.2 Genes showing restoration of expression in presence of glycation inhibitors.
A) Heat map of upregulated genes in NR and showing restoration in presence of glycation inhibitors. B) Heat map of downregulated genes in NR and showing restoration in presence of glycation inhibitors.

Pathway analysis using DAVID software suggested that upregulated genes in NR showing restored expression were ribosomal genes and many of them involved in metabolic pathways, oxidative phosphorylation, amino acid biosynthesis, etc (Table 4.1). Whereas, downregulated genes were from meiosis, cell cycle, mismatch repair, regulation of autophagy, etc (Table 4.2).

Table 4.1 Pathway analysis of Upregulated genes in NR showing restoration in presence of glycation inhibitors

Pathway	Number of genes	P-value
Ribosome	80	1.86E-08
Alanine, aspartate and glutamate metabolism	20	1.15E-05
Metabolic pathways	212	7.21E-05
Biosynthesis of secondary metabolites	103	1.22E-04
Oxidative phosphorylation	33	2.83E-04
2-Oxocarboxylic acid metabolism	19	7.86E-04
Biosynthesis of amino acids	46	3.20E-03
Endocytosis	28	1.18E-02
Biosynthesis of antibiotics	71	1.32E-02
Arginine biosynthesis	10	1.36E-02
Phagosome	16	2.34E-02
Butanoate metabolism	8	2.63E-02
Cyanoamino acid metabolism	6	2.91E-02
Riboflavin metabolism	6	2.91E-02
Starch and sucrose metabolism	17	2.93E-02
Protein processing in endoplasmic reticulum	31	2.98E-02
Carbon metabolism	38	5.12E-02

Table 4.2 Pathway analysis of Downregulated genes in NR showing restoration in presence of glycation inhibitors

Pathway	Number of genes	P-value
Meiosis - yeast	39	6.45E-05
Cell cycle - yeast	34	1.73E-03
mRNA surveillance pathway	17	1.85E-03
Mismatch repair	10	2.67E-02
Regulation of autophagy	8	1.38E-02
Nucleotide excision repair	12	3.18E-02
Endocytosis	19	3.58 E-02
Peroxisome	12	3.83E-02
N-Glycan biosynthesis	10	4.74E-02

4.3.2 Validation of microarray data by real-time PCR analysis

Microarray data was further validated by real-time PCR for longevity-related genes (LSG) selected based on earlier reports. Deletion of TOR/ Sch9 and Ras2 extends yeast CLS [75, 178]. Indeed, TOR/Sch9 and Ras/PKA signalling pathways were reported to be downregulated in CR mediated CLS extension [76, 166]. Autophagy is one of the anti-aging pathway in yeast. TOR/Sch9 and Ras/PKA pathway acts as negative regulators of autophagy [182]. In addition to these, *de novo* purine biosynthesis pathway negatively regulates yeast CLS [109]. Hence, we studied TOR/Sch9, Ras/PKA, autophagy and *de novo* purine biosynthesis pathway by RT-PCR.

4.3.2.1 Effect on TOR/Sch9 and Ras/PKA pathway

TORC1 complex regulates several aspects of cell growth and metabolism [183]. TOR1 gene negatively regulates yeast CLS. Main downstream effector of TOR, which is activated by TORC1, is protein kinase Sch9. Down regulation of Sch9 extends yeast CLS [184]. TOR1 and Sch9 was upregulated in NR condition, whereas glycation inhibitors were able to reduce its expression as like CR (Figure 4.3). Similarly, glycation inhibitors showed restoration of expression of Ras2 gene, which is reported to be negative regulator of yeast CLS [173] (Figure 4.3). All the three glycation inhibitors mimic CR condition.

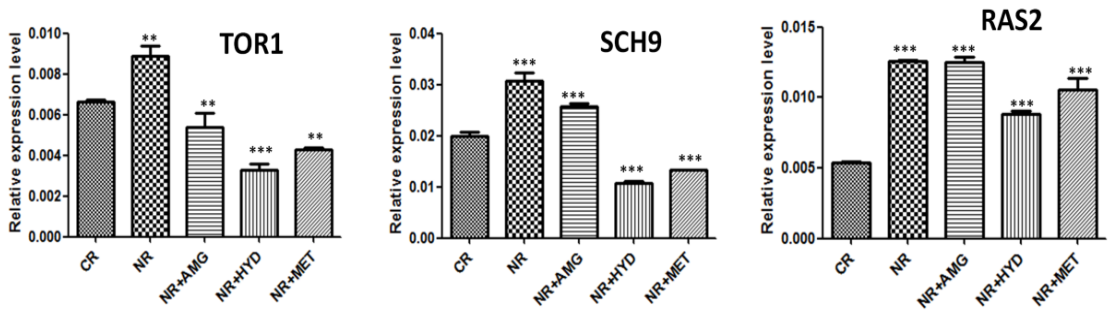


Figure 4.3 Glycation inhibitors mimic CR by inhibiting TOR/Sch9 and Ras2.
TOR1, Sch9 and Ras2 genes were upregulated in NR, whereas glycation inhibitors restore its expression. Experiments were performed with three biological and three technical replicates. All values are mean ±SEM. Statistical significance (*P< 0.05, ** P<0.01 and ***P< 0.001) was calculated by one-way ANOVA using GraphPad Prism 5.

4.3.2.2 Effect on autophagy (ATG pathway)

ATG (autophagy related) proteins are responsible to carry out process of autophagy [25]. ATG1, serine/threonine protein kinase is very essential for process of autophagy [185]. ATG7 and VPS30 are important during double membrane vesicle formation [186]. Quantitative PCR data revealed that autophagy genes such as ATG1, ATG7 and VPS30 were downregulated in NR condition resulting in chronological aging. However, presence of glycation inhibitors in NR condition restored the expression of those genes which is implicated in extension of yeast CLS (Figure 4.4).

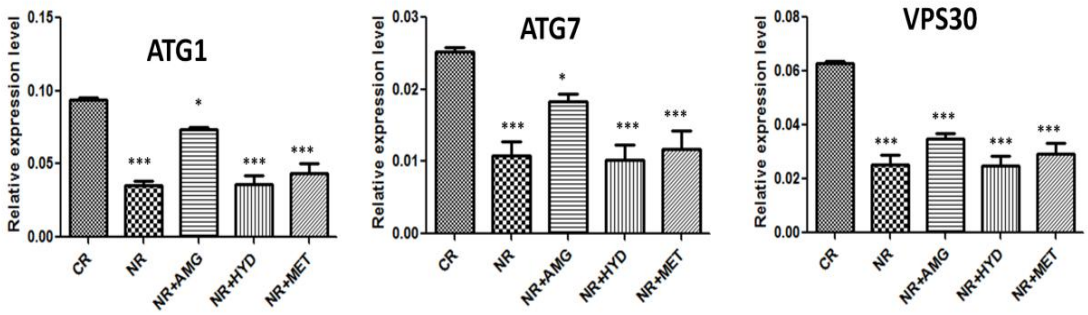


Figure 4.4 Glycation inhibitors induce genes required for autophagy
Autophagy genes ATG1, ATG7 and VPS30 were downregulated in NR condition and its expression was restored by gycation inhibitors. Experiments were performed with three biological and three technical replicates. All values are mean ±SEM. Statistical significance (*P< 0.05, ** P<0.01 and ***P< 0.001) was calculated by one-way ANOVA using GraphPad Prism 5.

4.3.2.3 Effect on *de novo* purine biosynthesis

De novo purine biosynthesis pathway generates IMP, AMP, and GMP. ADE genes are involved in this pathway. *De novo* purine biosynthesis plays important role in chronological lifespan of yeast. It acts as a negative regulator of yeast CLS [187]. ADE3 and ADE4 genes are important in *de novo* purine biosynthesis. Real time PCR analysis showed that these genes were upregulated in NR condition. However, treatment of glycation inhibitors reduced its expression similar to CR (Figure 4.5). Glycation inhibitors mimic CR by inhibiting *de novo* purine biosynthesis.

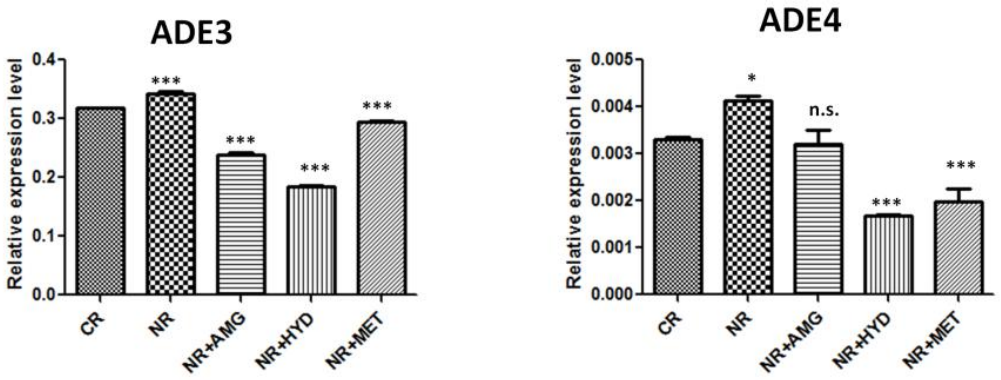


Figure 4.5 Glycation inhibitors inhibit De Novo purine biosynthesis
ADE3 and ADE4 genes were upregulated in NR condition and its expression was restored by gycation inhibitors. Experiments were performed with three biological and three technical replicates. All values are mean ±SEM. Statistical significance (*P< 0.05, ** P<0.01, ***P< 0.001 and n.s.- non significant) was calculated by one-way ANOVA using GraphPad Prism 5.

4.4 Discussion

In the previous chapter, it was reported that glycation inhibitors, AMG, MET and HYD, extends CLS of *S.cerevisiae* by reducing advanced glycation endproducts and back regulation of proteins involved in mitochondrial respiration [180]. These glycation inhibitors mimic CR condition by shifting metabolism from fermentation to respiration [180]. Protein glycation is non-enzymatic reaction which leads to formation of advanced glycation endproducts (AGEs). AGE modifications affect structure and functions of proteins involved in different cellular processes. It affects proteostasis [86, 90]. Loss of proteostasis is one of the hallmarks of aging and aging associated diseases [4]. Glycation is implicated in aging associated diseases, which accelerate aging process [87, 94, 113]. We have shown that glycation inhibitors can be used as anti-aging molecules as it extends yeast CLS[180].

This study examines effect of glycation inhibitors, at genomic level, on longevity related genes (LSG). Using DNA microarray-based transcriptional profiling, we observed that many of the ribosomal genes, genes which are involved in metabolic pathways, oxidative phosphorylation, and amino acid biosynthesis were upregulated, whereas genes involved in autophagy, meiosis, mismatch repair and cell cycle were downregulated in NR. Our results corroborates with the previous reports, where they showed that these genes are associated with accelerated aging [175, 188]. Interestingly, glycation inhibitors were able to restore the expression of these genes, which may be implicated in extension of yeast CLS. Genes involved in oxidative phosphorylation were upregulated in NR; however, their protein levels were decreased. Although, mRNA is translated into protein, there is a poor correlation between protein abundance and mRNA expression levels globally. This suggests the existence of additional levels of regulation after transcription which includes mRNA stability, ribosomal occupancy, and protein half-life [176].

Our microarray results were confirmed using RT-PCR of 8 selected longevity related genes. TOR/Sch9 and RAS/AC/PKA are the two important pro- aging signaling pathways in yeast [166]. Down regulation of TOR1 and Ras2 extends yeast CLS. Also, Sch9, downstream effector of TOR, is negative regulator of yeast CLS [178, 189]. RT-PCR data suggests that glycation inhibitors are effective in reducing

expression of upregulated TOR1, Sch9 and Ras2 genes in NR condition as like CR. This may contribute to glycation inhibitor mediated extension of yeast CLS.

In yeast, process of autophagy is essential for survival during starvation which recycles cellular components to get new nutrients [25]. ATG1 gene is essential for Cvt pathway and autophagy [185]. When TOR1/Sch9 and Ras/ PKA signaling pathways are active, it leads to phosphorylation of ATG proteins which in turn inhibit process of autophagy. It also inhibits expression of ATG genes through protein kinase Rim15 and transcription factor Msn2/4 [190-192]. As glycation inhibitors reduce the expression of TOR1, Sch9 and Ras2; this may be implicated into induction of autophagy. From RT-PCR analysis, it is observed that glycation inhibitors increased the expression of ATG1, ATG7 and VPS30 genes, which were downregulated in NR condition. Induction of autophagy by glycation inhibitors may lead to CLS extension in yeast.

Further, we have studied genes involved in *de novo* purine biosynthesis. Deletion of ADE4 gene, which carry out first and rate limiting step in *de novo* purine biosynthesis, extends yeast CLS [109, 163]. Deletion of any ADE gene is sufficient to extend yeast CLS significantly [109]. Interestingly, glycation inhibitors reduced the expression of ADE3 and ADE4 genes in NR condition, which explains the CLS extension in yeast.

4.5 Conclusion

Our results from microarray and RT-PCR analysis suggests that CLS extension mediated by glycation inhibitors, AMG, MET and HYD, is through inhibition of pro-aging signaling pathways, TOR/Sch9 and RAS/AC/PKA, and induction of anti-aging pathway that is autophagy.

Summary

Thesis aims to study glycation induced aging in yeast and use of glycation inhibitors as anti-aging molecules. It is reported that Non calorie restricted condition (NR) (high glucose) promotes aging via many signalling pathways. Glycation reaction can be one of the reasons of aging in high glucose condition as excess glucose reacts with proteins leading to formation of AGEs. AGEs can affect functions of many intracellular proteins which contribute to cellular damage. So it is important to study glycation induced aging. So, we have used yeast as a model system to elucidate the role of glycation in aging and used glycation inhibitors to delay glycation induced aging. For this, cells were grown in different concentrations of glucose (0.5%, 2%, and 4%) and effect of glucose on lifespan, AGE modification of proteins and ROS was checked. Viability assay showed reduced lifespan with increasing concentration of glucose. Also, AGE modification of proteins and ROS were increased with glucose concentration. Treatment of Aminoguanidine (AMG), glycation inhibitor, was able to reduce the effect of glucose which implied that Aminoguanidine extends yeast chronological lifespan by reducing AGE modification of proteins. Thus, yeast can be efficiently used as a model system to study glycation.

Glycation mainly affects structure and function of proteins. Hence, we investigated the effect of glycation inhibitors on yeast proteome to find out its role in progression of yeast aging. Proteomics analysis was done using SWATH-MS approach. Total 1500 proteins were identified, out of which 207 proteins were up regulated and 152 were down regulated in high glucose condition (NR). Proteins involved in cellular respiration were down regulated. Interestingly, Aminoguanidine showed back regulation of many differentially expressed proteins in NR. It restored expression of proteins involved in cellular respiration and reduced expression of glycolytic proteins mimicking CR condition. AMG induced back regulation of differentially expressed proteins could be possibly due to its chemical effect or indirectly by glycation inhibition. To delineate this, Metformin (MET), a structural analogue of AMG and a mild glycation inhibitor and Hydralazine (HYD), another potent glycation inhibitor but not structural analogue of AMG were used. All the three glycation inhibitors were able to back regulate deregulated proteins in NR. They back regulated expression of proteins involved in mitochondrial respiration which is reported to have role in aging.

Thus, glycation inhibitors extend yeast chronological lifespan by reducing AGE modification of proteins and restoring mitochondrial respiration.

Non calorie restriction led to identification of nutrient signaling factors such as TOR1, Sch9, RAS2 and PKA that contribute to reduce lifespan in yeast. In view of this, microarray analysis was done and data was validated with real time PCR. Microarray analysis showed that glycation inhibitors restored the expression of many genes involved in different pathways. DAVID analysis of microarray data suggested that glycation inhibitors restored the expression of genes involved in mitochondrial respiration and autophagy. They showed up regulation autophagy which is reported to delay aging and down regulation *de novo* purine biosynthesis which accelerates aging. TOR1, Sch9 and Ras2 are pro- aging genes. These were up regulated in NR leading to short lifespan, whereas glycation inhibitors restored the expression of these genes. Thus, glycation inhibition effect of molecules used showed activation of anti-aging pathways which may contribute to extension of yeast chronological lifespan. Hence, glycation inhibitors can serve as anti-aging molecules.

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Appendix

Appendix 1: List of differentially expressed proteins in NR

Accession	Protein Description	Gene Name	P-value	Max Fold Change
P01097	ATPase inhibitor, mitochondrial	INH1	1.4E-05	0.01
P37299	Cytochrome b-c1 complex subunit 10	QCR10	3.8E-05	0.02
P33303	Succinate/fumarate mitochondrial transporter	SFC1	3.3E-05	0.03
P22943	12 kDa heat shock protein	HSP12	0.00152	0.03
P40466	Fork head protein homolog 1	FKH1	3.1E-05	0.03
Q12233	ATP synthase subunit g, mitochondrial	ATP20	3.4E-05	0.03
Q08245	Protein ZEO1	ZEO1	3.1E-06	0.03
A6ZRW6	Malate synthase 1, glyoxysomal	MLS1	2E-05	0.05
P05747	60S ribosomal protein L29	RPL29	0.00953	0.05
Q12289	Mitochondrial carnitine carrier	CRC1	3.1E-06	0.05
Q12428	Probable 2-methylcitrate dehydratase	PDH1	0.00012	0.05
P05749	60S ribosomal protein L22-A	RPL22A	1.6E-07	0.06
P32316	Acetyl-CoA hydrolase	ACH1	3.1E-05	0.07
P11632	Non-histone chromosomal protein 6A	NHP6A	0.00091	0.07
P09232	Cerevisin	PRB1	3.7E-05	0.07
P00890	Citrate synthase, mitochondrial	CIT1	6.9E-05	0.07
P40185	Protein MMF1, mitochondrial	MMF1	1.1E-06	0.07
Q08179	Mitochondrial distribution and morphology protein 38	MDM38	0.00076	0.07
P18239	ADP,ATP carrier protein 2	PET9	7.3E-06	0.08
P08679	Citrate synthase, peroxisomal	CIT2	0.00016	0.08
Q03104	Meiotic sister chromatid recombination protein 1	MSC1	0.00101	0.09
P32796	Carnitine O-acetyltransferase, mitochondrial	CAT2	0.00028	0.09
P07143	Cytochrome c1, heme protein, mitochondrial	CYT1	9.9E-06	0.09
P00128	Cytochrome b-c1 complex subunit 7	QCR7	0.01713	0.09
P23641	Mitochondrial phosphate carrier protein	MIR1	8.4E-08	0.10
Q07451	Endoplasmic reticulum transmembrane protein 3	YET3	0.0003	0.10
P81449	ATP synthase subunit e, mitochondrial	TIM11	1.7E-06	0.10
P32191	Glycerol-3-phosphate dehydrogenase, mitochondrial	GUT2	7.5E-05	0.10
Q00711	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	SDH1	0.0002	0.10
P17505	Malate dehydrogenase, mitochondrial	MDH1	1.1E-06	0.10
P16547	Mitochondrial outer membrane protein OM45	OM45	0.0002	0.11
P09457	ATP synthase subunit 5, mitochondrial	ATP5	0.00056	0.12
O14455	60S ribosomal protein L36-B	RPL36B	1.1E-05	0.12
P32179	REVERSED 3'(2'),5'-bisphosphate nucleotidase	MET22	5.1E-06	0.12
P36015	Synaptobrevin homolog YKT6	YKT6	2.9E-05	0.12
Q12349	ATP synthase subunit H, mitochondrial	ATP14	1.9E-05	0.13
P07256	Cytochrome b-c1 complex subunit 1, mitochondrial	COR1	2.7E-07	0.13
P21801	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial	SDH2	3.4E-05	0.13
P30902	ATP synthase subunit d, mitochondrial	ATP7	3.5E-06	0.13
P43635	Citrate synthase 3	CIT3	2.5E-05	0.13
P05626	ATP synthase subunit 4, mitochondrial	ATP4	0.00121	0.13
P28240	Isocitrate lyase	ICL1	4.5E-05	0.13
Q06488	Chromatin structure-remodeling complex subunit RSC2	RSC2	0.00069	0.13
P25619	30 kDa heat shock protein	HSP30	0.00802	0.13
P07257	Cytochrome b-c1 complex subunit 2,	QCR2	4.3E-07	0.14

Accession	Protein Description	Gene Name	P-value	Max Fold Change
	mitochondrial			
P46367	Potassium-activated aldehyde dehydrogenase, mitochondrial	ALD4	8.4E-06	0.14
P10963	Phosphoenolpyruvate carboxykinase [ATP]	PCK1	0.00094	0.14
P17695	Glutaredoxin-2, mitochondrial	GRX2	0.03905	0.14
P07275	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial	PUT2	0.0353	0.14
Q12434	Rho GDP-dissociation inhibitor	RDI1	0.00742	0.15
P19262	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	KGD2	0.0016	0.15
P0CT05	Protease B inhibitor 1	PBI2	0.00106	0.15
P07251	ATP synthase subunit alpha, mitochondrial	ATP1	4.5E-07	0.15
P09201	Fructose-1,6-bisphosphatase	FBP1	2.1E-05	0.15
P20967	2-oxoglutarate dehydrogenase, mitochondrial	KGD1	5.5E-07	0.15
Q04947	Reticulon-like protein 1	RTN1	0.00099	0.16
Q02354	U3 small nucleolar RNA-associated protein 6	UTP6	0.03448	0.16
P28241	Isocitrate dehydrogenase [NAD] subunit 2, mitochondrial	IDH2	0.00046	0.17
P37302	Aminopeptidase Y	APE3	0.0022	0.17
P04650	60S ribosomal protein L39	RPL39	8.9E-05	0.18
P47001	Cell wall mannoprotein CIS3	CIS3	0.01648	0.18
P00431	Cytochrome c peroxidase, mitochondrial	CCP1	7E-05	0.18
P33317	Deoxyuridine 5'-triphosphate nucleotidohydrolase	DUT1	0.04345	0.18
P36046	Mitochondrial intermembrane space import and assembly protein 40	MIA40	0.00014	0.18
P02309	Histone H4	HHF1	3.8E-05	0.19
P40582	Glutathione S-transferase 1	GTT1	0.00184	0.19
P22803	Thioredoxin-2	TRX2	4.3E-06	0.19
P00830	ATP synthase subunit beta, mitochondrial	ATP2	1.8E-06	0.20
P08417	Fumarate hydratase, mitochondrial	FUM1	0.00114	0.20
P28834	Isocitrate dehydrogenase [NAD] subunit 1, mitochondrial	IDH1	4.5E-06	0.20
P38071	Enoyl-[acyl-carrier protein] reductase [NADPH, B-specific], mitochondrial	ETR1	2.5E-06	0.20
P00445	Superoxide dismutase [Cu-Zn]	SOD1	0.00015	0.21
Q02486	ARS-binding factor 2, mitochondrial	ABF2	0.00056	0.21
P47052	Succinate dehydrogenase [ubiquinone] flavoprotein subunit 2, mitochondrial	YJL045W	0.00621	0.21
Q3E841	Uncharacterized protein YNR034W-A	YNR034W-A	0.00014	0.21
P12695	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	LAT1	9.5E-05	0.21
P53312	Succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial	LSC2	7.2E-06	0.22
P28321	Monoglyceride lipase	YJU3	0.00956	0.22
P38077	ATP synthase subunit gamma, mitochondrial	ATP3	1.6E-07	0.22
P38910	10 kDa heat shock protein, mitochondrial	HSP10	0.00623	0.22
P15992	Heat shock protein 26	HSP26	7.9E-07	0.23
P61830	Histone H3	HHT1	3.6E-06	0.23
Q01574	Acetyl-coenzyme A synthetase 1	ACS1	0.00273	0.23
P04840	Mitochondrial outer membrane protein porin 1	POR1	7.1E-08	0.23
P09624	Dihydrolipoyl dehydrogenase, mitochondrial	LPD1	2.8E-06	0.23
P02992	Elongation factor Tu, mitochondrial	TUF1	0.00116	0.23
P22217	Thioredoxin-1	TRX1	3.1E-06	0.24
P38616	Protein YGP1	YGP1	0.00626	0.24
P36112	Formation of crista junctions protein 1	FCJ1	0.01378	0.26
P25372	Thioredoxin-3, mitochondrial	TRX3	0.00431	0.27

Accession	Protein Description	Gene Name	P-value	Max Fold Change
P53228	Transaldolase NQM1	NQM1	1.8E-05	0.27
P19414	Aconitate hydratase, mitochondrial	ACO1	3.4E-05	0.27
P32891	D-lactate dehydrogenase [cytochrome] 1, mitochondrial	DLD1	0.00303	0.28
P36059	ATP-dependent (S)-NAD(P)H-hydrate dehydratase	YKL151C	0.02507	0.30
P37012	Phosphoglucomutase-2	PGM2	0.00032	0.31
P53889	Uncharacterized mitochondrial hydrolase FMP41	FMP41	0.00789	0.31
P53598	Succinyl-CoA ligase [ADP-forming] subunit alpha, mitochondrial	LSC1	2.7E-05	0.31
P23542	Aspartate aminotransferase, cytoplasmic	AAT2	1.2E-05	0.32
P40513	Mitochondrial acidic protein MAM33	MAM33	0.0003	0.33
P22133	Malate dehydrogenase, cytoplasmic	MDH2	0.0459	0.34
P02294	Histone H2B.2	HTB2	3.1E-06	0.34
P04912	Histone H2A.2	HTA2	6.4E-06	0.35
Q12402	Protein YOP1	YOP1	0.00054	0.35
P36060	NADH-cytochrome b5 reductase 2	MCR1	4.3E-05	0.35
Q00764	Alpha,alpha-trehalose-phosphate synthase [UDP-forming] 56 kDa subunit	TPS1	0.00033	0.36
P14904	Vacuolar aminopeptidase 1	LAP4	4.7E-06	0.36
P16387	Pyruvate dehydrogenase E1 component subunit alpha, mitochondrial	PDA1	0.00037	0.36
P38891	Branched-chain-amino-acid aminotransferase, mitochondrial	BAT1	0.00069	0.37
P36010	Nucleoside diphosphate kinase	YNK1	1.6E-05	0.37
P32628	UV excision repair protein RAD23	RAD23	0.00596	0.37
P06780	GTP-binding protein RHO1	RHO1	6.4E-06	0.40
Q04728	Arginine biosynthesis bifunctional protein ArgJ, mitochondrial	ARG7	0.00025	0.40
P07267	Saccharopepsin	PEP4	2.5E-05	0.41
P0CX83	60S ribosomal protein L19-B	RPL19B	6.9E-05	0.42
P22146	1,3-beta-glucanosyltransferase GAS1	GAS1	0.00331	0.42
P04806	Hexokinase-1	HXK1	3.5E-06	0.43
P32352	C-8 sterol isomerase	ERG2	0.00745	0.44
P40017	Carnitine O-acetyltransferase YAT2	YAT2	0.017	0.44
P19882	Heat shock protein 60, mitochondrial	HSP60	8.9E-05	0.44
P39726	Glycine cleavage system H protein, mitochondrial	GCV3	0.00017	0.45
P41921	Glutathione reductase	GLR1	1.2E-05	0.45
P16603	NADPH--cytochrome P450 reductase	NCP1	0.00257	0.45
P06168	Ketol-acid reductoisomerase, mitochondrial	ILV5	6.1E-07	0.45
P32473	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	PDB1	8.5E-06	0.46
P07246	Alcohol dehydrogenase 3, mitochondrial	ADH3	0.00044	0.47
P25373	Glutaredoxin-1	GRX1	0.00014	0.49
P33416	Heat shock protein 78, mitochondrial	HSP78	0.00138	0.50
P0CS91	Heat shock protein SSC1, mitochondrial	SSC1	0.00014	0.50
P15703	Glucan 1,3-beta-glucosidase	BGL2	0.00356	0.51
P0CH09	Ubiquitin-60S ribosomal protein L40	RPL40B	0.00017	0.52
P00427	Cytochrome c oxidase subunit 6, mitochondrial	COX6	0.00344	0.52
P04037	Cytochrome c oxidase subunit 4, mitochondrial	COX4	0.04039	0.53
P14020	Dolichol-phosphate mannosyltransferase	DPM1	4.6E-05	0.53
P32454	Aminopeptidase 2, mitochondrial	APE2	0.00334	0.53
P21954	Isocitrate dehydrogenase [NADP], mitochondrial	IDP1	0.0029	0.54
P47068	Myosin tail region-interacting protein MTI1	BBC1	0.0202	0.54
P32263	Pyrroline-5-carboxylate reductase	PRO3	0.01404	0.55
P48015	Aminomethyltransferase, mitochondrial	GCV1	0.00011	0.57
P22137	Clathrin heavy chain	CHC1	0.00019	0.57
Q12335	Protoplast secreted protein 2	PST2	0.0235	0.57

Accession	Protein Description	Gene Name	P-value	Max Fold Change
P00447	Superoxide dismutase [Mn], mitochondrial	SOD2	0.01804	0.57
P29704	Squalene synthase	ERG9	0.00967	0.57
P38523	GrpE protein homolog, mitochondrial	MGE1	0.00872	0.58
P41939	Isocitrate dehydrogenase [NADP] cytoplasmic	IDP2	0.04862	0.62
P25574	ER membrane protein complex subunit 1	EMC1	0.00502	0.62
O74700	Mitochondrial import inner membrane translocase subunit TIM9	TIM9	0.00144	0.63
P34227	Mitochondrial peroxiredoxin PRX1	PRX1	0.03804	0.63
P02400	60S acidic ribosomal protein P2-beta	RPP2B	6.4E-05	0.63
P43616	Cys-Gly metallodipeptidase DUG1	DUG1	0.00161	0.65
P32332	Mitochondrial oxaloacetate transport protein	OAC1	0.00148	0.66
P09938	Ribonucleoside-diphosphate reductase small chain 1	RNR2	0.01662	0.66
P40495	Homoisocitrate dehydrogenase, mitochondrial	LYS12	1.8E-05	0.67
Q04120	Peroxiredoxin TSA2	TSA2	0.00079	1.49
P06169	Pyruvate decarboxylase isozyme 1	PDC1	0.00352	1.49
P60010	Actin	ACT1	0.00162	1.49
Q3E754	40S ribosomal protein S21-B	RPS21B	4.6E-05	1.51
P0CX38	40S ribosomal protein S6-B	RPS6B	5.8E-05	1.51
P32598	Serine/threonine-protein phosphatase PP1-2	GLC7	0.01468	1.52
P06787	Calmodulin	CMD1	0.00121	1.52
P40217	Eukaryotic translation initiation factor 3 subunit I	TIF34	0.00454	1.53
P0CX32	40S ribosomal protein S24-B	RPS24B	0.0025	1.54
P07264	3-isopropylmalate dehydratase	LEU1	2.4E-05	1.55
P0CX44	60S ribosomal protein L1-B	RPL1B	0.001	1.55
P04802	Aspartate--tRNA ligase, cytoplasmic	DPS1	0.00368	1.56
P48589	40S ribosomal protein S12	RPS12	4.3E-06	1.56
P20081	FK506-binding protein 1	FPR1	0.00023	1.57
P02557	Tubulin beta chain	TUB2	0.02462	1.57
P16120	Threonine synthase	THR4	0.00146	1.57
P46654	40S ribosomal protein S0-B	RPS0B	9.1E-05	1.57
P32905	40S ribosomal protein S0-A	RPS0A	9.1E-05	1.57
P53981	Uncharacterized phosphatase YNL010W	YNL010W	0.00762	1.58
P19097	Fatty acid synthase subunit alpha	FAS2	0.00133	1.59
P39743	Reduced viability upon starvation protein 167	RVS167	0.03877	1.59
P13663	Aspartate-semialdehyde dehydrogenase	HOM2	0.00028	1.61
P02994	Elongation factor 1-alpha	TEF1	4.3E-05	1.61
P29311	Protein BMH1	BMH1	0.00058	1.62
P14540	Fructose-bisphosphate aldolase	FBA1	0.00081	1.62
P19146	ADP-ribosylation factor 2	ARF2	0.00083	1.62
P32497	Eukaryotic translation initiation factor 3 subunit C	NIP1	0.02313	1.62
P00942	Triosephosphate isomerase	TPI1	2.8E-05	1.63
P07149	Fatty acid synthase subunit beta	FAS1	0.00058	1.64
P40309	REVERSED K(+)/H(+) antiporter 1	KHA1	0.00012	1.65
P05694	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	MET6	0.00061	1.66
P11076	ADP-ribosylation factor 1	ARF1	7.6E-05	1.66
P14126	60S ribosomal protein L3	RPL3	0.02931	1.67
P00498	ATP phosphoribosyltransferase	HIS1	0.02472	1.67
P05740	60S ribosomal protein L17-A	RPL17A	7.8E-08	1.67
P47096	3-hydroxyanthranilate 3,4-dioxygenase	BNA1	0.0294	1.67
P20459	Eukaryotic translation initiation factor 2 subunit alpha	SUI2	0.00165	1.68
P46672	GU4 nucleic-binding protein 1	ARC1	0.00128	1.68
Q03148	Pyridoxine biosynthesis protein SNZ1	SNZ1	0.00776	1.69
P38720	6-phosphogluconate dehydrogenase, decarboxylating 1	GND1	0.0001	1.69

Accession	Protein Description	Gene Name	P-value	Max Fold Change
P02406	60S ribosomal protein L28	RPL28	0.00615	1.69
P46655	Glutamate--tRNA ligase, cytoplasmic	GUS1	3.9E-06	1.71
P32527	Zuotin	ZUO1	0.0073	1.71
P20606	Small COPII coat GTPase SAR1	SAR1	0.00096	1.71
P32589	Heat shock protein homolog SSE1	SSE1	0.00306	1.71
Q3E7Y3	40S ribosomal protein S22-B	RPS22B	0.00041	1.71
P38088	Glycine--tRNA ligase 1, mitochondrial	GRS1	0.00015	1.72
P32861	UTP--glucose-1-phosphate uridylyltransferase	UGP1	4.1E-05	1.73
P39954	Adenosylhomocysteinase	SAH1	1.5E-05	1.73
P02829	ATP-dependent molecular chaperone HSP82	HSP82	6.3E-05	1.73
P22768	Argininosuccinate synthase	ARG1	8.2E-05	1.74
P34730	Protein BMH2	BMH2	0.00068	1.74
P0C2H9	60S ribosomal protein L31-B	RPL31B	0.00133	1.75
P15108	ATP-dependent molecular chaperone HSC82	HSC82	5E-05	1.75
P53141	Myosin light chain 1	MLC1	0.00019	1.75
P38911	FK506-binding nuclear protein	FPR3	0.00017	1.77
P05755	40S ribosomal protein S9-B	RPS9B	6.1E-05	1.77
Q05911	Adenylosuccinate lyase	ADE13	0.00344	1.77
P21264	Phosphoribosylaminoimidazole carboxylase	ADE2	0.00202	1.77
Q05506	Arginine--tRNA ligase, cytoplasmic	YDR341C	0.01472	1.77
P07244	Bifunctional purine biosynthetic protein ADE5,7	ADE5,7	0.00283	1.78
P13517	F-actin-capping protein subunit beta	CAP2	0.01819	1.81
P05030	Plasma membrane ATPase 1	PMA1	5.1E-06	1.82
Q01560	Nucleolar protein 3	NPL3	0.00057	1.82
P10080	Single-stranded nucleic acid-binding protein	SBP1	0.00377	1.83
P38754	60S ribosomal protein L14-B	RPL14B	0.00031	1.84
P05750	40S ribosomal protein S3	RPS3	4.3E-06	1.85
P32836	GTP-binding nuclear protein GSP2/CNR2	GSP2	1.4E-05	1.87
P00560	Phosphoglycerate kinase	PGK1	0.00089	1.87
P49626	60S ribosomal protein L4-B	RPL4B	0.00142	1.88
P32471	Elongation factor 1-beta	EFB1	0.0258	1.89
P14120	60S ribosomal protein L30	RPL30	0.00409	1.89
P26786	40S ribosomal protein S7-A	RPS7A	0.00035	1.91
P15624	Phenylalanine--tRNA ligase beta subunit	FRS1	0.04505	1.91
P36105	60S ribosomal protein L14-A	RPL14A	0.00011	1.91
P32588	Nuclear and cytoplasmic polyadenylated RNA-binding protein PUB1	PUB1	0.00352	1.92
P26321	60S ribosomal protein L5	RPL5	0.00048	1.94
P14127	40S ribosomal protein S17-B	RPS17B	2.4E-05	1.95
P38219	Obg-like ATPase 1	OLA1	4E-05	1.96
P07806	Valine--tRNA ligase, mitochondrial	VAS1	0.00081	1.96
P13188	Glutamine--tRNA ligase	GLN4	0.03011	1.97
Q05775	Eukaryotic translation initiation factor 3 subunit J	HCR1	0.00387	1.98
P48164	40S ribosomal protein S7-B	RPS7B	0.00559	1.98
P38079	Protein YRO2	YRO2	0.00222	2.00
P31373	Cystathionine gamma-lyase	CYS3	0.00045	2.01
P05739	60S ribosomal protein L6-B	RPL6B	3.2E-06	2.01
P38845	Cruciform DNA-recognizing protein 1	CRP1	0.04455	2.03
P09436	Isoleucine--tRNA ligase, cytoplasmic	ILS1	0.00141	2.03
O43137	Uncharacterized protein YBR085C-A	YBR085C-A	0.01693	2.04
P19358	S-adenosylmethionine synthase 2	SAM2	2.4E-06	2.06
P10659	S-adenosylmethionine synthase 1	SAM1	2.9E-05	2.06
P38701	40S ribosomal protein S20	RPS20	0.01349	2.07
Q02326	60S ribosomal protein L6-A	RPL6A	1.5E-06	2.08
P38972	Phosphoribosylformylglycinamidine synthase	ADE6	1.7E-05	2.08
P01123	GTP-binding protein YPT1	YPT1	0.00585	2.09
P15646	rRNA 2'-O-methyltransferase fibrillarin	NOP1	0.00013	2.09
P27476	Nuclear localization sequence-binding protein	NSR1	0.0128	2.12

Accession	Protein Description	Gene Name	P-value	Max Fold Change
P16862	6-phosphofructokinase subunit beta	PFK2	0.0008	2.12
Q06252	Uncharacterized protein YLR179C	YLR179C	0.00028	2.13
P39015	Suppressor protein STM1	STM1	0.00042	2.13
P49090	Asparagine synthetase [glutamine-hydrolyzing] 2	ASN2	6.2E-05	2.14
P33322	H/ACA ribonucleoprotein complex subunit 4	CBF5	0.01115	2.14
P05743	60S ribosomal protein L26-A	RPL26A	2.1E-05	2.15
P32324	Elongation factor 2	EFT1	0.00013	2.15
P0CX40	40S ribosomal protein S8-B	RPS8B	8.3E-06	2.15
Q99383	Nuclear polyadenylated RNA-binding protein 4	HRP1	0.00748	2.16
P37291	Serine hydroxymethyltransferase, cytosolic	SHM2	8.4E-05	2.16
P07342	Acetolactate synthase catalytic subunit, mitochondrial	ILV2	0.00063	2.17
P16861	6-phosphofructokinase subunit alpha	PFK1	4.8E-05	2.18
P04147	Polyadenylate-binding protein, cytoplasmic and nuclear	PAB1	0.00034	2.18
Q3E757	60S ribosomal protein L11-B	RPL11B	0.00044	2.18
P00330	Alcohol dehydrogenase 1	ADH1	0.00054	2.18
P0CX54	60S ribosomal protein L12-B	RPL12B	3.4E-05	2.19
P10081	ATP-dependent RNA helicase eIF4A	TIF1	0.00491	2.19
P34223	UBX domain-containing protein 1	SHP1	0.00266	2.19
P53334	Probable family 17 glucosidase SCW4	SCW4	6.2E-05	2.20
Q04894	NADP-dependent alcohol dehydrogenase 6	ADH6	0.00839	2.21
Q12672	60S ribosomal protein L21-B	RPL21B	3.3E-05	2.21
P0CX48	40S ribosomal protein S11-B	RPS11B	8.7E-06	2.23
P49089	Asparagine synthetase [glutamine-hydrolyzing] 1	ASN1	1.2E-05	2.23
P54113	Bifunctional purine biosynthesis protein ADE16	ADE16	0.00331	2.23
P00950	Phosphoglycerate mutase 1	GPM1	2.6E-05	2.24
P38999	Saccharopine dehydrogenase [NADP(+), L-glutamate-forming]	LYS9	1.6E-05	2.24
P49167	60S ribosomal protein L38	RPL38	0.00568	2.25
P38711	40S ribosomal protein S27-B	RPS27B	7.4E-06	2.26
P40150	Heat shock protein SSB2	SSB2	9.6E-05	2.26
P11484	Heat shock protein SSB1	SSB1	9.6E-05	2.26
P20435	DNA-directed RNA polymerases I, II, and III subunit RPABC2	RPO26	0.01086	2.26
P40825	Alanine--tRNA ligase, mitochondrial	ALA1	0.00092	2.26
P25294	Protein SIS1	SIS1	0.00522	2.27
P17423	Homoserine kinase	THR1	0.00333	2.27
P38707	Asparagine--tRNA ligase, cytoplasmic	DED81	0.00042	2.27
P15454	Guanylate kinase	GUK1	0.00104	2.27
P40054	D-3-phosphoglycerate dehydrogenase 1	SER3	0.00018	2.28
P40024	ABC transporter ATP-binding protein ARB1	ARB1	0.03714	2.29
P07283	Phosphomannomutase	SEC53	0.00767	2.29
Q05022	rRNA biogenesis protein RRP5	RRP5	0.03476	2.31
Q12074	Spermidine synthase	SPE3	0.00046	2.32
Q3E7X9	40S ribosomal protein S28-A	RPS28A	4.6E-05	2.33
P25694	Cell division control protein 48	CDC48	1.3E-06	2.33
P00815	Histidine biosynthesis trifunctional protein	HIS4	0.00021	2.34
Q12117	Protein MRH1	MRH1	0.00063	2.35
P03965	Carbamoyl-phosphate synthase arginine-specific large chain	CPA2	0.00012	2.36
P0C2H7	60S ribosomal protein L27-B	RPL27B	4.2E-05	2.37
P38013	Peroxiredoxin type-2	AHP1	0.00035	2.37
P32481	Eukaryotic translation initiation factor 2 subunit gamma	GCD11	0.0048	2.38
P27616	Phosphoribosylaminoimidazole-succinocarboxamide synthase	ADE1	0.00079	2.39
P00931	Tryptophan synthase	TRP5	1.7E-05	2.39

Accession	Protein Description	Gene Name	P-value	Max Fold Change
P31688	Trehalose-phosphatase	TPS2	0.01651	2.41
Q12114	Chitin biosynthesis protein CHS5	CHS5	0.03701	2.41
P00331	Alcohol dehydrogenase 2	ADH2	2.3E-06	2.46
P42222	Enolase-related protein 3	ERR3	0.00508	2.49
P38708	Putative proline--tRNA ligase YHR020W	YHR020W	0.00069	2.52
P33734	Imidazole glycerol phosphate synthase hisHF	HIS7	0.01097	2.52
P47120	Deoxyhypusine hydroxylase	LIA1	0.03499	2.52
P80210	Adenylosuccinate synthetase	ADE12	0.00016	2.54
P0CX50	60S ribosomal protein L18-B	RPL18B	1.6E-05	2.55
P00549	Pyruvate kinase 1	CDC19	1E-06	2.56
P04801	Threonine--tRNA ligase, cytoplasmic	THS1	0.00084	2.56
P15625	Phenylalanine--tRNA ligase alpha subunit	FRS2	9.6E-05	2.57
P38061	60S ribosomal protein L32	RPL32	8.9E-06	2.58
P38009	Bifunctional purine biosynthesis protein ADE17	ADE17	4.1E-06	2.62
P54839	Hydroxymethylglutaryl-CoA synthase	ERG13	0.00622	2.63
P0CX24	60S ribosomal protein L20-B	RPL20B	5.2E-05	2.64
Q04062	26S proteasome regulatory subunit RPN9	RPN9	0.00142	2.67
Q12377	26S proteasome regulatory subunit RPN6	RPN6	0.01111	2.77
P04076	Argininosuccinate lyase	ARG4	0.00012	2.77
P38011	Guanine nucleotide-binding protein subunit beta-like protein	ASC1	1E-06	2.77
P52910	Acetyl-coenzyme A synthetase 2	ACS2	0.01272	2.85
P29952	Mannose-6-phosphate isomerase	PMI40	0.00074	2.89
P87262	60S ribosomal protein L34-A	RPL34A	1.1E-05	2.90
P38817	ADP-ribosylation factor-binding protein GGA2	GGA2	0.00983	2.93
P41920	Ran-specific GTPase-activating protein 1	YRB1	4.6E-05	2.93
Q12485	Transposon Ty1-GR2 Gag polyprotein	TY1A-GR2	0.00441	3.01
P07245	C-1-tetrahydrofolate synthase, cytoplasmic	ADE3	0.00197	3.08
P39939	40S ribosomal protein S26-B	RPS26B	9.2E-08	3.11
P07278	cAMP-dependent protein kinase regulatory subunit	BCY1	0.01246	3.12
P23254	Transketolase 1	TKL1	2.5E-05	3.16
P07172	Histidinol-phosphate aminotransferase	HIS5	0.01896	3.16
Q12470	Transposon Ty1-NL2 Gag polyprotein	TY1A-NL2	0.0042	3.20
P04046	Amidophosphoribosyltransferase	ADE4	0.00063	3.21
P32582	Cystathionine beta-synthase	CYS4	2.4E-05	3.25
P34167	Eukaryotic translation initiation factor 4B	TIF3	0.0178	3.25
P10127	Alcohol dehydrogenase 4	ADH4	0.02111	3.35
P15180	Lysine--tRNA ligase, cytoplasmic	KRS1	2.2E-05	3.38
P50095	Inosine-5'-monophosphate dehydrogenase 3	IMD3	0.00098	3.47
Q07478	ATP-dependent RNA helicase SUB2	SUB2	0.00038	3.48
P21734	Ubiquitin-conjugating enzyme E2 1	UBC1	0.00385	3.49
P39078	T-complex protein 1 subunit delta	CCT4	0.03457	3.60
P41807	V-type proton ATPase subunit H	VMA13	0.00706	3.64
Q12122	Homocitrate synthase, mitochondrial	LYS21	1.3E-05	3.88
P48570	Homocitrate synthase, cytosolic isozyme	LYS20	3.4E-05	4.04
P43594	Altered inheritance of mitochondria protein 13, mitochondrial	AIM13	0.00011	4.15
P39929	Vacuolar-sorting protein SNF7	SNF7	0.03562	4.22
P32288	Glutamine synthetase	GLN1	0.00016	4.33
P16521	Elongation factor 3A	YEF3	4E-07	4.34
P25381	Subtilase-type proteinase RRT12	RRT12	0.00382	4.41
P36132	tRNA N6-adenosine threonylcarbamoyltransferase	KAE1	0.02032	4.46
P07263	Histidine--tRNA ligase, mitochondrial	HTS1	0.00859	4.51
P38230	Probable quinone oxidoreductase	ZTA1	0.00112	4.77
P0CX26	60S ribosomal protein L43-B	RPL43B	2.1E-05	4.96
P14843	Phospho-2-dehydro-3-deoxyheptonate aldolase,	ARO3	0.00241	5.17

Accession	Protein Description	Gene Name	P-value	Max Fold Change
	phenylalanine-inhibited			
P39976	D-lactate dehydrogenase [cytochrome] 3	DLD3	3.9E-05	5.85
P32503	Major capsid protein	gag	6.7E-07	6.03
P07262	NADP-specific glutamate dehydrogenase 1	GDH1	1.2E-06	6.13
P33399	La protein homolog	LHP1	0.01589	6.86
P00899	Anthranilate synthase component 1	TRP2	0.0066	18.76

Journal of Proteomics 156 (2017) 104–112

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Glycation inhibitors extend yeast chronological lifespan by reducing advanced glycation end products and by back regulation of proteins involved in mitochondrial respiration

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ARTICLE INFO

Article history:
Received 27 October 2016
Received in revised form 27 December 2016
Accepted 23 January 2017
Available online 27 January 2017

Keywords:
Aging
Proteomics
Mass spectrometry
Glucose
Glycation

ABSTRACT

Advanced Glycation End products (AGEs) are implicated in aging process. Thus, reducing AGEs by using glycation inhibitors may help in attenuating the aging process. In this study using *Saccharomyces cerevisiae* yeast system, we show that Aminoguanidine (AMG), a well-known glycation inhibitor, decreases the AGE modification of proteins in non-calorie restriction (NR) (2% glucose) and extends chronological lifespan (CLS) similar to that of calorie restriction (CR) condition (0.5% glucose). Proteomic analysis revealed that AMG back regulates the expression of differentially expressed proteins especially those involved in mitochondrial respiration in NR condition, suggesting that it switches metabolism from fermentation to respiration, mimicking CR. AMG induced back regulation of differentially expressed proteins could be possibly due to its chemical effect or indirectly by glycation inhibition. To delineate this, Metformin (MET), a structural analog of AMG and a mild glycation inhibitor and Hydralazine (HYD), another potent glycation inhibitor but not structural analog of AMG were used. HYD was more effective than MET in mimicking AMG suggesting that glycation inhibition was responsible for restoration of differentially expressed proteins. Thus glycation inhibitors particularly AMG, HYD and MET extend yeast CLS by reducing AGEs, modulating the expression of proteins involved in mitochondrial respiration and possibly by scavenging glucose.

Significance: This study reports the role of glycation in aging process. In the non-caloric restriction condition, carbohydrates such as glucose promote protein glycation and reduce CLS. While, the inhibitors of glycation such as AMG, HYD, MET mimic the caloric restriction condition by back regulating deregulated proteins involved in mitochondrial respiration which could facilitate shift of metabolism from fermentation to respiration and extend yeast CLS. These findings suggest that glycation inhibitors can be potential molecules that can be used in management of aging.

1. Introduction

Aging is an irreversible degenerative process characterized by a general decline in cellular metabolic activity accompanied with progressive deterioration of cellular components resulting in enhanced mortality [1]. Short lived organisms, such as yeast is an excellent model for aging research [2]. Yeast displays two distinct lifespans, namely, replicative lifespan (RLS) and chronological lifespan (CLS), which serve as models for proliferating (mitotic) and non-proliferating (post-mitotic) tissues in higher eukaryotes, respectively [3]. RLS is defined as the

Abbreviations: AGEs, advanced glycation end products; CR, calorie restriction; NR, non-calorie restriction; CLS, chronological lifespan; AMG, aminoguanidine; MET, metformin; HYD, hydralazine; RLS, replicative lifespan; Sch9, threonine/threonine, protein kinase; TOR, Target of Rapamycin kinase; AKT, protein kinase B; IGF, insulin like growth factor; *C. elegans*, *Caenorhabditis elegans*; Sir2, silent information regulator 2; NAD^+, nicotinamide adenine dinucleotide; Pck1, phosphoenolpyruvate carboxykinase 1; YEPD, yeast extract peptone dextrose; SC, synthetic complete; CFUs, colony forming units; DTT, dithiothreitol; SDS, PAGE sodium dodecyl sulphate polyacrylamide gel electrophoresis; PVDF, polyvinylidine fluoride; PBS, phosphate buffer saline; PBS, T–phosphate buffer saline with Tween 20; IDA, information dependent acquisition; SWATH, sequential window acquisition of all theoretical masses; TOF, time of flight; ACN, acetonitrile; RP-HPLC, reverse phase high performance liquid chromatography; DAVID, Database for Annotation Visualization and Integrated Discovery; BINGO, Biological Network Gene Ontology; ROS, reactive oxygen species; MALDI, matrix assisted laser desorption ionization; iTRAQ, isobaric tag for relative and absolute quantitation; SILAC, stable isotope labeling with amino acids in cell culture.

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http://dx.doi.org/10.1016/j.jprot.2017.01.015
1874-3919/

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Education

2011-Present	PhD in Biotechnology, CSIR-National Chemical Laboratory, Pune, India
2008-2010	Masters in Biotechnology, Vidya Pratishthan's school of biotechnology, Baramati, India
2004-2008	Bachelors in Biotechnology, Vidya Pratishthan's school of biotechnology, Baramati, India

Research Experience

2010-Present	Doctoral fellow CSIR-National Chemical Laboratory, Pune, India Advisor: Dr. Mahesh J. Kulkarni Project: Regulation of aging in yeast by glycation inhibitors.

Skills and Techniques

- Proteomic techniques: one and two dimensional electrophoresis, Western blot, PTM analysis and ELISA
- Mass spectrometry: Protein digestion techniques, identification and label free quantification of proteins by mass spectrometer. Handled Q-exactive Orbitrap MS (LC-QTOF), Waters-SYNAPT HDMS (LC-QTOF) and AB SCIEX TOF/TOF 5800 system. Mass spectrometry related software Proteome Discoverer, Protein lynx Global Server, Protein Pilot, PeakView and MarkerView
- Molecular biology: mRNA isolation and cDNA preparation, PCR techniques and Real time PCR
- Bioinformatics: protein- protein interaction networks, Software used DAVID, Cytoscape, BINGO, STRING.

Awards and Fellowship

2010	University Grant Commission-Junior research fellowship (UGC-JRF) with266th national rank for five years Ph.D fellowship

2010	Graduate Aptitude Test in Engineering (GATE) Biotechnology fellowship

Conferences

- Targeted Proteomics Workshop held at IIT Bombay, Mumbai, India during 10th to 14th Dec 2015.
- International Symposium on Bio-organic Chemistry (ISBOC-10), 2015, IISER, India.
- 6th Annual meeting of the Proteomics Society of India PSI, 2014, Bombay, India.
- 4th Annual meeting of the Proteomics Society of India PSI, 2012, Pune, India.
- 2nd Annual Meeting of the Proteomics Society, India PSI and International Proteomic Conference, 201, Delhi, India.
- 94th Indian Science Congress, 2007, Chennai, India.

Publications

- **R. S. Kazi,** R. M. Banarjee, A. B. Deshmukh, G. V. Patil, M. G. Jagadeeshaprasad, M. J. Kulkarni, M. J. Glycation inhibitors extend yeast chronological lifespan by reducing advanced glycation end products and by back regulation of proteins involved in mitochondrial respiration, ***Journal of proteomics*** 156 (2017) 104-112.

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