

GENOME WIDE ANALYSIS OF SMALL HEAT SHOCK PROTEINS INVOLVED IN YEAST AGEING

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This thesis is the result of my own work.

The material contained in the thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree of other qualification.

The research was performed at the Physiological Laboratory, Institute of Translational Medicine, University of Liverpool.

The data presented here was obtained by myself.

I dedicate this thesis to my loving grandparents Beatrice and Leslie Jacks, who have provided me with unconditional love and support throughout my life, and helped to make me the person I am today.

I also dedicate this thesis in loving memory of Philip Bloxam (Senior), Julie Sherlock and Philip Bloxam (Junior).

*I feel a warmth around me like your presence is so near,
And I close my eyes to visualize your face when you were here,
I endure the times we spent together and they are locked inside my heart,
For as long as I have those memories we will never be apart,
Even though we cannot speak no more my voice is always there,
Because every night before I sleep I have you in my prayer.*

By Louise Bailey

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Ageing is a phenomenon common to almost all living organisms and is characterised by the accumulation of changes with time that are associated with the ever-increasing susceptibility to disease and inevitably death. The rate of ageing is species-specific, indicating a strong genetic component. It is now widely accepted that genes involved in basic cellular processes such as stress resistance, metabolic regulation and genomic stability determine longevity in divergent organisms from yeast to mammals. This suggests that there may be conserved universal mechanisms involved in ageing. Furthermore, lifespan can be increased in all these model organisms by reducing the nutrients consumed - a phenomenon known as dietary restriction (DR). *Saccharomyces cerevisiae* is a useful model organism in which to study ageing and has been at the forefront of recent pioneering work on the molecular mechanisms underlying lifespan extension by DR. DR can be studied by reducing the glucose concentration from the standard 2% down to 0.5% or below, or by using genetic mimics. However, the exact mechanisms of lifespan extension by dietary restriction remain unclear and highly controversial.

Research in our laboratory has identified two proteins in yeast that are induced in response to DR and thus correlate with longevity. Both proteins, Hsp12 and Hsp26, belong to the small heat shock protein (sHsp) family. Previous work by the Morgan laboratory has found that Hsp12 is essential for the longevity effect of DR and have solved the structure of the protein by NMR. Further studies have revealed a genetic interaction between *HSP12* and *HSP26*, as *hsp12/hsp26Δ* double knockouts show a strongly reduced mean and maximum replicative lifespan. Despite this, the *hsp12/hsp26Δ* double knockout is not defective in various processes associated with yeast ageing, including stress resistance, rDNA silencing or protein aggregation.

To shed light on the mechanisms by which Hsp12 and Hsp26 affect longevity, we employed unbiased approaches of synthetic genetic array (SGA) and quantitative fitness analysis (QFA) to identify genetic interactions of *HSP12* and *HSP26* on a genome-wide scale. This involved the generation of thousands of double mutant strains and analysis of their growth under various conditions, including DR. Results from the SGA analysis have revealed genetic interactions between *HSP12* and *HSP26* with the regulation of transcription from RNA

polymerase II and processes associated with the mitochondria and vacuoles. QFA data is still to be analysed but ultimately we hope that QFA will re-confirm the genetic interactions identified by SGA analysis. We hope both SGA analysis and QFA data will provide insight into the cellular functions of Hsp12 and Hsp26 and how these proteins affect ageing, in particular lifespan extension by DR.

TABLE OF CONTENTS

DECLARATION.....	1
ACKNOWLEDGMENTS.....	2
ABSTRACT.....	4
ABBREVIATIONS.....	10
CHAPTER 1. INTRODUCTION.....	13
1.1 AN INTRODUCTION TO AGEING.....	13
1.1.1 A basic introduction to ageing.	13
1.1.2 The evolution of ageing.	15
1.2 THEORIES OF AGEING.....	17
1.2.1 Mutation accumulation theory of ageing.	17
1.2.3 The Disposable soma theory of ageing.	18
1.2.4 The free radical theory of ageing.	18
1.2.5 DNA damage and genome stability theory.	20
1.2.6 Replicative senescence.	20
1.3 AGEING IN YEAST	22
1.3.1 A brief Introduction to yeast ageing.	22
1.3.2 Theories of yeast ageing.	23
1.4 DIETARY RESTRICTION IN YEAST.....	25
1.4.1 Methods of DR in yeast.	25
1.5 THEORIES OF DR-MEDIATED LIFESPAN EXTENSION IN YEAST.....	26
1.5.1 DR-mediated lifespan extension by activation of Sir2.	26
1.5.2 DR-mediated lifespan extension by nutrient sensing pathways.....	29
1.5 DIETARY RESTRICTION IN OTHER MODEL ORGANISMS.....	32
1.6.1 DR in <i>Drosophila melanogaster</i>	32
1.6.2 DR-mediated lifespan extension in <i>Caenorhabditis elegans</i>	33
1.6.3 DR-mediated lifespan extension in rodents and higher organisms.	35
1.7 THE SMALL HEAT SHOCK PROTEINS.....	38
1.7.1 Overview of the heat shock response and heat shock proteins.	38
1.7.2 Small heat shock proteins in <i>S. cerevisiae</i>	41
1.7.3 <i>S. cerevisiae</i> Hsp12.	41
1.7.3 <i>S. cerevisiae</i> Hsp26.	43
1.8 THE SHSPS IN AGEING AND DISEASE.	46
1.8.1 The sHsps and ageing.	46
1.8.2 Stress proteins and human disease.	47
1.9 AIMS OF THE THESIS.	50
CHAPTER 2. MATERIALS AND METHODS.....	51
2.1 YEAST.	51
2.1.1 Strains used in this study.	51
2.1.2 Standard yeast culture media.	51
2.1.3 Construction of knockout strains by PCR-mediated gene disruption.	52
2.1.4 Construction of knockout strains by replica plating.	55
2.1.5 Confirmation of knockout mutants.	57

2.1.7 Qualitative silencing assays.	59
2.1.8 Stress assays.	59
2.1.9 Aggregation assays.	60
2.1.10 Laemmli yeast whole cell extraction.	61
2.2 WESTERN BLOTTING.	62
2.2.1 SDS polyacrylamide gel electrophoresis (SDS-PAGE).....	62
2.2.2 Western blotting.....	62
2.3 CLONING.....	63
2.3.1 Cloning synthetic <i>HSP12</i> gene from plasmid into expression vector pE-SumoPro Kan.	63
2.3.2 Ligation.	66
2.3.3 Transformation into <i>E. coli</i> TOP10.	66
2.3.7 Cloning for overexpression of <i>HSP12</i>	69
2.4 AFFINITY PURIFICATION OF HSP12 ANTIBODY.....	70
2.5 SYNTHETIC GENETIC ARRAY (SGA) ANALYSIS.....	71
2.5.1 Construction of query strains for SGA.	71
2.5.2 Random Spore Analysis (RSA).	72
2.5.3 SGA analysis.	75
2.6 QUANTITATIVE FITNESS ANALYSIS (QFA).....	80
2.7 REPLICATIVE LIFESPAN ANALYSIS.	81
2.8 FLUORESCENT MICROSCOPY FOR DETERMINATION OF VACUOLAR ACIDIFICATION.	83
CHAPTER 3. THE <i>HSP12/HSP26Δ</i> DOUBLE MUTANT HAS A REDUCED REPLICATIVE LIFESPAN.....	85
3.1 INTRODUCTION.....	85
3.1.1 Using <i>Saccharomyces cerevisiae</i> to study ageing.	85
3.1.2 Small heat shock protein expression during ageing.	85
3.2 METHODS.	90
3.2.1. Construction of deletion strains.	90
3.2.2 Western blotting against Hsp12 and Hsp26.	90
3.3 RESULTS.....	92
3.3.2 Determining the reactivity of Hsp12 and Hsp26 custom made antibodies.	94
3.3.3 Heat shock protein expression during glucose depletion.....	96
3.3.4 Replicative lifespan analysis.	100
3.4 DISCUSSION.	107
3.4.1 Hsp12 and Hsp26 are up-regulated in response to glucose depletion.....	107
3.4.2 The roles of the Hsps in longevity.....	110
3.4.3 Conclusion.	114
CHAPTER 4. CHARACTERISATION OF THE <i>HSP12/HSP26Δ</i> DOUBLE MUTANT.....	115
4.1 INTRODUCTION.....	115
4.1.1 Theories of yeast ageing.	115
4.1.2 Protein aggregation, autophagy and mitochondrial dysfunction.....	117
4.2 METHODS.	119

4.2.1. Yeast strains.....	119
4.3 RESULTS.....	120
4.3.1 Deletion of <i>HSP12</i> and <i>HSP26</i> does not cause a general sickness to the cell.	120
4.3.2 Deletion of <i>HSP12</i> and <i>HSP26</i> does not increase susceptibility to stresses or affect silencing at the telomeres.	122
4.3.3 Double deletion of <i>HSP12</i> and <i>HSP26</i> does not affect silencing at the rDNA.....	125
4.3.4 Glucose depletion did not cause an observable increase in protein aggregation in the <i>hsp12/hsp26Δ</i> double mutant.....	128
4.3.5 Double deletion of <i>HSP12</i> and <i>HSP26</i> does not increase mitochondrial dysfunction or alter vacuolar acidity.	131
4.4 DISCUSSION	137
4.4.1 Double deletion of <i>HSP12</i> and <i>HSP26</i> does not increase susceptibility to stresses.	137
4.4.2 Double deletion of <i>HSP12</i> and <i>HSP26</i> does not affect telomeric or rDNA silencing.	138
4.4.3 The <i>hsp12/hsp26Δ</i> double mutant does not show increased protein aggregation.	140
4.4.4 The <i>hsp12/hsp26Δ</i> double mutant does not show a reduction in vacuolar acidity or any alterations in mitochondrial structure.....	142
4.4.5 Conclusion.	144
 CHAPTER 5. AN UNBIASED APPROACH TO IDENTIFY GENETIC INTERACTIONS OF <i>HSP12/HSP26</i>.....	 145
5.1 INTRODUCTION.....	145
5.1.1 Findings so far.....	145
5.1.2 Introduction to SGA analysis.....	145
5.2 METHODS.	147
5.2.1 Interpretation of SGA results.....	147
5.2.2. Construction of knockout strains by replica plating.	148
5.2.3 Spot tests.	149
5.3 RESULTS.....	150
5.3.1 Construction of query strains for synthetic genetic array analysis.....	150
5.3.2 Confirmation of query strains by random spore analysis (RSA).	150
5.3.3 Synthetic genetic array analysis of <i>HSP26</i> with standard conditions.	155
5.3.4 Confirmation of synthetic genetic interactions identified by SGA.	162
5.3.5 Synthetic genetic array analysis of <i>HSP26</i> with glucose depletion.....	164
5.3.6 Synthetic genetic array analysis of <i>HSP12</i> with standard conditions.	169
5.3.6 Synthetic genetic array analysis of <i>HSP12</i> with glucose depletion.....	174
5.3.7 SGA analysis with stress conditions, a comparison between <i>HSP12</i> and <i>HSP26</i>	178
5.3.8 Identification of genes associated with the mitochondria and vacuoles.	182
5.4 DISCUSSION	186
5.4.1 Comparison of findings with other genetic screens.	186
5.4.2 The genetic link between the <i>HSP12</i> and <i>HSP26</i> and genes involved in stress response.	188
5.4.3 The genetic link between <i>HSP26</i> and genes involved in transcription from RNA polymerase II.	189
5.4.4 The genetic link between <i>HSP12</i> and genes associated with lipid metabolism, protein lipidation, cytoskeleton organisation and membrane fusion.	191

5.4.5 The genetic link between <i>HSP12</i> and <i>HSP26</i> and genes associated with the mitochondria.	192
5.4.6 The genetic link between <i>HSP12</i> and <i>HSP26</i> and genes associated with the vacuoles.	195
5.7 CONCLUSION	198
CHAPTER 6. DISCUSSION AND FUTURE WORK.....	196
6.1 SHSPS EXPRESSION DURING DIETARY RESTRICTION.	199
6.1.1 Hsp12 and Hsp26 are strongly induced by DR.	199
6.2 THE ROLE OF HSP12 AND HSP26 IN DR-MEDIATED LIFESPAN EXTENSION.	199
6.2.1. Hsp12 and Hsp26 are essential for DR-mediated lifespan extension.	199
6.2.3 <i>Hsp12/hsp26Δ</i> double mutants do not appear to be defective in processes associated with yeast ageing.	201
6.2.4 Genome wide analysis of genetic interactions of <i>HSP12</i> and <i>HSP26</i>	202
6.3 CONCLUSION.	204
APPENDIX.....	202
REFERENCES.....	206

ABBREVIATIONS

<i>AAP1</i>	Arginine/alanine AminoPeptidase
AC	Adenylate Cyclase
<i>age-1</i>	AGEing alteration-1
AMP	Adenosine Monophosphate-activated Protein
<i>ASH1</i>	Asymmetric Synthesis of HO
<i>Atg5</i>	Autophagy gene 5
<i>ATG-7</i>	Autophagy-related gene 7
<i>ATG-12</i>	Autophagy-related gene 12
<i>BUD27</i>	BUD site selection
cAMP	cyclic Adenosine MonoPhosphate
<i>CDC3</i>	Cell Division Cycle
<i>CDC9</i>	Cell Division Cycle
<i>CDC45</i>	Cell Division Cycle
CDK	Cyclin Dependent Kinase
CTD	C-Terminal Domain
<i>CTK1</i>	Carboxy-Terminal domain Kinase
CTL	Control
<i>DAF-2</i>	abnormal Dauer Formation-2
<i>DAF-16</i>	Forkhead transcription factor
DMSO	DiMethylSulfoxide
DNA	DeoxyriboNucleic Acid
DR	Dietary Restriction
ECL	Enhanced ChemiLuminescence
<i>ECM30</i>	ExtraCellular Mutant
ERCs	Extrachromosomal rDNA Circles
<i>ERG6</i>	ERGosterol biosynthesis
<i>ESC2</i>	Establishment of Silent Chromatin
EtOH	Ethanol
FOA	5'-Fluororotic Acid
<i>FOB1</i>	FOrk Blocking less, replication fork barrier protein
FOXO	FOrkhead BoX transcription factors
FFL	FireFly Luciferase
GDP	Guanosine DiPhosphate
GFP	Green Fluorescent Protein
GH	Growth Hormone
<i>Glk1</i>	Glucokinase
Gly	Glycine
GO	Gene Ontology
<i>Gpa2</i>	G protein alpha subunit
GPD	Glyceraldehyde 3-phosphate dehydrogenase
<i>Gpr1</i>	G-protein coupled receptor
GTP	Guanosine TriPhosphate
H ₂ O ₂	Hydrogen Peroxide
HCl	Hydrogen Chloride
HCS	High Content Screening
HD	Huntington's Disease

<i>HXK2</i>	HeXoKinase
Hsc70	Heat shock cognate 70
HOG	High Osmolarity Glycerol pathway
HSPBs	Human small heat shock proteins
HSEs	Heat Shock Elements
<i>HSF1</i>	Heat Shock Factor 1
Hsp	inducible Heat Shock Protein
ICE	Ion Exchange Chromatography
IGF-1	Insulin-like Growth Factor
ILS	Insulin Signalling Pathway
IPTG	Isopropyl- β -D-ThioGalactopyranoside
kDa	kilo Dalton
LEU	Leucine
LB	Luria Bertani
LiAC	Lithium Acetate
<i>LRS4</i>	Loss of RDNA Silencing
MAP	Mitogen Activated Protein
MEP	Mother Enrichment Programme
Msn2	Multicopy suppressor of SNF1 mutation
Msn4	Multicopy suppressor of SNF1 mutation
mtDNA	mitochondrial DNA
MW	Molecular Weight
NAD ⁺	Nicotinamide Adenine Dinucleotide, oxidised form
NADH	Nicotinamide Adenine Dinucleotide, reduced form
NDLB	Non Denaturing Lysis Buffer
NaCl	Sodium Chloride
NEM	N-ethylmaleimide
<i>NPL3</i>	Nuclear Protein Localisation
<i>NPT1</i>	Nicotinate PhosphoribosylTransferase
<i>NPY1</i>	NADH PYrophosphatase
<i>NYV1</i>	New Yeast V-SNARE
<i>NUT1</i>	Negative regulation of URS Two
<i>MED1</i>	MEDIator complex
MeOH	Methanol
MMS	methanesulfonate
ORF	Open Reading Frame
OSH	Oxysterol binding protein family
<i>OST3</i>	OligoSaccharylTransferase
<i>PNC1</i>	pyranzinamidase/nicotinamidase
P2RT	Pellet 2 room temperature
P243	Pellet 2 43 °C
PBS	Phosphate Buffered Saline
<i>PBS2</i>	Polymyxin B Sensitivity
PEG	Polyethylene Glycol
PCR	Polymerase Chain Reaction
PIKK	Phosphatidylinositol Kinase-related Kinase
PKA	adenosine 3',5'-monophosphate-dependent protein kinase

Pnc1	pyranzinamidase/nicotinamidase
QFA	Quantitative Fitness Analysis
QUIN	Quinacrine
<i>RAD9</i>	RADiation sensitive
<i>RCY1</i>	ReCYcling
rDNA	ribosomal DNA
RAS	RAS proto-oncogene
RENT	Regulator of Nucleolar silencing and Telophase Exit
RLS	Replicative lifespan
ROS	Reactive Oxygen Species
<i>RPD3</i>	Reduced Potassium Dependency 3
<i>RPL22A</i>	Ribosomal Protein of the Large Subunit
RRP1	Ribosomal RNA Processing
<i>RSC2</i>	Remodel the Structure of Chromatin
S6K1	ribosomal S6 protein kinase
SDS	Sodium Dodecyl Sulfate
SGA	Synthetic Genetic Analysis
SDS-PAGE	SDS-PolyAcrylamide Gel Electrophoresis
sHsp	Small Heat Shock Protein
<i>SCH9</i>	ortholog of mammalian S6 kinase
<i>SGS1</i>	Slow Growth Suppressor
SN1RT	Supernatant 1 room temperature
SN2RT	Supernatant 2 room temperature
SN143	Supernatant 1 43 °C
SN243	Supernatant 2 43 °C
<i>SSA1</i>	Stress-Seventy subfamily A
<i>SSD1</i>	Suppressor of SIT4 Deletion
<i>SIR2</i>	Silent Information Regulator
<i>STE11</i>	STERile
<i>SRB8</i>	Suppressor of RNA polymerase B
STRE	Stress Response Elements
<i>SUP35</i>	SUPpressor
Thr	Threonine
TIM50	Translocase of the Inner Mitochondrial membrane
TOM70	Translocase of the Outer Mitochondrial membrane
<i>TOR1</i>	Target Of Rapamycin 1
TORC1	Target of Rapamycin Complex 1
<i>TRX2</i>	ThioRedoXin
TRIZMA	Tris(hydroxymethyl)methylamine
<i>TSA1</i>	Thiol-Specific Antioxidant
TSS	Transcription Start Sites
<i>URE3</i>	UREidosuccinate transport
Val	Valine
WT	Wildtype
YCP	Yeast Centromere Plasmid
<i>YDJ1</i>	Yeast dnaJ
YPD	Yeast Peptone Dextrose

AN INTRODUCTION TO AGEING.

1.1.1 A basic introduction to ageing.

Ageing or senescence can usually be defined as a progressive increase in the age-specific death rate even when the population is maintained under conditions that are ideal for survival (Kirkwood and Rose, 1991). The progressive increase in age-specific death rate is due to deterioration in a wide range of physiological and metabolic functions (Kirkwood and Rose, 1991). The deterioration in cellular functions can be attributable to an eventual failure of maintenance, which has also been used as a definition for ageing (Holliday, 2006). The definition of ageing as the failure of maintenance was suggested from evidence showing that long-lived individuals have much more efficient maintenance mechanisms than short-lived animals (Holliday, 2006). The combined decline in cellular functions and maintenance mechanisms ultimately leaves the organism susceptible to intrinsic and extrinsic factors which may cause death (Kirkwood and Rose, 1991).

Rates of ageing are species-specific and the characteristics of ageing are different between individuals (Madden and Cloyes, 2012, Kirkwood and Rose, 1991). This therefore reflects how ageing is a multidimensional process, complicated further by genetic and environmental influences (Madden and Cloyes, 2012). The rate of ageing appears to be determined by a complex interplay between an individual's genetic makeup and environmental influences (Bartke, 2011). For example, there are some genetic diseases that lead to premature ageing including Hutchinson-Gilford progeria syndrome (HGPS) and Werner syndrome (WS) (Plasilova et al., 2011, Monnat, 2010). HGPS is caused by mutations in the lamin A/C gene affecting the adipose tissue, bone, cartilage and the cardiovascular system (Plasilova et al., 2011). WS is caused by mutations that lead to loss of function of RECQ helicases; this results in abnormalities in DNA repair, replication and telomere maintenance (Monnat, 2010, Muftuoglu et al., 2008).

In contrast to gene mutations that shorten human lifespan, there are genes that are associated with human longevity such as apolipoprotein E (*APOE*) and forkhead box O3A (*FOXO3A*) (Wheeler and Kim, 2011). The ϵ 4 allele of *APOE* is associated with an increased risk of Alzheimer's disease and cardiovascular disease, whereas the ϵ 2 allele is thought to offer a protective effect against Alzheimer's

disease and cardiovascular disease and is enriched in long-lived individuals (Wheeler and Kim, 2011). The forkhead (FOXO) transcription factor is negatively regulated by the insulin signalling pathway and contains alleles associated with longevity in European and Asian populations (Wheeler and Kim, 2011).

In addition to genetic influences, environmental influences also affect the rate of ageing (Ordovas and Smith, 2010). Factors such as air pollution, sun radiation, stresses such as smoking, alcohol abuse, and internal factors such as metabolism, fatigue, inflammation and illness have all been suggested to impact on ageing since they produce free radicals in human tissues and skin (Lademann et al., 2011). Free radicals provide a protective role against viral and bacterial infection. In response to a variety of pathogens, neutrophils, a family of immune cells, release large quantities of superoxide anion ($O_2^{\bullet-}$), in a process known as respiratory burst (Oliveira et al., 2010). However, inappropriate activation of respiratory burst can cause both tissue injury and prolonged infection (Oliveira et al., 2010). Hydrogen peroxide (H_2O_2) is produced as a by-product from the reduction of $O_2^{\bullet-}$ by superoxide dismutase (SOD). Superoxide anions and H_2O_2 are classified as reactive oxygen species (ROS) as they contain unpaired electrons which are highly reactive (Oliveira et al., 2010). Both $O_2^{\bullet-}$ and H_2O_2 can attack biomolecules causing oxidative damage to mitochondrial DNA (Oliveira et al., 2010) (Nonaka and Hasegawa, 2011). It is thought that ROS and oxidative damage contribute to the ageing process, however studies to investigate this hypothesis have reported conflicting results (discussed later) (Gems and Partridge, 2013, Perez et al., 2009).

Not all environmental factors have a negative effect on the rate of ageing; Dietary restriction (DR), for example, has been shown to have a positive effect on ageing. DR is defined as a reduction in total nutrient intake without malnutrition, and has been shown to significantly increase lifespan and reduce the incidence of age-related diseases in many model organisms including *Saccharomyces cerevisiae* (Baker's yeast), *Caenorhabditis elegans*, *Drosophila melanogaster*, rodents and primates (Colman et al., 2009, Colman and Anderson, 2011, Smith et al., 2010).

1.1.2 The evolution of ageing.

The science of cellular ageing originated in 1881, at this time August Weismann was considering the role of natural selection in regulating the duration of an organism's life (Kirkwood and Cremer, 1982). Weismann was an important follower of Charles Darwin, and his ideas on ageing included the theory that if long-lived individuals of a species obtain some advantage in the struggle for existence, they gradually become dominant, in contrast, those with the shortest lives will become exterminated (Rose et al., 2008). From this, Weismann predicted that the somatic cells of higher organisms have limited division potential (Kirkwood and Cremer, 1982). Sadly, Weismann's work on ageing was neglected until the early 1960s when Hayflick and Moorhead demonstrated that diploid human fibroblasts are restricted to a finite number of divisions *in vitro*, a phenomenon termed the 'Hayflick limit' (Hayflick and Moorhead, 1961).

Despite considerable research during much of the 20th century the underlying mechanisms of ageing remained unclear (Holliday, 2006). However, towards the end of the 20th century it became apparent that for animals to survive they need to develop to an adult, but not invest resources for maintaining the body or soma, indefinitely (Holliday, 2006). Instead, there is a trade-off between the investment of resources for reproduction and for lifespan (Holliday, 2006). This theory can help explain the different rates of ageing between species. Those that develop and reproduce fast have a short lifespan. In contrast, those that develop and reproduce more slowly have a long lifespan (Holliday, 2006).

In the 1940's Peter Medawar started to pursue the idea that the force of natural selection declines with age (Rose et al., 2008, Ljubuncic and Reznick, 2009). This observation was founded since all organisms eventually die from different causes (Ljubuncic and Reznick, 2009). From this, Medawar proposed that genes beneficial early in life are favored by natural selection over genes beneficial late in life (Charlesworth, 2000). Medawar continued with the idea that natural selection declines with age, thus making it possible for hazardous late acting genes to exist, a theory known as mutation accumulation (Medawar, 1952). The mutation accumulation theory was later modified by Charlesworth, in a model which suggests

that mortality plateaus are predicted to occur at late ages if alleles affecting fitness do so for more than one specific age range (Charlesworth, 2001).

Also working independently on the theory of natural selection at this time was an American scientist, George Williams (1957). Williams focused on the idea that natural selection actively favours genes that have a beneficial effect during youth, however are harmful at later ages and cause ageing as a side-effect (Rose et al., 2008). This theory was later named antagonistic pleiotropy (Rose et al., 2008). The major difference between the mutation accumulation theory and antagonistic pleiotropy theory of ageing, is in the latter, genes with negative effects are actively kept in the gene pool by selection, whereas in the mutation accumulation theory, genes with negative effects at old age accumulate in one generation to the next (Le Bourg, 2001).

Interestingly, some animals show negligible ageing/senescence and therefore form contradictions to existing theories of ageing (Finch and Austad, 2001). The term negligible senescence was first described by Caleb Finch and was used to describe animals which show very slow or negligible ageing (Finch and Austad, 2001). Finch described the criteria for negligible senescence as those subjects that do not show any observable age-related increase in mortality rate or decrease in reproduction rate after sexual maturity, and no observable age-related decline in physiological capacity or disease resistance (Finch and Austad, 2001, Guerin, 2004). Animals, which show negligible ageing, include rockfish, sturgeon, turtles and lobsters (Guerin, 2004). These animals therefore provide important models to study genetic and biochemical processes involved in slow ageing which may then be applied to human ageing (Guerin, 2004).

1.2 THEORIES OF AGEING.

1.2.1 Mutation accumulation theory of ageing.

The mutation accumulation theory of ageing proposes that natural selection does not occur in long-lived animals and ageing is an inevitability of the declining force of natural selection with age (Ljubuncic and Reznick, 2009, Gavrilov and Gavrilova, 2002, Medawar, 1952). In general, natural selection will bring forward the time of expression of 'good' genes and delay the time of expression of 'bad' genes (Kirkwood and Cremer, 1982). Therefore, a mutant gene, which kills young children, will be strongly selected against, while a lethal mutation which effects people over the age of 80 will experience no selection as this mutation will have already passed onto their offspring (Gavrilov and Gavrilova, 2002). The theory of mutation accumulation therefore suggests that over time we accumulate a 'genetic dustbin' of deleterious mutations with very late age-specific effects which are responsible for ageing (Kirkwood and Cremer, 1982). This theory does not attribute any benefit or any advantage to the process of senescence; instead ageing is a result of the decline of natural selection (Kirkwood and Cremer, 1982). The mutation accumulation theory of ageing was modified after the discovery of late-life mortality plateaus (Charlesworth, 2001). Experiments in *Drosophila*, have found that allelic effects are age-specific with effects extending over 1-2 weeks across all age classes, consistent with the modified mutation accumulation theory of ageing (Reynolds et al., 2007).

1.2.2 Antagonistic pleiotrophy.

The theory of antagonistic pleiotrophy is very similar to the mutation accumulation theory of ageing and also recognises that the force of natural selection declines with age (Gavrilov and Gavrilova, 2002, Kirkwood and Cremer, 1982). However, in this theory ageing is a result of genes that have good effects in early life but become harmful later on, owing to its alternative name of the 'pay later' theory (Gavrilov and Gavrilova, 2002, Kirkwood and Cremer, 1982). For example, a gene that increases survival to reproductive age will be favoured by natural selection if it decreases the chances of dying at age 10-20 (Ljubuncic and Reznick, 2009). Therefore, harmful late-acting genes can remain in a population if

they have a beneficial effect early in life. Replicative cellular senescence (discussed later) is a good example of antagonistic pleiotropy. The cell division limit ('Hayflick limit') is known to suppress tumorigenesis by arresting cell growth (Krtolica et al., 2001). The suppression of tumorigenesis early in life, may promote cancer later in life as senescent cells stimulate other premalignant cells to proliferate and form tumours (Krtolica et al., 2001).

1.2.3 The Disposable soma theory of ageing.

The disposable soma theory of ageing proposes that there will be a progressive accumulation of unrepaired cellular damage that causes frailty, disease and ultimately death (Kirkwood, 1977, Kirkwood and Holliday, 1979). In this theory, longevity is determined through 'longevity assurance' genes that control somatic maintenance and repair and provide an optimal compromise between investments in somatic maintenance and in reproduction (Kirkwood et al., 2000). The disposable soma theory predicts that the optimum level of investment in repair, which maximizes fitness, will ultimately be less than the minimum required for indefinite survival (Kirkwood and Cremer, 1982). The exact optimum is species-specific and the level of maintenance invested depends upon the degree of extrinsic hazard in the species' niche (Kirkwood and Cremer, 1982, Kirkwood et al., 2000). Since there are so many maintenance mechanisms it is also likely that many different genes control ageing and longevity. This theory provides an explanation as to why higher metazoans have not followed an evolutionary path which have permitted them indefinite survival (Kirkwood and Cremer, 1982).

1.2.4 The free radical theory of ageing.

Denham Harman first postulated the free radical theory of ageing in 1956 (Harman, 1956). In this theory, oxygen free radicals formed from normal metabolic processes are thought to increase oxidative damage to macromolecules thereby contributing to the ageing process and its associated degenerative diseases (Perez et al., 2009, Vina et al., 2013). As free radicals are continuously generated during the life of the cell, the antioxidant systems become overburdened, this results in oxidative damage in the cell and tissues (Vina et al., 2007). Harman also suggested that mitochondria are a key organelle of the ageing process (Vina et al., 2013). The

role of the mitochondria in the ageing process is supported by the fact that these organelles are responsible for the continuous generation of reactive oxygen species (ROS) (Golden et al., 2002). Furthermore, mitochondrial dysfunction is a characteristic of age in many eukaryotes and associated with this dysfunction is an increase in ROS (Wang et al., 2013).

The free radical theory of ageing has also been termed the oxidative stress theory of ageing (Perez et al., 2009). This modification was brought about by the fact that oxygen species such as peroxides and aldehydes, which are not free radicals, also play a role in causing oxidative damage to cells (Perez et al., 2009). A vast amount of research into the free radical theory/oxidative stress theory of ageing has raised controversial results, with some experiments favouring this theory and others showing results against this theory (Perez et al., 2009, Vina et al., 2013).

Experiments on mice studying 18 genetic manipulations in the antioxidant defense system and their effects on lifespan have shown strong evidence against the free radical/oxidative stress theory in playing a major role in ageing in mice (Perez et al., 2009). In this study only 1 mouse model null for superoxide dismutase (*Sod1*) had an effect on lifespan which could be attributable to the free radical/oxidative stress theory of ageing (Perez et al., 2009). Early experiments in *Drosophila* and *C. elegans* appeared to support the free radical/oxidative stress theory of ageing (Gems and Partridge, 2013). In *Drosophila* and *C. elegans* increasing the activity of SOD by genetic manipulation or pharmacological means has been shown to increase lifespan (Orr and Sohal, 1994, Melov et al., 2000). However, it has since been shown that the conclusions drawn from these two studies appear to have been the result of experimental artifact (Gems and Partridge, 2013). Furthermore, recent studies in *C. elegans* have found strong evidence against the free radical/oxidative stress theory of ageing (Gems and Partridge, 2013). Notably, studies that abolish the *sod* gene function do not result in a reduced lifespan and other studies that increase ROS and/or oxidative damage have reported an increase in lifespan (Gems and Partridge, 2013). In contrast, some mammalian studies have shown a correlation between oxidative stress and cognitive functions (Golden et al., 2002). Supplementing the diet of rats with extracts of fruits and vegetables containing high levels of anti-oxidants has been

shown to reverse age-related cognitive defects (Joseph et al., 1998). Furthermore, adding extracts of blueberry, spinach or strawberry to the diet of old animals for 8 weeks was shown to reverse the age-related functional decline and reduce oxidative stress (Joseph et al., 1999).

1.2.5 DNA damage and genome stability theory.

Genome instability has been implicated as a cause of ageing since the discovery that low doses of radiation shorten lifespan and promote the accumulation of pathological lesions later on in life (Curtis, 1963). Genome instability occurs naturally and is the tendency of the genome to undergo alterations under physiological conditions (Vijg and Suh, 2013). These alterations include; chemical damage to the DNA, mutations and epimutations (Vijg and Suh, 2013).

When the chemical structure of the DNA becomes different to the otherwise normal structure, this is referred to as DNA damage (Vijg and Suh, 2013). DNA damage can be caused by both endogenous and exogenous sources. Endogenous sources include depurination and depyrimidination due to spontaneous hydrolysis and oxidation, which cause chemical lesions. Exogenous sources include UV light, ionizing radiation and mutagens present in cigarette smoke and diesel exhaust fumes (Vijg and Suh, 2013). A cell can respond to DNA damage by arresting mitosis temporarily to allow repair or arrest mitosis permanently leading to cellular senescence (Vijg and Suh, 2013). Occasionally, repair is erroneous or the cell fails to correctly replicate the genome during cell division leading to mutations and epimutations (Vijg, 2000). It is now known that mutations accumulate in various tissues of model organisms including flies, mice and humans (Vijg and Suh, 2013). It is thought that accumulation of DNA damage and mutations leads to genome instability and results in a functional decline in cellular processes leading disease susceptibility in old age (Vijg and Suh, 2013).

1.2.6 Replicative senescence.

For most of the 20th century it was thought that all cultured cells if provided with proper conditions would replicate indefinitely (Hayflick, 2003). However, more than 50 years ago it was discovered that fibroblasts have a finite proliferation

capacity in cell culture, a phenomenon known as the 'Hayflick limit' (Hayflick and Moorhead, 1961). Hayflick and Moorhead concluded that normal, mortal human cells contain a replication counting mechanism. This conclusion was made since fibroblasts from different embryonic donors underwent a finite number of population doublings and cells frozen at any population doubling level retained 'memory' of that level (Hayflick, 2003). In the 1970s it was hypothesized that the replication counting mechanism could be related to the 'end-replication problem', which is the inability of the DNA polymerase to completely replicate the 3' end of the linear double stranded DNA (Olovnikov, 1971, Olovnikov, 1973). In the late 1970s it was found that telomeres in the protozoan, *Tetrahymena*, contain a sequence of hexameric nucleotide repeats of TTGGGG (Blackburn and Gall, 1978). Ten years later it was discovered that human telomeres contain very similar repeated sequence that differs in one position: TTAGGG (Moyzis et al., 1988). In the late 1980s it was suggested that the limited proliferative capacity of cultured normal cells might be due to a shortening in telomere length and in 1990 it was reported that throughout the lifespan of cultured fibroblasts the telomere length decreases by 2-3 kilobase pairs (Harley et al., 1990). The discovery of telomere attrition as cells reach their replicative capacity has since been demonstrated *in vitro* and *in vivo* (Hayflick, 2003). Telomerase-deficient mice can be used as an *in vivo* model system. These mice are characterised by telomere loss and uncapping which leads to progressive tissue atrophy, stem cell depletion, impaired tissue injury responses and organ system failure (Jaskelioff et al., 2011). Notably, telomerase reactivation reverses tissue degeneration in these mice and supports the implication that telomere damage is a driver of age-associated organ decline and ultimately disease risk (Jaskelioff et al., 2011). Collectively, these results suggest that replicative senescence may contribute to longevity (Hayflick, 2003).

1.3 AGEING IN YEAST

1.3.1 A brief Introduction to yeast ageing.

Despite being one of the simplest model organisms, *Saccharomyces cerevisiae*, has contributed to the identification of many mammalian genes that affect ageing (Longo et al., 2012). *S. cerevisiae* has been used as a model for ageing since 1959, when it was first noted that yeast cells have a finite lifespan (Mortimer and Johnston, 1959). In *S. cerevisiae*, cell division is asymmetric and a mother cell gives rise to a daughter cells at each cell division (Sinclair and Guarente, 1997). The lifespan of a yeast cell can therefore be determined by following the mother cell through multiple rounds of cell division until cell division stops and the mother cell undergoes senescence (Mortimer and Johnston, 1959).

Yeast mother cells have an average lifespan of 20-30 divisions and as yeast cells get older the probability of senescence increases exponentially (Sinclair and Guarente, 1997, Sinclair et al., 1998). Notable characteristics of old yeast cells include: increased size, decreased metabolic functions and a loss of fertility, with the latter being the most reliable marker of yeast ageing to date (Sinclair and Guarente, 1997, Sinclair et al., 1998).

There are two primary assays for yeast ageing, replicative and chronological lifespan (Longo et al., 2012). Replicative lifespan (RLS) was first described in 1959 by Mortimer and Johnston and is focused in mitotically active cells (Mortimer and Johnston, 1959). The RLS of a mother cell is defined by the number of daughter cells produced before cell division stops (Mortimer and Johnston, 1959). During RLS virgin mother cells are isolated and daughter cells physically removed using a microscope equipped with a micromanipulator (Kaeberlein, 2010). The chronological model of yeast ageing is focused in post-mitotic cells and the lifespan of the yeast cell is defined by the amount of time the cell can survive in a non-dividing state (Kaeberlein, 2010). Chronological lifespan is measured by growing a culture of yeast into a postdiauxic state where extracellular glucose is depleted (Longo et al., 2012). During this time the yeast switches from fermentation to respiration and growth is dramatically reduced (Longo et al., 2012). The viability of the yeast cell is then measured by its ability to resume mitotic growth once plated onto fresh media (Kaeberlein, 2010).

1.3.2 Theories of yeast ageing.

As mentioned previously, at each cell division the mother cell gives rise to a new daughter cell. Both cells remain attached to one another until the boundary between them constricts thereby separating the daughter cell (Sinclair et al., 1998). Each daughter cell leaves a circular-chitin-containing remnant on the surface of the mother cell, this is termed the bud scar (Sinclair et al., 1998). It was once thought that accumulation of bud scars might limit the RLS of yeast cells. However, this hypothesis has been widely discredited since bud scars occupy approximately 1% of the available cell surface therefore allowing at least 100 divisions to occur (Sinclair et al., 1998). Yet in laboratory strains of yeast the average lifespan is 20-30 divisions and therefore much fewer than 100 (Jazwinski, 1993, Kennedy et al., 1994, Sinclair et al., 1998). Furthermore, increasing the cell size does not extend lifespan and increased cell size is a characteristic of old yeast cells (Muller, 1985, Sinclair et al., 1998). In fact, increased cell size has also been proposed to contribute to yeast cell senescence (Mortimer and Johnston, 1959). However results suggest that cell size or surface to volume ratio is not related to yeast ageing since cell size does not limit lifespan (Sinclair et al., 1998, Mortimer and Johnston, 1959). Typically after a few cell divisions the yeast mother cells grow bigger in size; therefore the mother cells are easily distinguished from their smaller daughter cells by microscopic examination. However, very old mother cells tend to produce large daughter cells which have a short lifespan (Kennedy et al., 1994). This loss of asymmetry supports the theory that there is a diffusible senescence factor that can be distributed to the daughter cell leading to premature ageing (Sinclair et al., 1998). The accumulation of damaged mitochondria and oxidized proteins within the mother cell contribute to replicative senescence (Kaeberlein, 2010). However these damaged macromolecules are inherited by the mother cell and only passed onto daughter cells in very old mother cells when asymmetry has broken down (Kaeberlein, 2010).

The best-characterised type of molecular damage associated with replicative ageing is the accumulation of extra-chromosomal rDNA circles (ERCs) (Kaeberlein, 2010). ERCs are formed due to alterations in the ribosomal DNA (rDNA), which causes instability and rDNA recombination (Sinclair and Guarente, 1997). The yeast nucleolus is a crescent shaped region of the nucleus containing rDNA and other

components for ribosome assembly (Sinclair and Guarente, 1997, Sinclair et al., 1998). The yeast rDNA locus on chromosome XII consists of a repeated array of 100-200 copies of a 9.1 kb repeat (Sinclair and Guarente, 1997). In ageing mother cells the nucleolus becomes enlarged and fragmented (Sinclair and Guarente, 1997). Homologous recombination within the rDNA results in the formation of a self-replicating circular DNA molecule, known as the ERC (Kaeberlein, 2010). ERCs are asymmetrically retained within the nucleus of the mother cell during cell division and this is thought to be responsible for nucleolar changes seen in old yeast cells (Sinclair and Guarente, 1997, Kaeberlein, 2010).

Proteins that influence ERC formation have also been shown to influence RLS (Kaeberlein, 2010). Overexpression of the histone deacetylase, Sir2 represses ERC formation by inhibiting rDNA recombination and extends RLS (Kaeberlein et al., 1999). Whereas deletion of the replication fork block protein, Fob1, has been shown to promote rDNA recombination and ERC formation thereby reducing RLS (Steinkraus et al., 2008).

1.4 DIETARY RESTRICTION IN YEAST.

1.4.1 Methods of DR in yeast.

Dietary restriction (DR), also known as calorie restriction, is the most widely researched environmental manipulation known to extend lifespan in a wide variety of model organisms (Lin et al., 2002). Historical studies focused on DR and longevity were performed in rodents (McCay, 1935). However, because of their shorter lifespan and ease of housing, invertebrates, are much more suitable model organisms for studying large-scale lifespan experiments under many different conditions and for genetic analysis (Piper and Partridge, 2007). Normally laboratory yeast cells are grown on media containing 2% glucose and abundant amino acids (Kaeberlein et al., 2007). In yeast reducing the glucose concentration of the growth media to 0.5% or 0.05% or reducing the amino acid concentrations (or both) has been shown to increase RLS and CLS, and is a way to mimic DR (Fabrizio et al., 2005, Lin et al., 2000, Powers et al., 2006). An alternative method of testing DR in yeast includes reducing the activity of the glucose-sensing cyclic-AMP-dependent kinase (PKA) (Lin et al., 2002). In yeast glucose enters the cell via glucose-sensing transporters, this is a highly regulated process (Lin et al., 2000). The signal for glucose availability is transduced through the G-protein-coupled receptor Gpr1 and the G α protein Gpa2 (Dilova et al., 2007). Glucose is phosphorylated by glucokinase (GIK1) and hexokinases (Hxk1 or Hxk2) which then activates adenylate cyclase (AC) Cyr1 (Cdc35). The Cyr1 is then stimulated by GTP/GDP binding Ras proteins (Ras1, Ras2) and produces cAMP thereby activating PKA promoting dissociation of the regulatory subunits from the catalytic subunits (Figure 1) (Dilova et al., 2007). Glucose activates PKA and genetic mutations leading to inhibition of the PKA pathway mimics DR and extends yeast lifespan. Genetic models of DR include the hexokinase mutant (*hvk2 Δ*) and mutations in the adenylate cyclase (*cdc35-1*) or the RAS GTP/GDP exchange protein (*cdc25-10*) and deletions of the Gpa2 and Gpr1 proteins or Ras2 protein (Dilova et al., 2007, Wei et al., 2009). Alternatively, mutations that increase PKA activity shorten yeast lifespan (Lin et al., 2000).

1.5 THEORIES OF DR-MEDIATED LIFESPAN EXTENSION IN YEAST.

1.5.1 DR-mediated lifespan extension by activation of Sir2.

There are many proposed theories for how DR extends yeast lifespan; many implicate the activity of the silencing protein, silent information regulator 2 (Sir2) (Lin et al., 2002). Sir2 is a nicotinamide adenine dinucleotide (NAD⁺)-dependent histone deacetylase (Anderson et al., 2003) and a member of a complex of proteins that mediate transcriptional silencing at selected loci in the yeast genome (Guarente, 2011). Silencing of the rDNA is a characteristic feature of yeast ageing, yet homologues of yeast Sir2 have been found in nematodes, fruit flies and mammals (Guarente, 2011). There are seven sirtuins in mammals which share significant sequence homology to the *S. cerevisiae* gene *SIR2* in addition to NAD-dependent protein deacetylation activity (Yu and Auwerx, 2009). SIRT1 is the mammalian orthologue of Sir2 (Guarente, 2011).

In yeast, deletion of the *SIR2* gene is known to shorten lifespan by increasing recombination at the rDNA locus and is characterised by reduced silencing in the rDNA (Smith et al., 1999). Reduced silencing and increased rDNA recombination is thought to cause increased ERC accumulation in the nucleus of the cell, thereby having detrimental effects (Sinclair and Guarente, 1997). In contrast, cells with an extra copy of *SIR2* live 30% longer than wildtype strains through increased silencing activity at the rDNA repeats (Lin et al., 2002, Anderson et al., 2003, Guarente, 2011). Increased rDNA silencing is thought to increase rDNA stability, reducing rDNA recombination leading to yeast longevity (Sinclair and Guarente, 1997).

One theory suggests that Sir2 is activated upon the shift from anaerobic fermentation to aerobic respiration during moderate DR, as this increased respiration raises the cellular NAD⁺/NADH ratio (Figure 1) (Bishop and Guarente, 2007). This theory was proposed since physiological or genetic mimics of DR were shown to increase yeast lifespan, however, this increase was abolished in mutants lacking *SIR2* (Lin et al., 2000). The link to NAD⁺ was highlighted since DR is also unable to extend the lifespan of yeast mutants lacking *NPT1* gene, which is part of the pathway involved in the synthesis of NAD⁺ (Lin et al., 2000). The levels of Sir2

proteins do not increase in response to DR, therefore increased lifespan is thought to be a result of increased Sir2 enzymatic activity (Figure 1) (Anderson et al., 2003).

The theory that Sir2 is regulated by changes in nicotinamide levels, led to an alternative proposal; that DR induces the expression of pyranzinamidase/nicotinamidase gene (*PNC1*) thus increasing the activity of Sir2 (Figure 1) (Anderson et al., 2003). This theory was suggested since increased expression of *PNC1* was shown to be necessary and sufficient for lifespan extension by DR (Anderson et al., 2003). The *PNC1* gene product converts nicotinamide, an inhibitor of Sir2, to nicotinic acid in the NAD⁺ salvage pathway (Anderson et al., 2003). In fact it has been shown that activation of Sir2 can occur either by depleting levels of its inhibitor nicotinamide or by increasing the availability of its co-substrate NAD⁺ (Anderson et al., 2003). Activation of Sir2 by *PNC1* is thought to increase rDNA stability by reducing rDNA recombination (Figure 1) (Anderson et al., 2003). Interestingly DR has also been shown to extend lifespan in some *SIR2* knockout strains leading to the proposal of an alternative theory which is independent of Sir2 (Kaeberlein et al., 2004b).

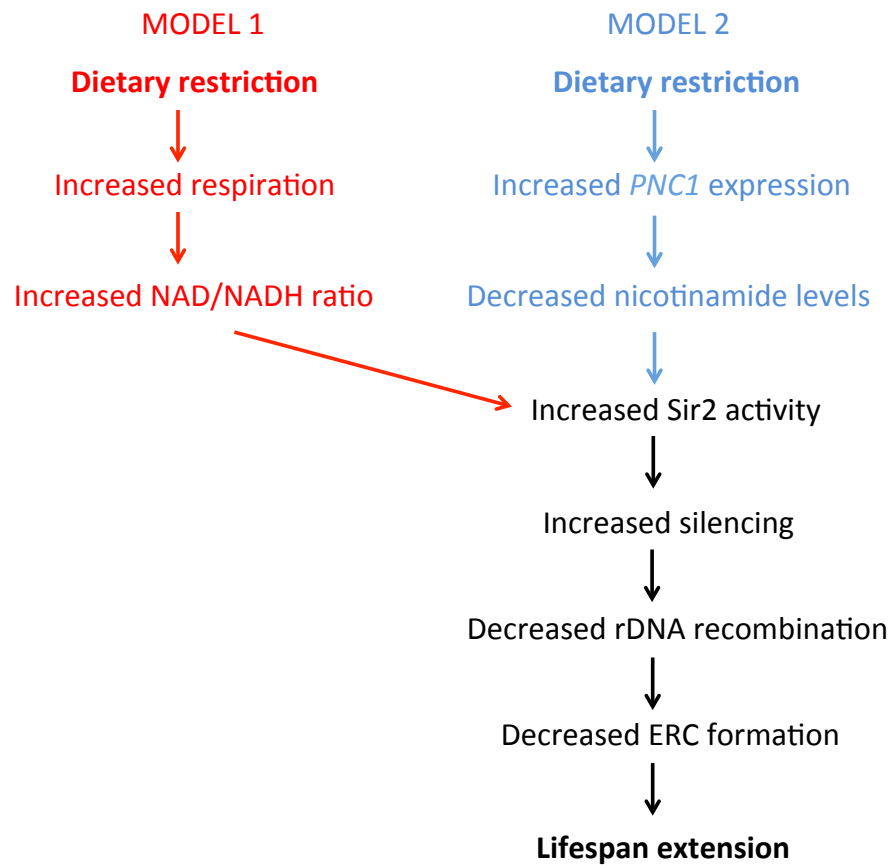


Figure 1. Models of DR-mediated lifespan extension through activation of Sir2. Both models act via the same downstream pathway of activation of the silencing protein, Sir2 (highlighted in black). But differ in the upstream pathways which lead to increased activation of Sir2. Model 1 proposes increased respiration leads to Sir2 activation (highlighted in red). While model 2 suggests activation of Sir2 is caused by increased expression of the *PNC1* gene (highlighted in blue) (Riesen and Morgan, 2009). Figure adapted from Riesen and Morgan, 2009.

1.5.2 DR-mediated lifespan extension by nutrient sensing pathways.

The target of rapamycin (TOR) pathway was originally shown to mediate the effects of DR in yeast and has since become a major player in the field of ageing and DR (Katewa and Kapahi, 2010). TOR was originally identified in *S. cerevisiae* by mutations that confer resistance to the growth inhibitory properties of rapamycin (Wullschleger et al., 2006). In yeast, a large-scale analysis of 564 single gene mutants identified 10 gene deletions that increased RLS (Kaeberlein et al., 2005). Notably, 6 of these genes were linked to the TOR and Sch9 pathway (Kaeberlein et al., 2005). Sch9 is a yeast orthologue of the mammalian S6 protein kinase (S6K1) (Kaeberlein et al., 2005). In yeast, TOR deletion or prolonged rapamycin treatment causes phenotypic changes characteristic of starved cells, these include: an altered transcription pattern, down-regulation of protein synthesis and acquisition of thermo-tolerance and accumulation of carbohydrates (Barbet et al., 1996). In yeast, reducing TOR signalling by rapamycin treatment, deletion of *TOR1* or inhibiting the glutamine synthetase enzyme increases RLS and CLS (Powers et al., 2006, Kaeberlein et al., 2005). Yet DR is unable to further extend the lifespan of *tor1Δ* yeast mutants, suggesting that TOR is a target of DR (Kaeberlein et al., 2005).

In yeast two TOR genes exist, *TOR1* and *TOR2*, only *TOR2* is required for growth (Kunz et al., 1993). TOR signalling is known to regulate cell growth and metabolism in response to environmental factors (Wullschleger et al., 2006). In *S. cerevisiae*, TOR promotes cell growth and proliferation when activated by nutrients in the form of amino acids or carbohydrates (McCormick et al., 2011). In yeast, the major growth processes controlled by TOR are ribosome biogenesis and translation (Dilova et al., 2007). Every eukaryote genome examined contains a *TOR* gene; TORs are large proteins that belong to a group of kinases known as phosphatidylinositol kinase-related kinase (PIKK) family (Wullschleger et al., 2006). Consistently, studies have linked lifespan extension of reduced TOR signalling to that of DR (McCormick et al., 2011). Like PKA, Sch9, is a nutrient sensing protein kinase that regulates replicative ageing in yeast (Kaeberlein et al., 2005). Sch9 is thought to act in a pathway parallel to TOR and PKA (Kaeberlein et al., 2005). Like that described for *tor1Δ* mutants, DR is also unable to further extend the lifespan of *sch9Δ* mutants (Kaeberlein et al., 2005). Collective results suggest that PKA, TOR and Sch9 all

appear to be a target for DR in yeast (Figure 2). Decreased signalling by PKA, TOR and Sch9 has been shown to result in increased transcriptional activity of Msn2 and Msn4, which then increase the expression of *PNC1* gene and other stress responsive genes (Medvedik et al., 2007). As mentioned earlier, *PNC1* is a regulator of Sir2, and this finding links the nutrient sensing pathways to the sirtuins (Medvedik et al., 2007). Furthermore, regulation controlled by Tor1, Sch9 and Ras2 involves a protein kinase Rim15 (Wei et al., 2008). Rim15 activates the transcription of genes involved in cellular protection (Figure 2) (Wei et al., 2009). Consistent with this finding other studies have shown that down-regulation of nutrient signalling pathways activates stress-resistance transcription factors that regulate expression of genes involved in oxidative stress, heat stress, DNA repair and metabolism (Parrella and Longo, 2010). However, the exact molecular mechanisms underlying the effects of TOR and Sch9 on yeast lifespan are still not clear, yet one hypothesis suggests the effects of TOR signalling are related to reduced ribosomal protein biogenesis (Kaeberlein et al., 2005).

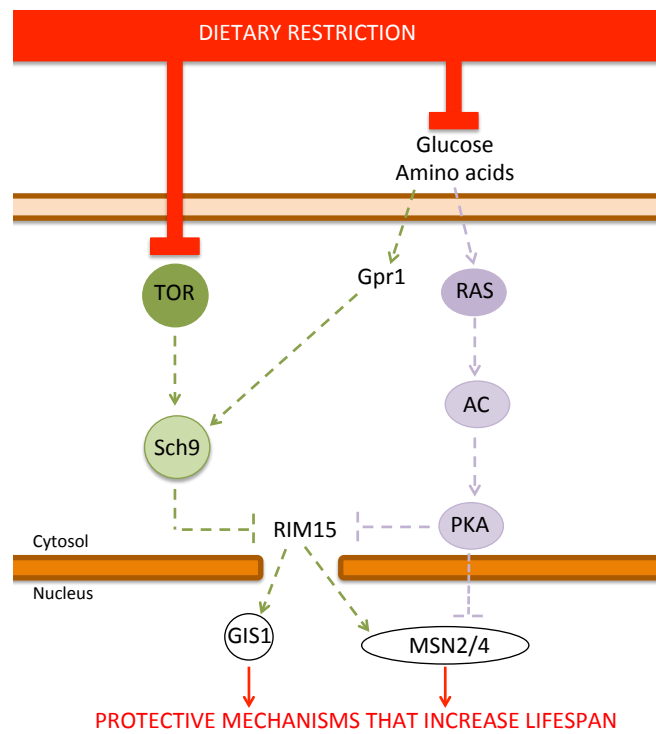


Figure 2. Nutrient signalling pathways that regulate longevity in yeast. Dietary restriction reduces the activity of TOR, PKA and Sch9 converging on the protein kinase Rim15. Rim15 activates transcription factors Gis1, Msn2 and Msn4 which induce the expression of genes involved in cellular protection. Reduced nutrient sensing is thought to decrease free radical superoxide, induce the expression of chaperones, decrease protein translation and activate autophagy (Fontana et al., 2010). Figure based on Fontana et al, 2010.

1.5 DIETARY RESTRICTION IN OTHER MODEL ORGANISMS.

1.6.1 DR in *Drosophila melanogaster*.

The fruitfly, *Drosophila melanogaster*, has been used as a model organism to study ageing for more than 80 years (Pearl, 1921). In *Drosophila*, DR can be achieved by different methods but the most practical method involves dilution of the food medium to which the flies have *ad libitum* access (Partridge et al., 2005). The first reports that DR could extend *Drosophila* lifespan date back to 1928 and a study by Kopec, who concluded that a 6-hour period of starvation out of every 24 hours, increases the lifespan of flies (Kopec, 1928). However, more recent analysis of Kopec's work has revealed the results to be less than convincing with an average extension of 2% over ten trials (Piper and Partridge, 2007). In addition, recent studies of intermittent feeding in various fly species to achieve lifespan extension by DR have been unsuccessful (Carey et al., 2002, Cooper et al., 2004). Notably, in these studies flies had access to water that did not contain sucrose (Carey et al., 2002, Cooper et al., 2004). In contrast, a study by Partridge et al, reported a 30% extension in flies fed yeast intermittently (every sixth day) with constant access to sugar-water in comparison to flies fed yeast and sugar *ad libitum* (Piper and Partridge, 2007). The inconsistencies in results reported for DR in *Drosophila* have highlighted the necessity of a carbohydrate-enriched diet and specific nutritional effects of yeast in mediating fly lifespan extension by DR (Piper and Partridge, 2007).

In *Drosophila* the reproductive activity responds rapidly to nutrition (Piper et al., 2005). Increased nutrient availability triggers an elevation in reproductive activity increasing the daily and lifetime number of eggs (Chapman and Partridge, 1996). Whereas DR arrests egg laying in *Drosophila*, but is still able to extend the lifespan of female flies containing mutations to prevent egg production (Mair et al., 2004, Piper et al., 2005). In addition to a reduced fecundity, DR flies are also able to survive longer than control-fed flies during periods of starvation and have an elevated lipid content (Bradley and Simmons, 1997). Notably, DR flies do not show any difference in mass-specific metabolic rate or ROS-production rates compared to control flies (Hulbert et al., 2004, Miwa et al., 2004).

Like that reported in yeast, the insulin/IGF-like signalling (IIS) and target of

rapamycin (TOR) signalling pathways in flies are known to control lifespan and couple growth to nutrition (Kennedy, 2008). Testing of specific gene mutations that reduce signalling through these pathways gives the same outcome as DR, notably, slow growth, reduced fecundity and lifespan extension (Piper and Partridge, 2007, Kennedy, 2008). Furthermore, the maximum lifespan achieved by DR cannot be further extended by mutations in these pathways (Piper et al., 2005). These findings suggest that IIS and TOR signalling pathways are involved in DR-mediated lifespan extension.

The exact mechanisms underlying lifespan extension by DR in *Drosophila* remain unclear. Yet the fact that DR is able to mediate lifespan extension in a wide variety of organisms suggests the pathways are evolutionarily conserved. DR can be achieved in *Drosophila* through different methods and this has led to conflicting results (Piper and Partridge, 2007). Future research should be focused on the development of a defined diet that is suitable for *Drosophila* DR lifespan studies (Piper et al., 2005).

1.6.2 DR-mediated lifespan extension in *Caenorhabditis elegans*.

As mentioned previously DR can mediate lifespan extension in many model organisms including the lifespan of the nematode *Caenorhabditis elegans* (*C. elegans*) (Partridge, 2007). As with yeast and *Drosophila*, there are a number of methods to achieve DR in *C. elegans*, some include the use of a genetic mutation (*eat-2*) that leads to pharyngeal pumping defects, another includes reduction of the growth rate or density of the *E. coli* bacteria on the surface of the agar and an alternative method includes the use of special chemical media that induces a DR-like phenotype (Greer and Brunet, 2009, Partridge, 2009). Reducing the growth rate or density of the *E. coli* or using genetic mutants in pharyngeal pumping extends *C. elegans* lifespan by 30–60% (Klass, 1977, Lakowski and Hekimi, 1998). Furthermore, in studies where DR is achieved by the use of a chemical media (axenic medium) the largest extension up to 100% has been reported (Houthoofd et al., 2002).

Similar to that described for yeast and *Drosophila*, many of the gene mutations that extend lifespan in *C. elegans* are homologues of the mammalian insulin/IGF-1 pathway (Braeckman et al., 2006). Mutations in the *C. elegans* gene

encoding the worm insulin/IGF-1 receptor, *daf-2* (abnormal Dauer Formation-2), increase lifespan (Kenyon et al., 1993). DAF-2 mutations lead to Dauer formation, which is a diapause-like alternative larval stage which enables the worms to withstand harsh conditions such as starvation (Riddle et al., 1981). Both IIS and TOR signalling influence dauer formation in worms (McCormick et al., 2011). Mutations in *daf-2* are thought to cause the down-regulation of the *age-1* gene (AGEing alteration-1) and result in activation of the forkhead transcription factor DAF-16 (Friedman and Johnson, 1988). However, the role of the insulin/IGF-1 signalling pathway in DR-mediated lifespan extension of *C. elegans* has shown conflicting results (Mair et al., 2009, Greer and Brunet, 2009). Some studies have shown that DAF-16, a key IIS-responsive forkhead transcription factor, is not required for lifespan extension by DR (Mair et al., 2009). In contrast, other studies using different methods of DR have shown DAF-16 to be required for lifespan extension (Dorman et al., 1995).

Another nutrient responsive pathway important in longevity regulation of *C. elegans* includes the TOR signalling pathway (Dilova et al., 2007). *C. elegans* and yeast share 6 gene orthologues linked to the TOR pathway whose reduced expression increases both yeast RLS and worm lifespan (Smith et al., 2008). In worms, activation of the TOR kinase can occur in response to amino acids, in response to glucose via the IIS pathway, in response to the AMP (adenosine monophosphate-activated protein) and MAP (mitogen activated protein) kinase pathways or in response to stress (McCormick et al., 2011). Similar to that described in yeast, the longevity benefits of reduced TOR signalling are linked to DR-mediated lifespan extension of *C. elegans* (Wei et al., 2008). However, studies focused on testing the targets of TOR have yielded inconsistent results (Fontana et al., 2010). Collective results suggest an overlap between IIS and TOR signalling in the regulation of longevity in *C. elegans*. However, the exact mechanisms in which these nutrient responsive pathways play in DR-mediated lifespan extension of worms remain unknown. Although the precise mechanisms of DR-mediated lifespan extension are still unclear, results suggest that the pathways may be evolutionarily conserved. It is therefore important to continue research in short-lived model organisms such as yeast, flies and worms to give insights into ageing in longer-lived

higher organisms.

1.6.3 DR-mediated lifespan extension in rodents and higher organisms.

In rodents there are two ways to study DR (Anderson et al., 2009). In the first type of study, DR is started on one-month old rodents, this study is termed 'early-onset' and is the best model for studying dietary-induced decelerated ageing in mammals (Anderson et al., 2009). 'Early-onset' DR studies are not applicable to young humans or primates as restricting calories so early on may actually be detrimental to development and maturity (Anderson et al., 2009). In the second type of study DR is introduced at middle age, this model is the most relevant for potential human application and is termed 'adult-onset' (Anderson et al., 2009).

Early studies showing the effectiveness of DR in mediating lifespan extension date back to the 1930s to studies focused on rats (McCay, 1934, McCay, 1939, McCay, 1935). McCay and Crowell, reported that feeding laboratory rats a reduced calorie diet while maintaining micronutrient levels resulted in lifespan extension double that of rats fed *ad libitum* (McCay, 1934). In addition to extending the mean and maximum lifespan of female and male rats, DR also delayed the onset of age-associated pathologies (McCay, 1935). It has since been shown that DR in rats reduces the incidence of obesity, cancer, diabetes, autoimmune diseases, sarcopenia and cardiovascular disease (Anderson et al., 2009, Weindruch and Walford, 1982). However, DR in mice can also impair immunity and wound healing and increase susceptibility to bacterial, viral and parasitic infections (Fontana et al., 2010).

As described for yeast, flies and worms, reducing the activity of the nutrient sensing pathways can also increase lifespan in mice (Fontana et al., 2010). Evidence that IGF-1 plays a role in longevity stems from the finding that growth hormone (GH)-impaired mice live longer and have very low circulating IGF-1, reduced insulin and glucose levels (Al-Regaiey et al., 2005). Also consistent with that reported for yeast, flies and worms, mouse lifespan can be increased by inhibiting the mTOR pathway (Harrison et al., 2009). When rapamycin is administered to mice at 600 days of age this results in an increase in mean and maximum lifespan (Harrison et al., 2009). Mouse lifespan can also be extended by deleting the ribosomal S6

protein kinase (S6K1) which also inhibits the mTOR pathway (Selman et al., 2009). Inhibition of mTOR by S6K1 deletion also reduces the incidence of bone, immune and motor dysfunction and loss of insulin sensitivity (Selman et al., 2009). The authors from this study propose that deletion of S6K1 is a mimic of DR (Selman et al., 2009). In mice, the inverse relationship between calorie intake and lifespan extension has suggested a role for regulators of energy metabolism in the mechanisms underlying DR (Colman et al., 2009). Studies in yeast, flies, worms and mice have indicated a role for the nutrient sensing pathways including insulin/IGF-1 and mTOR in longevity and ageing. The relevance of results from invertebrate and mice studies on human ageing depends upon conservation of the effects of DR on ageing in primates (Colman et al., 2009).

To date, there have been two studies investigating the effects of DR on primates (Kemnitz, 2011). A 20-year longitudinal 'adult-onset' DR study in rhesus monkeys has shown 80% of the DR fed (30% restriction) animals survived in comparison to 50% of control fed (13% restriction) animals (Colman and Anderson, 2011). In this study DR was reported to reduce the incidence of age-associated pathologies including cancer, diabetes, cardiovascular disease and brain atrophy (Colman and Anderson, 2011). However, a separate study investigating DR implementation in young and older rhesus monkeys has reported no improvement in survival outcomes (Mattison et al., 2012). In the second study the health benefits of DR are also inconsistent showing no reduced incidence of cardiovascular disease or insulin-dependent diabetes (Mattison et al., 2012). DR was reported to reduce the incidence of cancer and improve immunity (Mattison et al., 2012). Differences in results may be due to differences between the two studies. Notable differences between these studies include study design, diet composition and animal husbandry, which may be attributable to the different effects reported for DR (Mattison et al., 2012, Kemnitz, 2011). Study design, method of DR and husbandry, have also been reported to cause differences in the reported effects of DR in rodents (Forster et al., 2003). Even within a single organism different methods of DR can involve different mechanisms and give conflicting results (Partridge, 2009).

DR in human volunteers has shown consistent findings to that reported in rodents and includes beneficial effects against obesity, insulin resistance,

inflammation and oxidative stress (Fontana et al., 2010). Furthermore, DR in humans has been shown to reduce the risk of cardiovascular disease by reducing cholesterol, C-reactive protein and the intima-media thickness of the carotid arteries (Fontana et al., 2010). Research in rhesus monkeys is now focused on investigating cell metabolism, gene expression, insulin signalling pathways and mTOR pathways to help understand how DR attenuates the negative consequences of ageing (Mattison et al., 2012).

1.7 THE SMALL HEAT SHOCK PROTEINS.

1.7.1 Overview of the heat shock response and heat shock proteins.

It has now been 50 years since the discovery that elevated temperatures could mount very strong transcriptional activity, a phenomenon known as the heat shock response (Ritossa, 1962). The heat shock response was discovered by an Italian scientist, Ferruccio Ritossa, who was analysing chromosomal puffs in *Drosophila* larvae (Ritossa, 1962). Ritossa noticed a novel pattern of chromosomal puffs that was uncharacteristic of that stage of larval development. Notably this larvae had been mistakenly incubated at a higher temperature than normal delivering a heat shock to the tissue (Ritossa, 1996). Ritossa then incubated more larvae at this elevated temperature and reproduced the same chromosomal puffing pattern (Ritossa, 1996). It is now known that exposure to elevated temperatures evokes a similar heat shock response in all living organisms (Lanks, 1986). This response includes inhibition of general protein synthesis, cessation of proliferation, enhanced synthesis of a limited set of proteins and eventual cell death (Lanks, 1986). We now know that the heat shock response is not just limited to exposure to heat and is a universal response induced by exposure to a large array of stresses (De Maio et al., 2012).

As mentioned earlier, the heat shock response leads to enhanced synthesis of a limited set of proteins (Lanks, 1986). These proteins correspond to the chromosomal puffs discovered by Ritossa and were later identified by Alfred Tissières as the heat shock proteins (Hsps) (Tissieres et al., 1974). The Hsps are highly conserved from plants to animals and divided into families based on their molecular weight and sequence conservation (Lindquist and Craig, 1988, Hightower, 1991). The Hsp families include Hsp100, Hsp90, Hsp70, Hsp60 and the small Hsps (sHsps) (Ferreira et al., 2006). The Hsps act as molecular chaperones and facilitate the correct folding of nascent proteins, refolding of denatured proteins and can also assist in the degradation of misfolded and aggregated proteins (Murshid et al., 2013). Misfolded proteins can be degraded by the proteasome or transported to the lysosome and degraded by chaperone mediated autophagy (CMA) or degraded

by an alternative autophagy pathway known as aggrephagy (Figure 3) (Murshid et al., 2013). The Hsps act to prevent proteotoxicity and favor stress resistance.

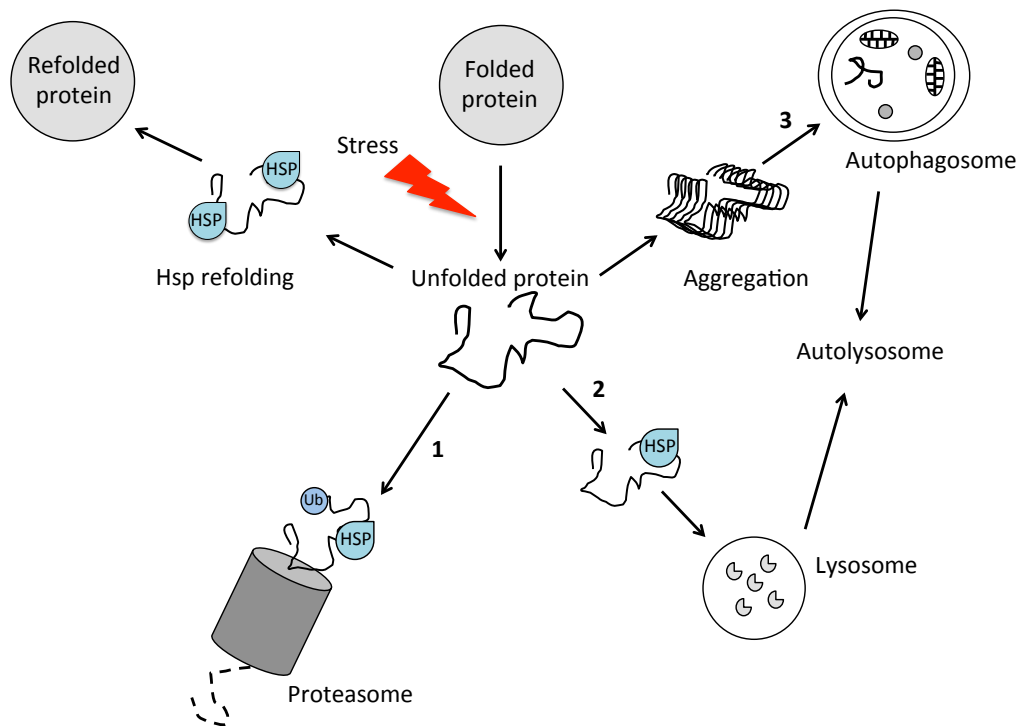


Figure 3. Mechanisms of proteostasis by the Hsps. Exposure to stresses can lead to proteins misfolding exposing their hydrophobic residues. Hsps can bind to misfolded proteins and assist in refolding. If proteins cannot be correctly refolded, misfolded proteins can be ubiquitinated (Ub) and degraded by the proteasome (1). Alternatively misfolded proteins can be transported to the lysosomes and degraded by chaperone-mediated autophagy (CMA) (2). Misfolded proteins that have formed aggregates can be degraded by aggrephagy (3). Schematic based on Murshid et al, 2013 (Murshid et al., 2013).

1.7.2 Small heat shock proteins in *S. cerevisiae*.

Like that described for other organisms, *S. cerevisiae* demonstrates a stress response when exposed to a wide range of environmental insults (Nisamedtinov et al., 2008). The most striking feature of this evolutionarily conserved stress response is the induced synthesis of the Hsps (Nisamedtinov et al., 2008). Like their larger relatives, the sHsps are a superfamily of molecular chaperones providing protection from stresses and suppressing protein aggregation (White et al., 2006). In addition to assisting in the refolding process, the sHsps can also assist in the removal of misfolded proteins by the cellular proteolytic systems (Proctor and Lorimer, 2011). However, unlike the Hsps, the sHsps show only limited sequence homology between species (Praekelt and Meacock, 1990). In yeast the sHsp family range in size from 10-42 kDa, they are synthesized during different developmental stages and contain a conserved C-terminal α -crystallin domain (Haslbeck et al., 2004, Hohfeld and Hartl, 1994, Praekelt and Meacock, 1990). The α -crystallin domain of yeast sHsps shows sequence homology to α A and α B-crystallins of vertebrate eye lens (Franzmann et al., 2005). In *S. cerevisiae*, Hsp12 and Hsp26 are components of the heat shock response and are amongst the best studied sHsps in yeast.

1.7.3 *S. cerevisiae* Hsp12.

The *S. cerevisiae* *HSP12* gene was first isolated and identified by Praekelt and Meacock in 1990 (Praekelt and Meacock, 1990). In *S. cerevisiae*, the *HSP12* gene encodes a protein of ~12 kDa and is undetectable in non-stressed cells but induced several hundred fold by heat shock and entry into stationary phase (Praekelt and Meacock, 1990). Since its discovery Hsp12 has been shown to accumulate massively in yeast cells exposed to osmostress, oxidative stress, glucose starvation, cell wall stress and high concentrations of alcohol (Varela et al., 1995, Welker et al., 2010). In fact, Hsp12 appears to be the sHsp in yeast whose expression is up-regulated the highest fold in response to a variety of stresses (Welker et al., 2010). Despite its massive up-regulation, studies analysing mutants with deletion of the *HSP12* gene have reported no effect on growth or viability of yeast cells when grown at various temperatures (Praekelt and Meacock, 1990). More recent studies have reported an increased susceptibility of yeast cells with

HSP12 gene disruptions when exposed to caffeine and H₂O₂ (Motshwene et al., 2004, Welker et al., 2010).

In *S. cerevisiae*, the *HSP12* gene is activated by the high-osmolarity glycerol (HOG) pathway and negatively regulated by Protein Kinase A (PKA) (Varela et al., 1995). Mutations in the *HOG1* and *PBS2* genes coding for components of the osmosensing pathway lead to a dramatic decrease in *HSP12* expression in osmostressed cells (Varela et al., 1995). Furthermore, mutations resulting in high PKA activity reduce or abolish the accumulation of *HSP12* mRNA in stressed cells (Varela et al., 1995). Similarly the *HSP12* gene of *Candida albicans* (*CaHSP12*) is also regulated at least in part by the Hog1 and cAMP-PKA signalling pathway (Fu et al., 2012).

During exposure to stress conditions the transcriptional regulators Msn2 and Msn4 in *S. cerevisiae* are involved in the activation of *HSP12* gene expression (de Groot et al., 2000). Msn2 and Msn4 shuttle between the cytosol and nucleus during unstressed conditions allowing normal growth. However, Msn2 and Msn4 accumulate in the nucleus during stress conditions and during low PKA activity (de Groot et al., 2000). Msn2 and Msn4 bind to the 5 stress-responsive elements (STREs) present in the promoter of *HSP12* and mediate transcriptional activation of the *HSP12* gene (Varela et al., 1995, de Groot et al., 2000).

Recent studies have identified the structure and function of Hsp12. It is now known that Hsp12 exists in the cytosol and associated with the plasma membrane (Welker et al., 2010). Hsp12 is intrinsically unstructured however in the presence of certain lipids adopts a 4-helical conformation (Welker et al., 2010, Herbert et al., 2012). These studies have shown that Hsp12 differs structurally and functionally to other studied sHsps. Differences between Hsp12 and other sHsps include; Hsp12 is natively unfolded, Hsp12 does not form large homo-oligomeric structures, it does not contain a conserved α -crystallin domain and has negligible anti-aggregation activity (Welker et al., 2010). The finding that Hsp12 has very little if any anti-aggregation activity has ruled out a functional role as a chaperone like its family member Hsp26 (Herbert et al., 2012). Recent structural work has led to the proposal that Hsp12 acts as a membrane stabilising lipid chaperone (Welker et al., 2010). In the presence of certain lipids Hsp12 forms a 4-helical conformation. Each

α -helix has been shown to be amphipathic with hydrophobic residues lying on one face and charged residues lying on the opposite face (Herbert et al., 2012). It is thought that the hydrophobic residues insert into the lipidic component of membranes while the charged residues interact with negatively charged head groups projecting away from the membrane (Herbert et al., 2012). During exposure to stresses Hsp12 expression is massively induced, Hsp12 is then thought to bind to lipids within the plasma membrane, endosomes and possibly vacuoles to stabilise the membrane (Welker et al., 2010).

S. cerevisiae Hsp12 shows 47% sequence homology to *C. albicans* WH11 which is also an Hsp12 family member (Praekelt and Meacock, 1990). *S. cerevisiae* also shows sequence homology to *Schizosaccharomyces pombe* Hsp9 (Welker et al., 2010). However, outside the kingdom of fungi there are no proteins with striking sequence homology to Hsp12 (Ahn et al., 2012, Welker et al., 2010). Hsp12 displays low level homology to two mammalian proteins, ageing-associated protein 2 (HIP) and α -synuclein (Welker et al., 2010, Herbert et al., 2012). α -synuclein shows striking similarities to Hsp12. Notably, α -synuclein is also natively unfolded in human neurons and becomes structured once it attaches to the membrane (Ulmer et al., 2005). Furthermore, it has also been suggested that α -synuclein organizes and stabilises lipids in synaptic vesicle membranes (Madine et al., 2006). This suggests that Hsp12 and α -synuclein share functional properties. A potential explanation for lack of Hsp12 homologues in higher eukaryotes may be that the properties described for Hsp12 do not require a conserved amino acid sequence but rather a pattern of side-chain properties (Welker et al., 2010).

1.7.3 *S. cerevisiae* Hsp26.

In *S. cerevisiae*, Hsp26 has been described as the principal sHsp and is also the best characterised family member (Susek and Lindquist, 1990, Petko and Lindquist, 1986). The *S. cerevisiae* *HSP26* gene encodes a 213 amino acid protein that contains a conserved α -crystallin domain (Bossier et al., 1989). Like that described for Hsp12, the Hsp26 mRNA transcript is undetectable in unstressed cells but strongly induced in response to heat shock, ethanol exposure, entry into stationary phase and glucose starvation (Carmelo and Sa-Correia, 1997, Lindquist,

1986, Lanks, 1986). Despite its strong up-regulation in response to different stresses the *HSP26* gene is not essential for thermotolerance, resistance to ethanol, spore development or survival after long-term storage in stationary phase (Petko and Lindquist, 1986).

The transcriptional activation of *HSP26* in response to different stresses requires the heat shock factor (HSF) and Msn2 and Msn4 transcription factors (Amoros and Estruch, 2001). In response to stresses Hsf1 binds to the heat shock element (HSE) and Msn2 and Msn4 bind to STRE to regulate expression of stress-responsive genes (Amoros and Estruch, 2001). As described for *HSP12* earlier, in response to stresses Msn2 and Msn4 accumulate within the nucleus and bind to STREs within the *HSP26* promoter to mediate transcriptional activity (Varela et al., 1995). In contrast Hsf1 exists as a monomer within the cytosol and forms a trimer upon exposure to stresses (Murshid et al., 2013). The activated Hsf1 trimer then translocates to the nucleus and binds to HSEs in the *HSP26* promoter thereby activating transcription (Murshid et al., 2013). The promoter of *HSP26* contains both HSEs and STREs and differs to *HSP12* which contains STREs only (Amoros and Estruch, 2001, Varela et al., 1995).

A key feature of the sHsps is their existence as dynamic oligomers (Franzmann et al., 2005). *S. cerevisiae* Hsp26 is a cytosolic protein that exists as a hollow sphere of 24 subunits assembled from 12 dimers (Franzmann et al., 2005). Unlike Hsp12, which has negligible anti-aggregation activity, Hsp26 is able to suppress the aggregation of a variety of substrate proteins and has 100-fold more chaperone activity (White et al., 2006, Haslbeck et al., 2004). Hsp26 is able to switch from inactive to an active chaperone state by a temperature induced conformational change within its middle domain (Franzmann et al., 2008). Hsp26 shares a 90% overlap in substrate proteins with another cytosolic sHsp, Hsp42, and yeast cells with *HSP26* deletions have been shown to have a significant increase in protein aggregation (Haslbeck et al., 2004). Hsp26 is known to bind to non-native proteins during stress conditions preventing aggregation and enabling spontaneous or chaperone-assisted refolding (Haslbeck et al., 2004).

The Hsp26 protein of *S. cerevisiae* contains a conserved C-terminal α -crystallin domain which shows sequence homology with wheat Hsp16.9,

Methanocaldococcus jannaschii (*M. jannaschii*) Hsp16.5, *Mycobacterium tuberculosis* (*M. tuberculosis*) Hsp16.3 and human α A and α B crystallins (White et al., 2006). The α A and α B crystallins are the two major eye lens proteins in vertebrates and appear to be crucial for preventing cataract formation by suppressing protein aggregation (Franzmann et al., 2005, White et al., 2006). *S. cerevisiae* Hsp26 shares sequence homology with higher organisms in addition to conserved functions as a molecular chaperone.

1.8 THE SHSPS IN AGEING AND DISEASE.

1.8.1 The sHsps and ageing.

The sHsps protect the cell against a variety of stresses by preventing protein misfolding and the accumulation of damaged proteins, both of which are characteristics of ageing (Wadhwa et al., 2010). The sHsps have gained prominence in ageing research as they are thought to be important for maintenance of proteome stability with increasing age (Swindell, 2009). In fact the sHsps have been implicated in ageing in invertebrate models including *C. elegans*, *Drosophila* and *S. cerevisiae* (Morrow et al., 2004a, Morrow et al., 2004b, Walker and Lithgow, 2003). In *S. cerevisiae*, transcriptome analysis has shown the up-regulation of *HSP12* and *HSP26* in old yeast cells (Lesur and Campbell, 2004, Lin et al., 2001). In *Drosophila*, *Hsp22* has been shown to be up-regulated with ageing and experiments overexpressing *Hsp22* result in lifespan extension (Morrow et al., 2004b). Consistent with the hypothesis that *Hsp22* is linked with *Drosophila* longevity, flies with *Hsp22* deletions have a reduced lifespan (Morrow et al., 2004a). Furthermore, exposure of flies to a non-lethal heat treatment extends lifespan and increases tolerance to more severe heat stress (Khazaeli et al., 1997). In *C. elegans*, mutations in the IIS signalling pathway extend adult lifespan (Walker and Lithgow, 2003). This lifespan extension has been linked with an overexpression of Hsp genes and an enhanced ability to cope with macromolecular damage (Walker and Lithgow, 2003). In fact, in *C. elegans* extra copies of the HSP-16 gene increases lifespan and stress resistance (Walker and Lithgow, 2003).

The sHsps have also been linked with ageing due to the fact that they play a role in autophagy. Autophagy is an important survival pathway in response to many stresses and has been linked with DR and ageing (Murshid et al., 2013). Due to the accumulation of various deleterious changes and a subsequent decline in cellular functions, aged cells accumulate damaged macromolecules and organelles (Yen and Klionsky, 2008). Autophagy deficiency is also a characteristic of aged cells and has been proposed as the main cause of damaged macromolecular and organelle accumulation (Cuervo et al., 2005). The importance of autophagy in regulating ageing has been highlighted in invertebrate and mice studies. In yeast,

chronological lifespan extension by DR and by reduced TOR signalling both require autophagy (Yen and Klionsky, 2008). When autophagy genes are inactivated in *Drosophila*, flies become short lived and accumulate protein aggregates within their nervous system (Jaiswal et al., 2012). In contrast, increasing the activity of autophagy genes rescues aged cells from mitochondrial dysfunction and increases flies lifespan (Simonsen et al., 2008). In *C. elegans* knock down of autophagy genes, ATG-7 and ATG-12 partially suppresses the lifespan extension in *daf-2* mutants (Hars et al., 2007). In mice deficient in the autophagy gene, *Atg5*, show accumulation of damaged proteins and organelles leading to accelerated ageing and a shortened lifespan (Kuma et al., 2004).

1.8.2 Stress proteins and human disease.

Protein homeostasis results from coordinated quality control systems that govern protein synthesis, protein folding and protein disassembly and ensure continual renewal under normal physiological conditions (Morimoto and Cuervo, 2009). Alterations in proteostasis leads to the accumulation of misfolded and aggregated proteins. In fact, the accumulation of protein aggregates in neurons is the hallmark of several neurodegenerative diseases (Qi et al., 2012). An example includes Huntington's disease, which is caused by an abnormal expansion of a CAG trinucleotide repeat also known as the polyQ (polyglutamine) tract in the huntingtin protein (Vacher et al., 2005, Qi et al., 2012). In addition to Huntington's disease, there are 8 other known diseases caused by CAG/polyQ repeat expansions, the pathological hallmark of these diseases is the presence of intracellular aggregates formed by the mutant proteins (Qi et al., 2012, Vacher et al., 2005). Aggregates recruit chaperones in cell and in mouse disease models and Hsps have been shown to co-localise with the pathological protein aggregates (Wytttenbach et al., 2000, Vacher et al., 2005, Dimant et al., 2012). Notably, transgenic mice overexpressing the yeast Hsp104 chaperone crossed with mice expressing the mutant huntingtin protein showed reduced aggregate formation and a 20% prolonged lifespan (Vacher et al., 2005). Other studies have shown a role for CMA and the heat shock cognate Hsc70 in degradation of the mutant huntingtin protein and suggest enhanced CMA-mediated clearance may be a beneficial target for disease therapy (Qi et al., 2012).

Chaperones such as Hsp40 and Hsp70 have also been shown to suppress polyQ aggregation and rescue cell death in tissue culture models (Wytenbach et al., 2002).

In addition to Huntington's disease, the formation of protein aggregates within neurons is also a characteristic feature of Alzheimer's disease and Parkinson's disease (Vacher et al., 2005). Alzheimer's disease is characterised by the deposition of Amyloid β peptides and intracellular neurofibrillary tangles of hyperphosphorylated Tau protein (Morawe et al., 2012). The molecular chaperones have been implicated in the pathogenesis of Alzheimer's disease, since loss of proteostasis is a feature of the disease (Morawe et al., 2012). Parkinson's disease is caused by the accumulation of proteins including the presynaptic protein α -synuclein which forms Lewy bodies (Dimant et al., 2012). The molecular chaperones have been implicated in Parkinson's disease since the finding that Hsp40 and Hsp70 co-localise in Lewy bodies (Auluck et al., 2002). A viral vector Parkinson's disease mouse model has also shown up-regulation of Hsp27, Hsp40 and Hsp70 in response to α -synuclein overexpression (Dimant et al., 2012). It is thought that investigating the roles of the chaperones in protein aggregation will help develop a better understanding of neurodegenerative disease mechanisms and highlight potential therapies.

In humans there are 10 family members of the sHsps (HSPB1-HSPB10) which play a crucial role in maintaining the integrity and functions of tissues (Kampinga and Garrido, 2012). Many of the HSPBs have ATP-independent chaperone activity and recent studies have shown mutations in HSPBs to have implications in human disease (Kampinga and Garrido, 2012). For example, a missense mutation in the HSPB5/ α B-crystallin gene causes congenital cataracts (Litt et al., 1998). Another group have shown a different missense mutation in the HSPB5/ α B-crystallin gene to cause desmin-related myopathy, a neuromuscular disorder (Vicart et al., 1998). In addition, single nucleotide polymorphisms in the HSPB7 gene have been associated as a risk for a structural heart disease termed dilated cardiomyopathy (Stark et al., 2010). The HSPBs have also been linked with cancer, with HSPB1 and HSPB5 implicated in tissue fibrosis and metastasis (Kampinga and Garrido, 2012). Elevated expression of HSBPs has been linked with resistance to cancer therapies and

increased tumorigenesis and metastasis (Zoubeidi and Gleave, 2012). Furthermore, Hsp27 and Hsp70 expression has been shown to be deregulated in cancer, with decreased lymphocyte Hsp27 levels associated with a higher risk of lung cancer (Wang et al., 2009).

The protective roles of the sHsps in cellular processes such as proteostasis, neurodegenerative diseases and cancer suggest these proteins are important in the regulation of ageing and longevity. These proteins may also provide useful targets for disease therapies (Kampinga and Garrido, 2012).

1.9 AIMS OF THE THESIS.

The principal aims of this thesis were to determine if two members of the sHsp family, Hsp12 and Hsp26, play a role in processes associated with DR-mediated lifespan extension in *S. cerevisiae*. Published data report the induced expression of *HSP12* and *HSP26* mRNA in response to glucose deprivation (Lindquist, 1986, Lanks, 1986, Kasambalides and Lanks, 1983). Furthermore, transcriptome analysis has revealed *HSP12* and *HSP26* up-regulation in old yeast cells (Lin et al., 2001, Lesur and Campbell, 2004). Both findings may suggest that Hsp12 and Hsp26 play a role in longevity regulation in response to DR.

The first objective was to determine if Hsp12 and Hsp26 are up-regulated at the protein level in response to moderate (0.5% (w/v) glucose) and severe (0.05% (w/v) glucose) DR. The second objective was to determine if *hsp12Δ*, *hsp26Δ* and *hsp12/hsp26Δ* deletion mutants have any effect on RLS when grown on media supplemented with 2% (w/v) glucose and 0.05% (w/v) glucose. The third objective was to determine if *hsp12Δ*, *hsp26Δ* and *hsp12/hsp26Δ* deletion mutants have any defects in processes associated with yeast ageing including stress resistance, Sir2 activity and protein aggregation. Defects in any of these processes may reveal a role for Hsp12 and Hsp26 in yeast longevity.

The fourth objective was to perform an unbiased genome wide approach, synthetic genetic array analysis (SGA), to identify genetic interactions of *HSP12* and *HSP26*. SGA analysis involves the generation of thousands of double mutants and allows their growth under various conditions including DR to be analysed. The rationale behind this approach was that SGA analysis should provide insights into the cellular functions of Hsp12 and Hsp26 and help to develop an understanding of how these proteins may effect ageing and DR-mediated lifespan extension in yeast.

2.1 YEAST.

2.1.1 Strains used in this study.

The yeast strains used in this study included JS128 (a kind gift from Professor Jeffrey Smith, University of Virginia, Charlottesville, VA) which carries a *Ty::mURA3* marker with the reporter module inserted into the non-transcribed spacer region (NTS1) of the ribosomal DNA (rDNA) and AEY1017 (a kind gift from Dr Jessica A. Downs, University of Sussex) which has a *URA3* gene inserted in the left telomere of chromosome VII (Smith and Boeke, 1997, Meijsing and Ehrenhofer-Murray, 2001). The BY4741, BY4742 and BY4743 wildtype and single mutants were purchased from Abgene (Fisher Scientific UK LTD, Leicestershire, UK). A BY4741 *hsp12/hsp26Δ* double mutant was constructed at the University of Liverpool previous to this study by Dr Michèle Riesen (Appendix table 1).

The DLY7325 starter strain, as well as the DLY7388 *his3Δ* control and DLY7325 *hsp26Δ* and *hsp12Δ* single mutants were obtained from Professor David Lydall (Newcastle University). The DLY7325 and DLY7388 strains are a derivative of S288C. In addition single mutants from the KANMX deletion collection were also obtained from Professor David Lydall (Appendix table 1).

2.1.2 Standard yeast culture media.

Standard yeast culture media used in this study included yeast peptone dextrose (YPD) media, made by adding 2% peptone, 1% yeast extract and D-glucose to the required concentration. Yeast peptone dextrose (YPD) agar was made by adding 2% peptone, 1% yeast extract, 2% agar and D-glucose to the required concentration. Synthetic complete (SC) Kaiser mixture was made by adding 0.67% yeast nitrogen base supplemented with complete or drop-out amino acid mixture according to the manufacturer instructions and adding D-glucose to the required concentration. A 20% (w/v) stock of D-glucose was prepared in MilliQ H₂O, filtered sterilised and then added to molten media to the required concentration.

Yeast liquid cultures were routinely grown overnight or until they reached the required OD₆₀₀ in a shaking incubator at 30 °C and 200 rpm. Solid agar plates were routinely incubated at 30 °C for 2 to 4 days, however, yeast strains exposed to severe stresses often required longer incubation up to 12 days.

2.1.3 Construction of knockout strains by PCR-mediated gene disruption.

Generally, deletion mutants were constructed by PCR-mediated gene disruption. For this, a deletion cassette carrying a resistance marker was integrated into the yeast genome by homologous recombination. Flanking sequences either end of the deletion cassette were designed to be homologous to sequences either side of the targeted open reading frame (ORF). When transformed into competent yeast cells homologous recombination allows the targeted ORF to be replaced by the deletion cassette (Figure 2.1). Subsequent transformations were then screened for by selective antimicrobial resistance or by nutritional selection. The deletion cassettes used in this study include NATMX, conferring resistance to clonNAT (nourseothricin), KANMX4, conferring resistance to G418 (geneticin) and HIS3MX6, which allows growth on media lacking histidine (Goldstein and McCusker, 1999, Baudin et al., 1993). The deletion cassettes were either PCR-amplified from plasmid DNA e.g. NATMX from PDL1222 plasmid DNA or PCR amplified from deletion strains, e.g. KANMX4 and HIS3MX6. Primers were designed to amplify the deletion cassette and contain flanking sequences homologous to regions either side of the target ORF. PCR amplification of the deletion cassette was set up in a final volume of 50 μ l as follows: 22 μ l of MilliQ H₂O, 1 μ l of forward primer (100 μ M), 1 μ l of reverse primer (100 μ M), 1 μ l of plasmid DNA or 1 μ l of purified gDNA from deletion strain and 25 μ l of 2X My Taq master mix (Bioline, London, UK).

The following PCR conditions were used:

1 cycle:	94 °C	5 minutes
35 cycles:	94 °C	45 seconds
	55 °C	60 seconds
	72 °C	3 minutes
1 cycle:	72 °C	10 minutes

To make cells competent to take up exogenous DNA (Gietz and Woods, 2002), a fresh 50 ml culture was grown overnight in YPD 2% (w/v) glucose to a density of 2×10^7 cells/ml. Cultures were centrifuged at 5,000 g for 5 minutes and

harvested in 25 ml MilliQ H₂O. Cells were then washed in 1 ml 100 mM lithium acetate (LiAC), centrifuged again and resuspended in 400 µl 100 mM LiAC.

To create gene knockouts, 50 µl of competent cells were added to 240 µl 50% (w/v) polyethylene glycol (PEG), 36 µl 1 M LiAC, 50 µl salmon testes single stranded DNA and 34 µl of the deletion cassette PCR-product. Cells were vortexed for 1 minute, incubated at 30 °C for 30 minutes, heat shocked at 42 °C for a further 30 minutes, centrifuged at 7,000 g for 15 seconds and then resuspended in 1 ml of MilliQ H₂O and added to 4 ml YPD and incubated overnight at 30 °C, 200 rpm. The following day successful transformants were selected for by growth of colonies on appropriate media. After 2-3 days at 30 °C, 3 colonies of each strain were picked and freshly grown on individual solid agar plates. After 2 days incubation at 30 °C, DNA was extracted and purified and the knockout strain confirmed by PCR (see section 2.2.1). Glycerol stocks were also made by taking a large inoculum of growth from the agar plate and re-suspending in appropriately labelled cryotubes (Corning) containing 50% (w/v) glycerol tubes were stored at -80 °C.

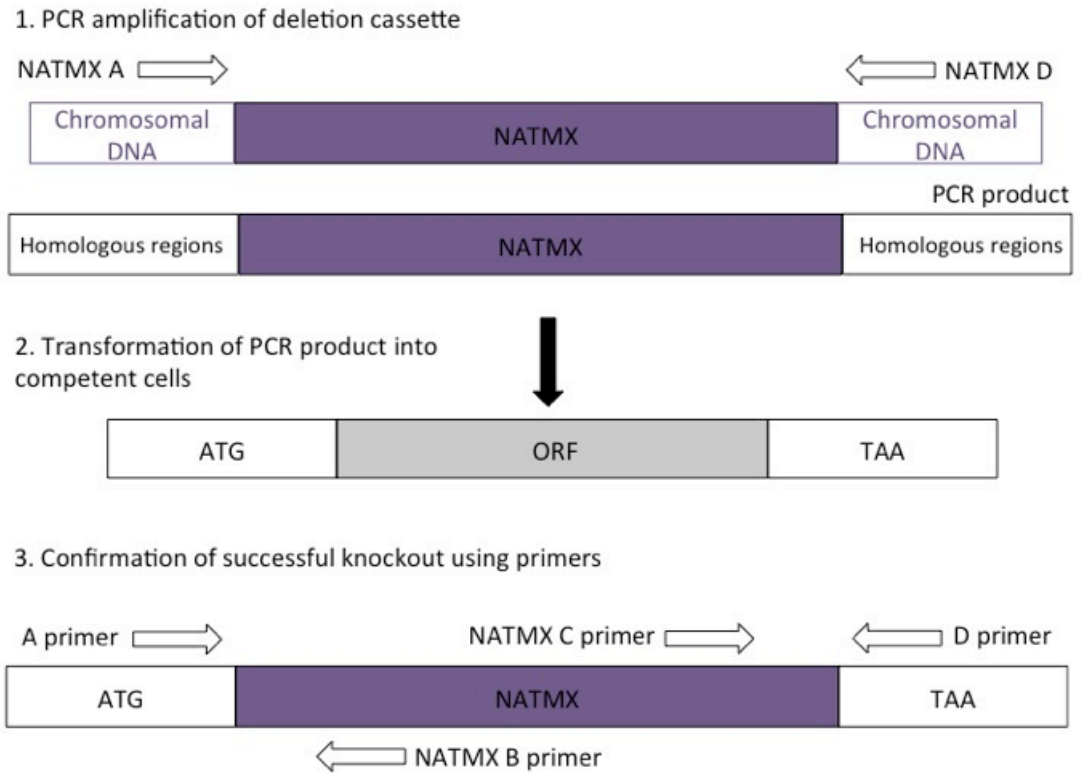


Figure 2.1. PCR mediated gene disruption. The NATMX deletion cassette is PCR-amplified from PDL1222 plasmid DNA using NATMX forward (A) and reverse primers (D), which anneal either side of the deletion cassette (step 1). The NATMX forward and reverse primers are also designed to have flanking sequences homologous to the sequences either side of the targeted ORF. The PCR product is then transformed into competent yeast cells, which use homologous recombination to replace the targeted ORF with the deletion cassette (step 2). DNA is purified from transformed colonies growing on selective media and confirmation of successful knockout mutants is confirmed by PCR using primers that anneal either side of the ORF (A and D primer) or within the deletion cassette (NATMX B and C) (step 3).

2.1.4 Construction of knockout strains by replica plating.

For construction of knockout strains by replica plating, one strain of yeast carrying a gene deletion linked to a NATMX deletion cassette, was mated with another strain of yeast of opposite mating type carrying a gene deletion linked to a KANMX deletion cassette. For the mating process, one colony of each yeast knockout strain was inoculated into a microcentrifuge tube containing 500 µl of YPD broth and incubated at 30 °C, 200 rpm overnight. The following day, resulting zygotes were selected by inoculating 100 µl of the mating culture on media containing both antibiotics, geneticin (G418) and clonNAT, to which the deletion cassettes are resistant. Plates were incubated at 30 °C for 3-5 days to allow diploids to form. Once formed, the heterozygous diploids were replica plated onto enriched sporulation media containing 10 g potassium acetate, 1 g yeast extract, 0.5 g glucose, 0.1 g amino acid supplement (0.1% leucine, 0.02% histidine, 0.02% methionine and 0.02% uracil), 20 g agar and 1 L MilliQ H₂O. Enriched sporulation media contains a reduced nitrogen content thereby inducing sporulation, plates were incubated at 22 °C for 5 days to allow for the formation of haploid meiotic progeny. Haploids of mating type a were selected by replica plating onto selective media lacking histidine. To obtain double mutants of the desired mating type, haploids were finally replica plated onto selective media lacking histidine containing both clonNAT and G418 (Figure 2.2). After 2 days' incubation at 30 °C, glycerol stocks were prepared and DNA was extracted and knockout strains confirmed by PCR (See section 2.1.5).

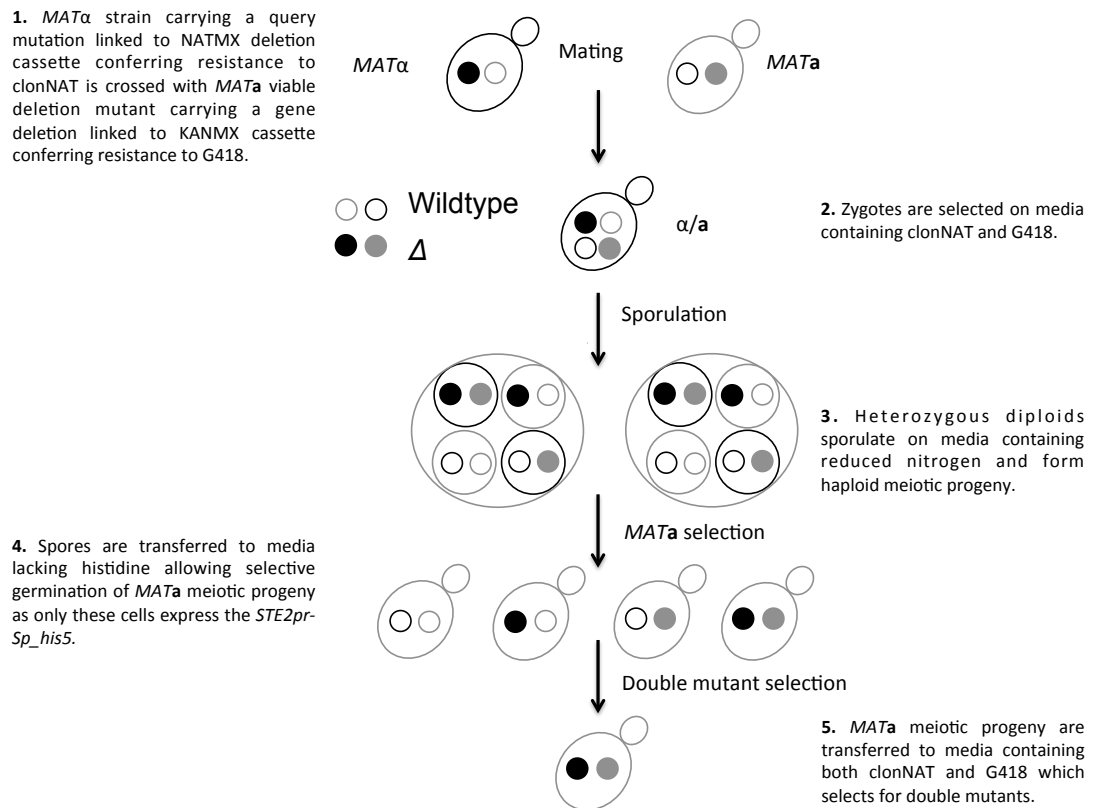


Figure 2.2. Schematic showing construction of knockout strains by replica plating. Yeast strains of opposite mating type each containing a single gene knockout linked to different deletion cassettes, in this example, KANMX4 and NATMX. Yeast strains were mated overnight and resulting zygotes were selected for on media containing both antibiotics to which the deletion cassettes were resistant, i.e. clonNAT and G418. Replica plating onto enriched sporulation media induced the formation of haploid meiotic progeny. Replica plating haploids onto media lacking histidine selected for *MAT α* haploids only, as the *HIS5* reporter gene from *S. pombe* is under control of an STE2 promoter (*STE2pr-SP_his5*) (Tong, 2007b). Finally *MAT α* double mutants were selected for by replica plating onto media lacking histidine and containing clonNAT and G418.

2.1.5 Confirmation of knockout mutants

To confirm correct construction of knockout strains genomic DNA (gDNA) was extracted and purified using Y-DER yeast DNA extraction kit (Perbio Science UK Ltd), in accordance with the manufacturer's instruction. Knockout strains were then confirmed by PCR using primers that anneal either side of the ORF and show a difference in band size in the knockout mutant compared to the wildtype gDNA (see Figure 2.3).

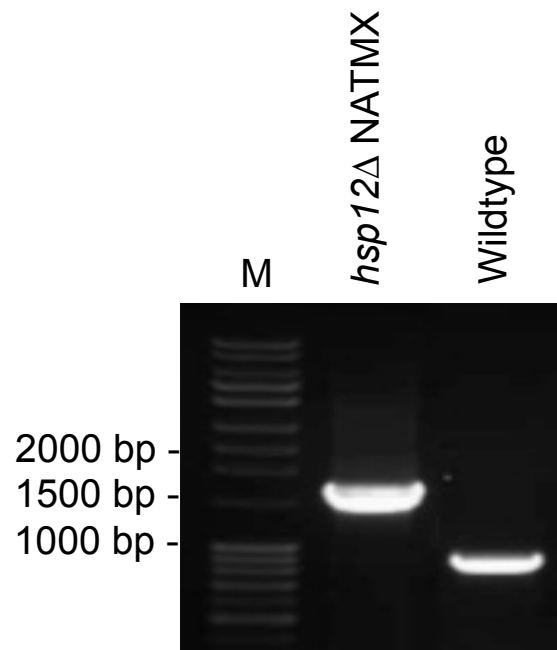


Figure 2.3. Confirmation of knockout mutants by PCR. Agarose gel showing amplified gDNA from an *hsp12Δ* NATMX knockout mutant (lane 1) with Hsp12 primers, which anneal either end of the *HSP12* ORF. The *hsp12Δ* knockout mutant gives a band ~1583bp when the *HSP12* ORF has been replaced with the NATMX deletion cassette. In contrast the wildtype gDNA gives a band ~913bp (lane 2).

2.1.6 Visualisation of DNA.

PCR products were separated on 0.8% (w/v) agarose gels in Tris-acetate/ethylenediaminetetraacetic acid (TAE) buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) and bands were visualised with SYBRSafe DNA stain (Invitrogen, Eugene, Oregon, USA). HighRanger Plus 100 bp DNA ladder (Norgen Biotech Corporation) was loaded as a visual reference for molecular size and 2 µl of 6X DNA loading buffer (0.35% (w/v) orange G, 30% (w/v) sucrose, 0.05% (w/v) bromophenol blue) was added to 10 µl of sample. Gels were run at 90 V for 50 to 60 minutes. Images of gels were taken using a BioRad ChemiDoc™ XRS (BioRad, Hemel Hempstead, UK).

2.1.7 Qualitative silencing assays.

For qualitative silencing assays single colonies of yeast strains were grown overnight in 5 ml YPD broth containing 2% (w/v) glucose. Cultures were adjusted to an OD₆₀₀ 1.0 and 50 µl of cultures were pipetted into column 1 of a 96 well plate containing 150 µl MilliQ H₂O and five-fold serially diluted in MilliQ H₂O to a final volume of 300 µl. Using a replica plater (Sigma-Aldrich, Poole, Dorset), yeast dilutions were inoculated onto synthetic complete (SC) media, synthetic uracil drop out (SC-URA) and SC supplemented with 5-fluororootic acid (FOA was dissolved in sterile-filtered dimethylsulfoxide (DMSO) and added to SC to give a final concentration of 1 mg/ml). Plates were incubated at 30 °C for 2 days and imaged in a BioRad ChemiDoc™ XRS.

2.1.8 Stress assays.

Yeast strains were grown overnight and stress assays prepared as detailed for qualitative silencing assays. Yeast strains were then replica plated onto YPD plates supplemented with the following drugs: 1 mM or 5 mM hydrogen peroxide (H₂O₂), 3 µM paraquat, 15 mM caffeine, 200 nM rapamycin, 0.4 M or 1 M sodium chloride (NaCl), 13% ethanol (EtOH), 0.04% methyl methanesulfonate (MMS) and 25 µM N-ethylmaleimide (NEM). For Dietary restriction (DR) and thermotolerance yeast strains were replica plated onto media with reduced glucose (0.5% and 0.05% (w/v)) or standard media and incubated at a range of temperatures. Other plates were incubated at 30 °C for 2 to 4 days. However, yeast strains exposed to severe

stresses often required longer incubation up to 12 days. Plates were imaged using a BioRad ChemiDoc™ XRS.

2.1.9 Aggregation assays.

For aggregation assays, yeast cultures were grown in the appropriate liquid broth to an OD₆₀₀ 0.6, and then centrifuged at 5000 g for 5 minutes. The pellet was resuspended in 1 ml MilliQ H₂O, divided equally between 2 microcentrifuge tubes, one of which kept at room temperature and the other heat shocked at 43 °C. After an hour's incubation, samples were centrifuged at 13,523 g for 5 minutes and the pellets resuspended in 500 µl non-denaturing lysis buffer (NDLB) (150 mM NaCl, 50 mM TRIZMA base pH 8, 10% glycerol and 1 tablet of protease inhibitor (Sigma) per 10 mls). Samples were transferred to labelled cryotubes (Corning) and acid-washed beads (450-600 µm) were added to the meniscus and tubes shaken in the Mikro Dismembrator S (Sartorius Stedim S.A) at 2000 rpm for 5 minutes. The bottom of the tubes were pierced three times with a 27 gauge hypodermic needle and immediately transferred to 15 ml Falcon tubes and centrifuged at 5000 rpm for 5 minutes. The lysates were transferred to microcentrifuge tubes and cleared at 13,523 g for 10 minutes. For the lysate previously heat shocked at 43 °C, an aliquot was stored for future BCA protein assays (Thermo Scientific). The remaining supernatant was transferred to a microcentrifuge tube labelled SN143 and an equal volume of 2X Laemmli (63 mM Tris HCL, 10% (w/v) glycerol, 2% (w/v) SDS, 0.0025% (w/v) bromophenol blue pH 6.8) added. The same volume of 2X Laemmli was added to the pellet and this tube labelled P143. Samples were then boiled for 5 minutes.

For lysates previously incubated at room temperature the supernatant was transferred to a fresh microcentrifuge tube. 2X Laemmli was added to the pellet, the tube was labelled P1RT and the sample boiled for 5 minutes. A 30 µl aliquot of the supernatant was stored for future BCA protein assay. The remaining supernatant was evenly divided between 3 microcentrifuge tubes. One tube was labelled SN1RT and an equal volume of 2X Laemmli was added and the sample boiled for 5 minutes. The two remaining tubes were then incubated for 1 hour, one tube at room temperature and one tube at 43 °C. Samples were cleared by centrifugation at 13,523 g for 10 minutes. 30 µl aliquots from each sample were

transferred to fresh microcentrifuge tubes labelled either SN2RT or SN243 accordingly, and stored for future BCA protein assay. The remaining supernatants were transferred to fresh microcentrifuge tubes labelled SN2RT or SN243, an equal volume of 2X Laemmli was added and the samples were boiled for 5 minutes. 2X Laemmli was added to the pellets and the tubes labelled P2RT or P243 accordingly, samples were then boiled for 5 minutes.

Protein assays were performed using Thermo Scientific Pierce BCA Protein Assay Kits in accordance with the manufacturers instructions. Equal amounts of proteins were loaded and separated on Bis-Tris precast gels (Invitrogen). 20 μ l of samples were loaded and as a reference 10 μ l of Novex[®] Sharp pre-stained protein standard marker (Invitrogen) was used.

2.1.10 Laemmli yeast whole cell extraction.

Single colonies of yeast strains were grown in the appropriate liquid culture to an OD₆₀₀ 0.6. Cell pellets were harvested at 5,000 g for 5 minutes, washed twice in 10 ml MilliQ H₂O, weighed and adjusted to approximately 30 mg/ml and resuspended in 100 μ l 2X Laemmli buffer (63 mM Tris HCl, 10% (w/v) glycerol, 2% (w/v) SDS, 0.0025% (w/v) bromophenol blue pH 6.8). The suspension was transferred to a 2 ml cryotube (Corning), acid-washed glass beads (450-600 μ m) were added to the meniscus and samples boiled at 100 °C for 5 minutes. The tubes were inserted into a Mikro Dimembrator S and shaken at 2,000 rpm for 2 minutes and then boiled for a further 5 minutes and shaken in the Mikro Dismembrator at 2,000 rpm for 2 minutes. The bottom of the tubes were pierced three times with a 27 gauge hypodermic needle and immediately transferred to 15 ml Falcon tubes and centrifuged at 5,000 rpm for 5 minutes at 4 °C. The cryotubes were removed and the lysates transferred to microcentrifuge tubes and cleared at 13,000 rpm for 20 minutes. The supernatant was carefully transferred to a fresh tube and 10 μ l of each sample was diluted in 40 μ l 4X Laemmli buffer.

2.2 WESTERN BLOTTING.

2.2.1 SDS polyacrylamide gel electrophoresis (SDS-PAGE).

Proteins were separated on Bis-Tris precast gels (Invitrogen). 20 µl of samples were loaded and as a reference 10 µl of Novex® Sharp pre-stained protein standard marker (Invitrogen) was used. The samples were run in 1X 2-(N-morpholino)ethanesulfonic acid (MES) buffer (Invitrogen) at 180 V until the lowest marker band had reached the bottom of the gel. The gels were either stained with SimplyBlue™ SafeStain (Invitrogen) or transferred to nitrocellulose membrane.

2.2.2 Western blotting.

SDS-PAGE gels were transferred to nitrocellulose (Whatman Protran BA83, 0.2 µm) at 100 V in low molecular weight transfer buffer (25 mM Tris, 192 mM glycine, 40% methanol). The membrane was stained with Ponceau S solution (Sigma) to check the efficiency of the transfer. The membrane was then washed with 1X PBS to remove the stain and blocked in blocking buffer (3% (w/v) milk in PBS) for 1 hour on an orbital shaker. The nitrocellulose was incubated for 1 hour with the primary antibody (rabbit anti-Hsp26 C-terminal or rabbit anti-Hsp12 C-terminal, custom designed from Genosphere biotechnologies) diluted 1:1000 in 1X PBS and then washed three times in 1X PBS for a total of 15 minutes. A horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody was used at 1:1000 in 3% (w/v) milk solution with 0.5% (w/v) Tween and incubated for 1 hour on an orbital shaker. The membrane was washed once in 1X PBS with 0.5% (w/v) Tween and three times in 1X PBS. Bands were visualised by mixing enhanced chemiluminescence (ECL) reagents solution A (2.5 mM luminol, 0.4 mM p-coumaric acid, 100 mM Tris HCl pH 8.5) and solution B (100 mM Tris HCl pH 8.5, 0.02% H₂O₂) 1:1.

2.3 CLONING.

2.3.1 Cloning synthetic *HSP12* gene from plasmid into expression vector pE-SumoPro Kan.

A custom synthesised *HSP12* gene which was codon optimised for expression in *Escherichia coli* (*E. coli*) was ordered from Geneart (Invitrogen, Paisley, UK) (Figure 2.4). The pE-SumoPro Kan vector (2 µg) (Figure 2.5) was digested with BsaI, in a 20 µl reaction and incubated at 37 °C for 90 minutes and heat inactivated at 65 °C for 20 minutes. 17 µl (1.7 µg) of the *HSP12* gene was double digested using BsaI and XbaI in a 20 µl reaction incubated at 37°C for 90 minutes and heat inactivated at 65°C for 20 minutes.

```

                                KpnI NdeI BsaI
CGAATTGGCGGAAGGCCGTCAAGGCCACGTGTCTTGTCCAGGTACCATATGAGGTCTCCA
1 -----+-----+-----+-----+-----+-----+-----+
GCTTAAACCGCCTTCCGGCAGTTCGGGTGCACAGAACAGGTCCATGGTATACTCCAGAGGT

                                BspMI
GGTATGAGTGATGCAGGTTCGTAAGGTTTTGGTGAAAAAGCAAGCGAAGCACTGAAACCG
61 -----+-----+-----+-----+-----+-----+
CCATACTCACTACGTCCAGCATTTCAAAAACCACCTTTTTCGTTCGCTTCGTGACTTTGGC
    M S D A G R K G F G E K A S E A L K P

GATAGCCAGAAAAGCTATGCAGAACAGGGCAAAGAATATATTACCGATAAAGCGGATAAA
121 -----+-----+-----+-----+-----+-----+
CTATCGGTCTTTTCGATAAGTCTTGTCCCGTTTCTATATAATGGCTATTTTCGCCTATTT
    D S Q K S Y A E Q G K E Y I T D K A D K

                                BspMI
GTGGCAGGTAAAGTTCAGCCGGAAGATAATAAAGGTGTTTTTCAGGGTGTGCATGATAGC
181 -----+-----+-----+-----+-----+-----+
CACCGTCCATTTCAAGTCGGCCTTCTATTATTTCCACAAAAAGTCCACACGTACTATCG
    V A G K V Q P E D N K G V F Q G V H D S

GCAGAAAAGGTAAGATAATGCCGAAGGTCAGGGTGAAAGCCTGGCAGATCAGGCACGT
241 -----+-----+-----+-----+-----+-----+
CGTCTTTTCCATTCTATTACGGCTTCCAGTCCCACTTTTCGGACCGTCTAGTCCGTGCA
    A E K G K D N A E G Q G E S L A D Q A R

GATTATATGGGTGCAGCAAAAAGCAAACGAATGATGCCGTTGAAATATGTTAGCGGTCTGT
301 -----+-----+-----+-----+-----+-----+
CTAATATACCCACGTCTTTTCGTTTGACTTACTACGGCAACTTATACAATCGCCAGCA
    D Y M G A A K S K L N D A V E Y V S G R

                                XbaI BamHI SacI
GTTTCATGGTGAAGAAGATCCGACCAAAAAATAATCTAGAGGATCCGAGCTCTGGAGCACA
361 -----+-----+-----+-----+-----+-----+
CAAGTACCACTTCTTCTAGGCTGGTTTTTTTATTAGATCTCCTAGGCTCGAGACCTCGTGT
    V H G E E D P T K K *

AGACTGGCCTCATGGGCCTTCCGCTCACTGC
421 -----+-----+-----+-----+-----+
TCTGACCGGAGTACCCGGAAGGCGAGTGACG

```

Figure 2.4. Geneart synthesised HSP12 sequence. Sequence showing Kpn1, Nde1 and Xba1 restriction enzyme sites at the 5' end and Xba1, BamH1 and Sac1 restriction enzyme sites at the 3' end.

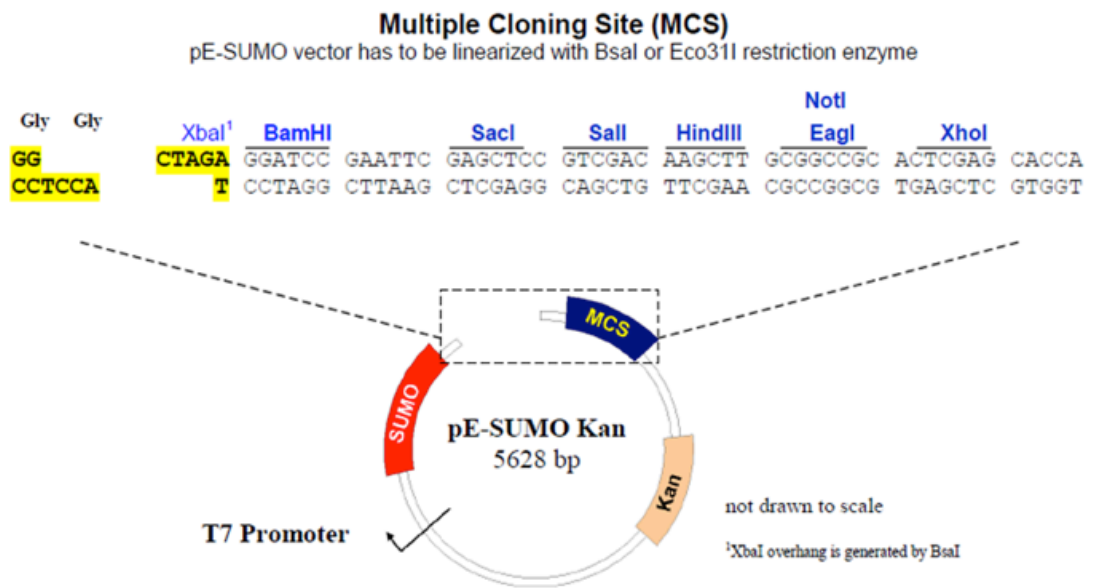


Figure 2.5. Cloning map of pE-SUMO Kan.

http://www.lifesensors.com/pdf/1000K_1001K_Polylinker_map_Kan-1.pdf

2.3.2 Ligation.

The insert was ligated into the vector using Roche rapid ligation kit according to manufacturer's instructions. The ligation reaction was prepared as follows: 0.5 µl linearised pE-SumoPro Kan (25 ng), 1.5 µl of linearised synthetic gene plasmid (250 ng), 1 µl 5X DNA dilution buffer, 5 µl 2X T4 DNA ligation buffer, 0.5 µl T4 DNA ligase, 1.5 µl MilliQ H₂O and incubated at room temperature for 5 minutes.

2.3.3 Transformation into *E. coli* TOP10.

Chemically competent *E. coli* TOP10 cells were thawed on ice; 2 µl of the ligation reaction was added to the competent cells and then incubated on ice for 30 minutes. The cells were heat shocked at 42 °C for 30 seconds before placing back on ice for 1 minute, 250 µl of SOC media (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 8.56 mM NaCl, 2.5 mM KCl, 10 mM MgCl and 20 mM glucose) was added to the ligation reaction and incubated at 37 °C, 200 rpm for 1 hour. After 1 hour, 20 µl and 200 µl of culture was inoculated onto two LB agar plates containing kanamycin (30 µg/ml). To screen out false positives, 10 individual colonies were inoculated into 5 ml of LB broth containing kanamycin and with the same loop inoculated into 5 ml LB broth containing ampicillin (100 µg/ml), cultures were grown at 37 °C and 200 rpm until they became turbid or overnight. The following morning, kanamycin resistant cultures that were unable to grow in LB plus ampicillin were minipreped using GenElute HP Plasmid miniprep kit (Sigma) in accordance with manufacturer's instructions. To check the cloning had been successful a test digest was performed with Xba1. The Xba1 restriction enzyme digests the plasmid twice, once at the end of the gene and another just before the sumo tag giving an insert band ~700 bp.

2.3.4 Transformation into *E. coli* BL21 (DE3) cells.

Microcentrifuge tubes utilised for the transformation were chilled, and BL21 (DE3) cells thawed on ice. 2.5 µl of miniprep DNA (Hsp12 pE-SumoProKan) was added to 50 µl of BL21 (DE3) cells and left on ice for 30 minutes. Cells were then heat shocked at 42 °C for 30 seconds before placing back on ice for a further 1 minute 250 µl of SOC media was added and vials were shaken for 1 hour at 37 °C and 200 rpm. After incubation 20 µl and 200 µl of culture was inoculated onto 2 LB plates containing kanamycin (30 µg/ml) and incubated at 37 °C overnight. The

following morning, colonies of transformants were grown in 10 ml LB broth plus kanamycin (30 µg/ml) to OD₆₀₀ 0.8.

2.3.5 Expression and purification of recombinant Hsp12.

To test protein expression, 10 ml cultures of BL21 (DE3) cells transformed with Hsp12-pE-SumoPro Kan were grown in LB containing kanamycin (30 µg/ml) to OD₆₀₀ 0.8. A 250 µl aliquot was removed and stored at 4 °C as a non-induced sample and to the remaining culture isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce protein expression. Cultures were incubated at 37 °C for 4 hours. After 4 hours, 250 µl of the culture was removed and a final OD₆₀₀ reading was taken, this was kept as the induced sample. An equal volume of 2X Laemmli buffer was added to induced and non-induced samples which were boiled and then analysed by SDS-PAGE to check protein expression. Once protein expression had been confirmed in the induced sample, overnight starter cultures of Hsp12-pE-SumoPro Kan were grown in 10 ml LB containing kanamycin. 8 ml of the starter culture was then added to 1 L LB plus kanamycin and incubated at 37 °C to an OD₆₀₀ of 0.8. IPTG (1 mM final) was then added to the culture and protein expression was induced for 4 hours at 37 °C and 200 rpm. After incubation the culture was centrifuged at 5,000 g at 4 °C for 10 minutes and the cell pellet resuspended in 8 ml buffer 1 (50 mM HEPES, 150 mM sodium chloride pH 7.5). Bacterial cells were lysed using a One Shot Cell Disrupter (Constant Systems, Daventry, UK).

The recombinant Hsp12 contains a short affinity-tag consisting of six histidine residues known as a Hexa His-tag (Terpe, 2003). Histidine exhibits a strong interaction with immobilised metal ion matrices such as nickel (Terpe, 2003), hence the Hsp12 containing the His-tag will bind to the nickel immobilised on affinity resin. Recombinant proteins containing a His-tag can be eluted by the addition of free imidazole which has a higher affinity for nickel and will displace the protein from the affinity resin (Hochuli et al., 1987). The His-tag therefore provides an excellent means for purifying recombinant proteins. His-SUMO-Hsp12 was thus purified by affinity chromatography to separate the protein of interest from other bacterially expressed proteins on the basis of this highly specific interaction. His-

SUMO-Hsp12 was passed through a nickel affinity column also known as a His-trap (GE Healthcare) on a ÄKTA fast protein liquid chromatography system (GE Phamacia). His-SUMO-Hsp12 was eluted with the addition of Buffer 2 (same as Buffer 1 but includes 0.5 M imidazole). The recombinant His-SUMO-Hsp12 is released from the column as the imidazole concentration increases. Fractions of 2.5 ml were collected and analysed by SDS-PAGE to identify the fractions containing the recombinant protein. An aliquot of protein was saved for cleavage of the His-SUMO tag and the remaining aliquots were subjected to ion exchange chromatography (IEC).

Before IEC, fraction 20 containing a high quantity of His-SUMO-Hsp12 was dialysed overnight in 2 L of 20 mM Hepes pH 7.5 at 4 °C to remove the imidazole. IEC separates proteins based on charge and was used to further purify Hsp12 away from other protein contaminants. The pI of Hsp12 is 5.2; therefore anionic exchange was utilized with Buffer A (20 mM HEPES pH 7.5). Since the protein has a net negative charge at this pH it will bind to the positively charged resin at the anion exchanger. The gradual addition of Buffer B (20 mM HEPES, 1 M NaCl pH 7.5) increases the salt concentration of the buffer. IEC was used as a final purification step as proteins with weaker ion interactions will be eluted at lower salt concentrations and those with stronger charges will remain bound for longer and will only be displaced at higher salt concentrations; this therefore provides a purer protein product. IEC fractions were analysed by SDS-PAGE.

2.3.6 Cleavage of His-SUMO tag with SUMO protease.

A test cleavage was set up using 10X 2 µl aliquots of purified His-SUMO-Hsp12 diluted 1:10 with 50 mM HEPES, 150 mM NaCl pH 7.5, and SUMO protease added at 1:4 dilution series. Tubes were incubated at 30 °C for 1 hour. Samples were then analysed by SDS-PAGE to check cleavage of the His-SUMO-tag to determine the appropriate concentration of SUMO protease required. 2.5 ml of a fraction containing a large amount of His-SUMO-Hsp12 was then dialysed overnight at 4 °C in 2 L of 20 mM HEPES, at pH 7.5. The cleaved protein was then passed over a His-trap column; since the recombinant protein no longer contains a His-tag it no longer binds to the column and is collected in the flow-through from the column.

The His-SUMO tag binds to the nickel and are only eluted with the addition of a second buffer containing 0.5 M imidazole. Fractions were analysed by SDS-PAGE.

2.3.7 Cloning for overexpression of *HSP12*.

The ORF of *HSP12* was PCR amplified from S288C purified gDNA using primers that anneal either side of the ORF and contain Pst1 and Kpn1 restriction enzyme overhangs (Hsp12/Pst1 forward 5'-GTCCAGGTCTGCAGCGATTTGTTTCGTTATATGCC-3' and Hsp12/Kpn1 reverse 5'-CATGAGTAGGTACCAGGAGGGTAGGAAGCGCGAG-3'). A restriction digest was set up on the resulting PCR product and YCp:LEU shuttle vector (Sikorski and Hieter, 1989) in a total volume of 20 μ l using 2 μ l NE buffer 1, 0.5 μ l Pst1 and 0.5 μ l Kpn1. Reactions were incubated at 37 °C for 3 hours and 5 μ l of shrimp alkaline phosphatase was added in the final 15 minutes to catalyse the dephosphorylation of 5' phosphates in the DNA. Before ligation, a PCR clean up step was performed using a GenElute PCR clean up kit in accordance with manufacturer's instructions.

The ligation reaction was set up on ice in a total volume of 10 μ l as follows; 1 μ l YCp:LEU vector, 4 μ l S288C PCR product (or ddH₂O for control reaction), 2 μ l T₄ DNA ligation buffer, 1 μ l T4 ligase and 2 μ l ddH₂O. Reactions were incubated at room temperature for 2 hours. Following incubation 3 μ l of the ligation reaction was transformed into 80 μ l of DH5 α cells, samples were incubated on ice for 30 minutes and heat shocked at 42 °C for 2 minutes. 400 μ l of SOC media was added and the samples transferred to 15 ml Falcon tubes and incubated for 1 hour at 37 °C. 45 μ l of the samples was inoculated onto LB agar supplemented with ampicillin and incubated at 37 °C overnight. The following day 5 colonies were miniprepped using GenElute HP Plasmid miniprep kit (Sigma) and a test digest performed using Kpn1 and Pst1 enzymes to confirm successful cloning. The YCp:LEU:pHsp12:Hsp12 plasmid DNA was then utilised to transform BY4741 wildtype cells (Gietz and Woods, 2002) to achieve overexpression of *HSP12* under the control of its endogenous promoter.

2.4 AFFINITY PURIFICATION OF HSP12 ANTIBODY.

10 ml of Hsp12 N-terminal antiserum was incubated with 1 ml of His-SUMO-Hsp12 protein (0.45 mg/ml) bound to a nickel affinity column, known as a His-trap (GE Healthcare) and agitated on a rocker for 1 hour at room temperature. Following incubation the sample was centrifuged at 1,000g for 1 minute and the unbound supernatant was transferred to a labelled 15 ml falcon tube and kept on ice. The pellet was washed 6 times by centrifuging at 1,000 g for 1 minute and resuspending the pellet in HEPES buffered saline (50 mM HEPES, 150 mM NaCl, pH 7.5). The pellet was resuspended in 5 M LiCl, 10 mM KH₂PO₄ pH 7 and incubated for 10 minutes with agitation. The antibody was eluted by centrifuging at 1,000 g and transferred to a labelled tube. The LiCl eluted antibody was dialysed overnight in HEPES buffered saline using a Slide-A-Lyser Dialysis cassette 10K MWCO (Thermo Scientific, UK). 0.5% sodium azide in PBS was added to the affinity column and stored with the unbound fraction at 4 °C.

2.5 SYNTHETIC GENETIC ARRAY (SGA) ANALYSIS.

SGA analysis was performed in collaboration with Professor David Lydall at Newcastle University. For this, a *MAT α* strain carrying a query mutation (*hsp12 Δ* or *hsp26 Δ*) linked to a NATMX deletion cassette conferring resistance to clonNAT was crossed to an ordered array of ~5000 *MAT α* viable deletion mutants (*xxx Δ*) each carrying a gene deletion mutation linked to a KANMX deletion cassette conferring resistance to geneticin (G418). Through multiple replica plating steps the meiotic progeny carrying both gene deletions were selected and then scored for fitness defects.

Construction of *hsp12 Δ* and *hsp26 Δ* query strains was performed at the University of Liverpool. Confirmation of the query strains by PCR and random spore analysis (RSA) was performed at the university of Liverpool. Once confirmed, the *hsp12 Δ* and *hsp26 Δ* query strains were sent to Newcastle University and SGA experiments were performed. The results from the SGA analysis were then sent to the University of Liverpool for analysis.

2.5.1 Construction of query strains for SGA.

The query mutants (*hsp12 Δ* and *hsp26 Δ*) were constructed in the DLY7325 yeast background strain, which is *MAT α* and a derivative of S288C. The NATMX deletion cassette was amplified from the PDLI222 plasmid DNA by PCR, and the query strains were constructed by PCR-mediated gene disruption (described earlier). Successful transformed colonies were selected for on media containing 100 $\mu\text{g/ml}$ of clonNAT and 300 $\mu\text{g/ml}$ of hygromycin B. The query strain contains a hygromycin marker (HPHMX) which shares homologous promoter and terminator sequences with the NATMX deletion cassette (Goldstein and McCusker, 1999). Therefore when transformed into competent yeast cells the NATMX cassette can be integrated at the HPHMX locus instead of the targeted ORF. Therefore both clonNAT and Hygromycin B were added to the media to select for only transformants with correctly integrated deletion cassettes. gDNA from 3 colonies was purified and query strains were confirmed by PCR using primers that anneal either side of the ORF and show a difference in band size in the knockout mutants compared to the wildtype gDNA.

2.5.2 Random Spore Analysis (RSA).

RSA was performed on query strains as a second confirmation step following PCR confirmation. RSA is a yeast genetic tool for the generation of recombinant progeny and determining gene linkage by genetic crosses (Khare et al., 2011). This procedure involves mating yeast strains of opposite mating type, then growing the resultant diploids in media with reduced nitrogen to achieve sporulation. Sporulation produces the formation of ascospores (four spores in an ascus) the sporulation culture is then incubated with a lytic enzyme, zymolyase, which lyses the vegetative cells without disrupting the spores. Sonication is performed to disperse the spores and the resultant culture is then plated onto selective media for semi-quantitative determination of linkage between different loci and recombinants among unlinked loci (Figure 2.6) (Khare et al., 2011).

To investigate through RSA fresh colonies of the query strain and tester strains were inoculated on YPD media supplemented with 2% (w/v) glucose and grown for 2 days at 30 °C. For the mating process, one colony of the query strain and one colony of the tester strain were inoculated into an appropriately labelled microcentrifuge tube containing 500 µl of YPD broth supplemented with 2% (w/v) glucose. This was repeated so that there were 8 tubes each containing one colony of the query strain and one colony of each tester strain. The tubes were then vortexed to resuspend the cells and then incubated overnight at 30 °C at 200 rpm. The following day 200 µl of the overnight mating culture was inoculated onto selective media containing clonNAT (final concentration of 100 µg/ml) and G418 (final concentration of 200 µg/ml), plates were incubated at 30 °C for 3 to 5 days to allow diploid colonies to form. After incubation, diploid colonies were inoculated into tubes containing 2 ml YPD broth supplemented with 2% (w/v) glucose and grown overnight at 30 °C, 200 rpm. Following incubation cells were centrifuged at 1,000 rpm for 3 minutes and washed in 4 ml MilliQ H₂O, then centrifuged and washed again. To induce sporulation the pellet was resuspended in 2 ml 1% potassium acetate (KOAc) (Sigma-Aldrich, UK) pH 7.6 supplemented with 1% uracil (Sigma-Aldrich, UK), 1% methionine (L-Methionine, Sigma-Aldrich, UK) and 1% leucine (L-Leucine, Sigma-Aldrich, UK) and incubated for 2 to 3 days at 30 °C, 150 rpm. Sporulation cultures were then centrifuged at 1,500 rpm for 3 minutes and

washed in 5 ml MilliQ H₂O, centrifuged and washed again for a further two times. Cells were resuspended in 500 µl of Zymolyase 20T solution (AMS Biotechnology (Europe) Limited, UK) at a final concentration of 1 mg/ml, 10 µl 2-β-mercaptoethanol at a stock concentration of 14.3 M (Sigma-Aldrich, UK) was added and cells were incubated overnight at 30 °C, 160 rpm. Following incubation 5 ml of 1.5% NP-40 (Tergitol, Sigma-Aldrich, UK) was added and the culture was transferred to a 15 ml centrifuge tube, vortexed and centrifuged at 3,000 rpm for 5 minutes. 5 ml of the supernatant was discarded and the pellet was resuspended in the remainder and transferred to a sterile 1.5 ml microcentrifuge tube and incubated on ice for 15 minutes. Spores were then sonicated on ice for 30 seconds, centrifuged at 13,000 rpm for 30 seconds. The supernatant was discarded and the pellet resuspended in 1 ml 1.5% NP-40, vortexed and sonicated on ice a final time. Spores were centrifuged at 13,000 rpm for 30 seconds, the supernatant was removed and spores resuspended in 1 ml MilliQ H₂O and vortexed. 100 µl of spores were inoculated onto an YPD agar plate supplemented with 2% (w/v) glucose and an YPD agar plate containing clonNAT and G418 and supplemented with 2% (w/v) glucose. Plates were incubated at 30 °C for 2 to 3 days. Following incubation, the number of G418 and clonNAT resistant colonies were counted and the percentage resistance calculated by comparing the number of colonies on the G418 and clonNAT selective plate to the corresponding YPD plate. For unlinked genes, 25% G418 and clonNAT resistant colonies were expected in comparison to the total number of colonies on the corresponding YPD plate. For linked genes, no G418 and clonNAT resistant colonies were expected representing the absence of a chromosomal crossover event. Once the query strains were confirmed by RSA they were sent for SGA analysis.

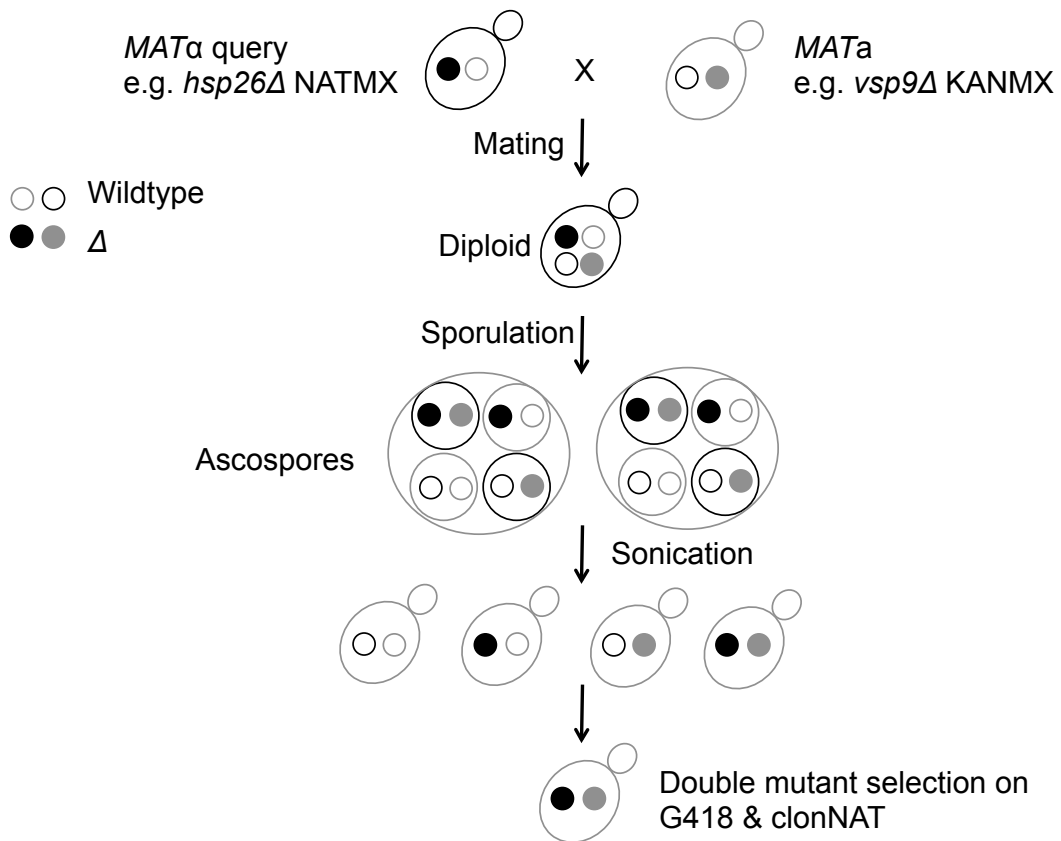


Figure 2.6. Schematic of RSA methodology. The *MAT α* query strain with a mutation linked to a NATMX deletion cassette is mated with a *MAT α* tester strain from the KANMX deletion collection. Diploids are selected for on media containing G418 and clonNAT. Diploids are transferred to reduced nitrogen medium to induce sporulation and the formation of haploids. Spores are treated with zymolyase and dispersed by sonication, then finally inoculated onto non-selective YPD media and selective YPD media containing G418 and clonNAT. The percentage of G418 and clonNAT resistant colonies were compared to the corresponding YPD plate. For unlinked genes, 25% G418 and clonNAT resistant colonies were expected representing double mutants. For linked genes no G418 and clonNAT resistant colonies were expected.

2.5.3 SGA analysis.

Briefly, SGA included mating the query strain with the deletion mutant array (DMA) using a Beckman Biomek FX robot pinning the resultant *MATa/α* diploids onto YPD media supplemented with G418 and clonNAT and incubating at 30 °C. As the resulting diploids are heterozygous for one or both deletion alleles they can lead to the generation of false negatives. To overcome this, *MATa* cells carry an SGA reporter, where a mating-type **a** specific promoter (STE2) is linked to a selectable marker, in this case, the *S. pombe HIS5* gene. This allows *MATa* cells to grow on media lacking histidine, whereas *MATα* and *MATa/α* cells are unable to grow.

To induce the formation of haploid meiotic progeny, the diploids were pinned onto enriched sporulation medium (10 g potassium acetate, 1 g yeast extract, 0.5 g glucose, 0.1 g amino acid supplement (0.1% leucine, 0.02% histidine, 0.02% methionine and 0.02% uracil), 20 g agar and 1 L MilliQ H₂O) and incubated for 5 days at 22 °C. Following incubation, *MATa* haploids were selected by pinning spores onto SD + MSG-HIS-ARG-LYS + canavanine + thialysine (0.17% yeast nitrogen base without amino acids and ammonium sulphate, 0.1% monosodium glutamate (L-glutamic acid monosodium salt hydrate), 2% agar, 0.2% -HIS-ARG-LYS drop out mixture, 300 µg/ml L-canavanine sulphate salt, 300 µg/ml thialysine (S-(2-aminoethyl)-L-cysteine hydrochloride) and 2% glucose) and incubated at 30 °C for 2 days. Canavanine, a toxic analogue for arginine, and thialysine, a toxic analogue for lysine, were added to the media to prevent the selection of *MATa/a* diploids that may have been generated by rare mitotic crossover events between homologous chromosomes (Tong, 2007a). *MATa/a* diploid cells behave like *MATa* haploids as they express the SGA reporter and carry both deletion alleles. The query strain contains *can1Δ* and *lyp1Δ* markers, which confer drug resistance and provides a mechanism to knockout *MATa/a* diploid cells. The *CAN1* gene encodes an arginine permease that allows canavanine to enter the cell and kill it. Likewise, the *LYP1* gene encodes a lysine permease that allows thialysine to enter and kill the cell (Tong, 2007a). Therefore *MATa/a* diploid cells are killed on media containing canavanine and thialysine as they can contain a wildtype copy of the *CAN1* and *LYP1* genes (Tong, 2007a). After 2 days' incubation, *MATa* meiotic progeny were pinned onto fresh SD + MSG-HIS-ARG-LYS + canavanine + thialysine and incubated for 1 day

at 30 °C. After the second round of haploid selection the *MATa* meiotic progeny were pinned onto SD + MSG-HIS-ARG-LYS + canavanine + thialysine + 200 µg/ml G418 and incubated at 30 °C for 2 days to select for *MATa* meiotic progeny carrying the *kan* resistant marker. This selection step was repeated once more and it was important that MSG was utilised in the media instead of ammonium sulphate, as the latter impedes the function of G418 and clonNAT (Cheng et al., 2000).

The *MATa* meiotic progeny carrying the *kan* resistant marker were then pinned onto SD + MSG-HIS-ARG-LYS-LEU + canavanine + thialysine + G418 + 300 µg/ml Hygromycin B (Formedium) and incubated for 2 days at 30 °C. The query strain contains a HPHMX and a LEU2 marker at the *lyp1* locus. Therefore, the media lacks leucine and contains hygromycin B to ensure the selection of strains with correctly integrated cassettes and prevents the selection of strains where the NATMX cassette has switched with the HPHMX cassette. Cassette switching can occur since the upstream sequences (P-TEF) and downstream (T-TEF) regulatory sequences in the NATMX and HPHMX deletion cassettes are identical (Goldstein and McCusker, 1999). Double mutants were finally selected by replica plating onto SGA final media as follows; SD + MSG-HIS-ARG-LYS-LEU + canavanine + thialysine + G418 + Hygromycin B + 100 µg/ml clonNAT to select for *MATa* meiotic progeny carrying both *kan* resistant and *nat* resistant markers. Plates were incubated at 30 °C for 2 days (Figure 2.7).

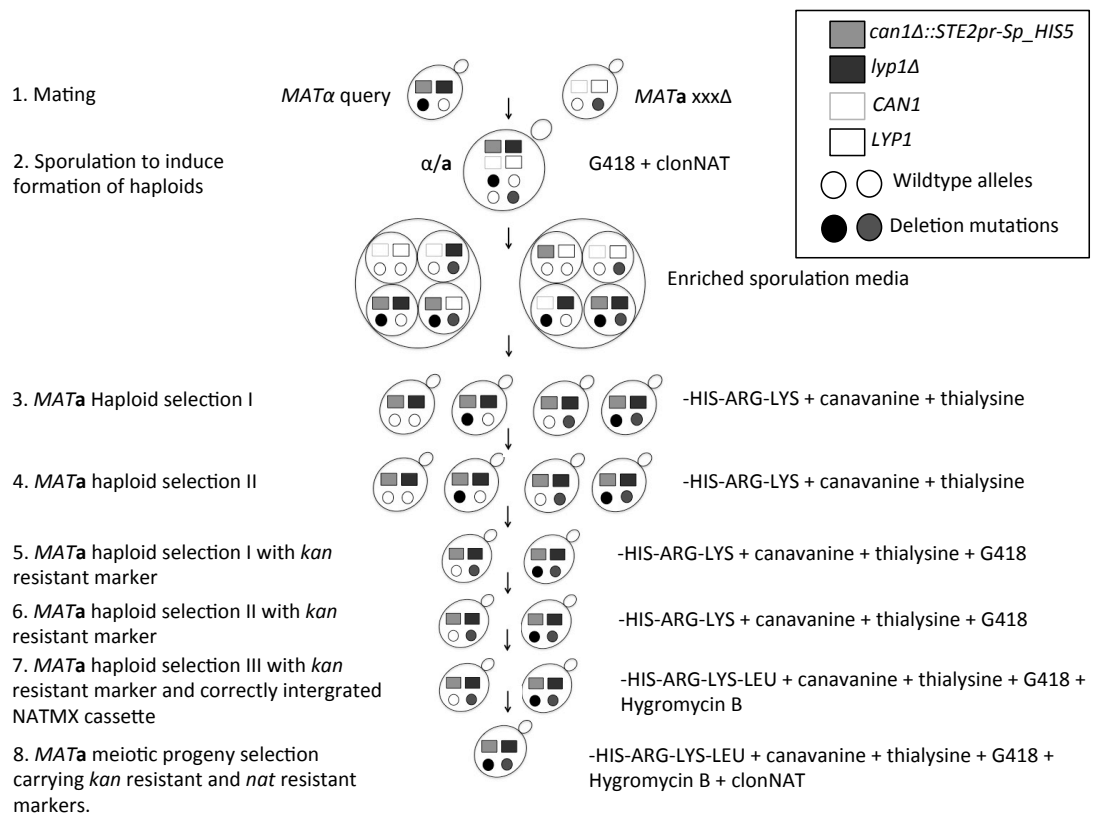


Figure 2.7. Schematic of SGA methodology (based on (Baryshnikova et al., 2010)). The *MATα* query strain carries a query mutation linked to NATMX (filled dark circle) deletion cassette and an SGA reporter, *can1Δ::STE2pr-Sp_his5*, which is integrated into the genome so that it deletes the ORF of the *CAN1* gene, which normally confers sensitivity to canavanine. The query strain also lacks the *LYP1* gene that normally confers sensitivity to thialysine. (Step 1) The query strain is mated with an ordered array of *MATa* deletion mutants (*xxxΔ*) each carrying a single gene deletion linked to a KANMX4 deletion cassette (filled grey circle). (Step 2) The resultant diploids that have been selected for on media containing G418 and clonNAT are pinned onto enriched sporulation media, which has a reduced nitrogen content and induces the formation of haploids. (Steps 3 and 4) Spores are transferred to media lacking histidine to select for *MATa* meiotic progeny owing to the expression of the SGA reporter, *can1Δ::STE2pr-Sp_his5*. Canavanine and thialysine are added to the media to improve the selection allowing cells carrying *can1Δ* and *lyp1Δ* markers to grow while killing *CAN1* and *LYP1* cells. (Steps 5 and 6) The *MATa* meiotic progeny are transferred to media containing G418 to select for cells carrying the *kan* resistant marker. (Step 7) The *MATa* meiotic progeny are transferred onto media lacking leucine and containing hygromycin B to ensure the selection of mutants with correctly integrated deletion cassettes. (Step 8) Double mutants are selected on final SGA media containing both clonNAT and G418.

To identify genetic interactions, the fitness of the double mutants were then scored and compared to the fitness of the corresponding single mutant. For this, double mutants and single mutants were plated onto the following media:

- SGA final media 2% (w/v) glucose and incubated at 30 °C (standard conditions)
- SGA final media 2% (w/v) glucose containing 1 mM H₂O₂ (stress condition)
- SGA final media 2% (w/v) glucose containing 0.8 M NaCl (stress condition)
- SGA final media 2% (w/v) glucose containing 10% ethanol (stress condition)
- SGA final media containing 0.5% (w/v) glucose (stress condition)
- SGA final media containing 0.05% (w/v) glucose (stress condition)
- SGA final media 2% (w/v) glucose incubated at 37 °C (stress condition)

Colonies were imaged using a Colonyzer tool which allows automated quantification of microorganism growth characteristics on solid agar (Lawless et al., 2010). Colonies were imaged at early (2 days' incubation) and late (6 days' incubation) time points. Using the data collected by the Colonyzer tool, a mean growth rate, a *t*-test, *p*-value and *q*-value were calculated and a genetic interaction score (GIS) determined for each strain (Figure 2.8).

A

ORF	Gene	P	Q	GIS	QueryFitnessSummary	ControlFitnessSummary
YCL032W	STE50	8.58E-06	0.00104388	-27668.164	27559.47126	79222.92561
YKL212W	SAC1	0.0036081	0.01960769	-27447.731	14611.13898	60332.59777
YBR057C	MUM2	1.94E-05	0.00140212	-27088.646	27009.89582	77603.26361
YBR099C	YBR099C	0.00096687	0.00920867	-26753.663	29733.89176	81030.25393
YGL151W	NUT1	6.89E-08	5.85E-05	-26394.84	26777.92575	76275.25649
YIR028W	DAL4	0.0001698	0.00410012	-26291.707	47455.80479	105789.3133
YOL146W	PSF3	0.08940844	0.17092668	-26236.895	34945.78312	87765.31374
YBR098W	MMS4	0.00088568	0.00875527	-25879.401	31098.57739	81733.75742

B

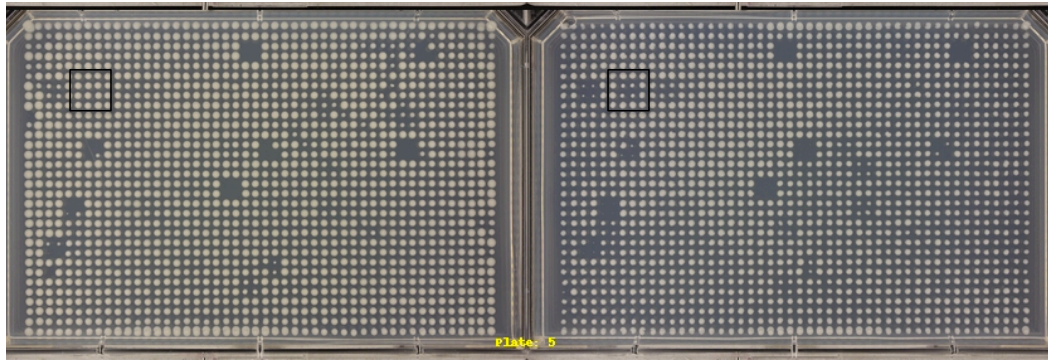


Figure 2.8. Interpretation of SGA data to identify genetic interactions. (A) Excel file from standard *HSP26* SGA, showing calculated p values, q values, genetic interaction scores (GIS), fitness values of the query strains and fitness values of the control strains. In the Excel file *NUT1* has been highlighted to show an example of a negative genetic interactor of *HSP26*. *NUT1* has a statistically significant q value (<0.05), a negative GIS, and a smaller fitness score for the query strain than that recorded for the control strain. (B) A Colonyzer late image of 2 SGA agar plates each containing 16 rows by 24 columns of colonies representing four replicas of 384 independent yeast strains. The control strains can be seen in left agar plate and query strains in the right. The square black box highlights a difference in growth between the control strain and corresponding query strain. A clear reduction in growth can be seen in the query strain (right agar plate) compared to the control strain (left agar plate) confirming a negative genetic interaction of *HSP26* and *NUT1*.

2.6 QUANTITATIVE FITNESS ANALYSIS (QFA).

QFA was performed in collaboration with Professor David Lydall at Newcastle University. For QFA cultures of yeast were heavily diluted before being inoculated onto agar, this allowed complete growth curves to be captured with much higher precision than that determined by SGA (Banks et al., 2012). The double mutants generated by SGA (in the background of our genes of interest, *HSP12* and *HSP26*) were subjected to QFA with the hope of generating more detailed information on their fitness measurements.

The final agar plates from SGA contained 16 rows by 24 columns of colonies representing four replicas of 384 independent yeast strains. 384 strains out of 1536 were transferred robotically using an S&P Robotics Inc BM3-SC robot with a 96 pin tool into four 96 well plates containing 200 μ l of selective media and grown to saturation. The pin tool was sterilised by washing in sterile MilliQ H₂O, then washing in 70% ethanol and finally in 100% ethanol. Using a Beckman Biomek FX, saturated cultures were resuspended by vortexing on a Variomag Teleshake (1000 rpm for 20 seconds) and diluted 1:70 by pinning into 200 μ l of sterile water using a sterile 96 robotic pin tool (V&P Scientific magnetic mounting pintool). The pin tool was sterilised by washing in sterile MilliQ H₂O, followed by 70% ethanol and then used to spot the diluted cultures onto solid agar plates in 384 format. Plates were imaged using an S&P Robotics automated imager on a Canon EOS Rebel Ti 35mm DSLR giving a zero time point growth measurement. Plates were then transferred to a Cytomat temperature controlled, humidified incubator with an automatic carousel and access hatch. Plates were imaged at zero time point and every 6 hours and incubated until colony growth has reached saturation (Banks et al., 2012).

2.7 REPLICATIVE LIFESPAN ANALYSIS.

Yeast strains from frozen stocks were streaked for single colonies onto YPD agar plates supplemented with the appropriate amount of glucose and incubated at 30 °C for 2 days. Cells from each strain were lightly patched along a vertical line on the left side of a fresh YPD plate, parafilmmed and incubated overnight at 30 °C (Figure 2.9). Using a MSM micromanipulator (Singer Instruments, Someset, UK), approximately 50 single cells from the first patched strain were transferred to a position on the plate distal to the patched cells (Figure 2.9). The needle was cleaned by repeatedly touching the agar surface and this step was repeated for the remaining yeast strains. The agar plate was parafilmmed and incubated at 30 °C for 2 hours to allow the cells to divide. Following incubation the cells were visualised to check they had undergone cell division, if further time was needed for cell division to be completed the plate was incubated for longer. Starting with the first yeast strain, the needle was used to detach the virgin daughter cells from the mother cell; the virgin cell was then transferred to a coordinate on the agar plate (Figure 2.9), this was repeated until approximately 20 virgin cells had been selected for lifespan analysis. The needle was cleaned by repeatedly touching the agar and virgin daughter cells were obtained from the remaining yeast strains. The plate was parafilmmed and incubated at 30 °C for 2 hours to allow the virgin daughter cells to divide. Following incubation the needle was used to detach the daughter cell from the mother cell and transfer it to an area near the patches (“the graveyard”). The number of daughter cells produced by the mother cell was recorded. The plate was parafilmmed and incubated at 30 °C for 2 hours, and the number of daughter cells produced recorded for 8-10 hours during the working day. The plate was then stored in the fridge overnight to halt the cell cycle. Cells were analysed for 8-10 hours daily until the mother cell had stopped dividing. Raw data from the replicative lifespan experiment was then submitted to OASIS online application (<http://sbi.postech.ac.kr/oasis/introduction/>) for survival analysis and a survival curve produced and the mean lifespan, median lifespan, standard deviation and Wilcoxon Rank-Sum test performed (Yang et al., 2011, Steffen et al., 2009).

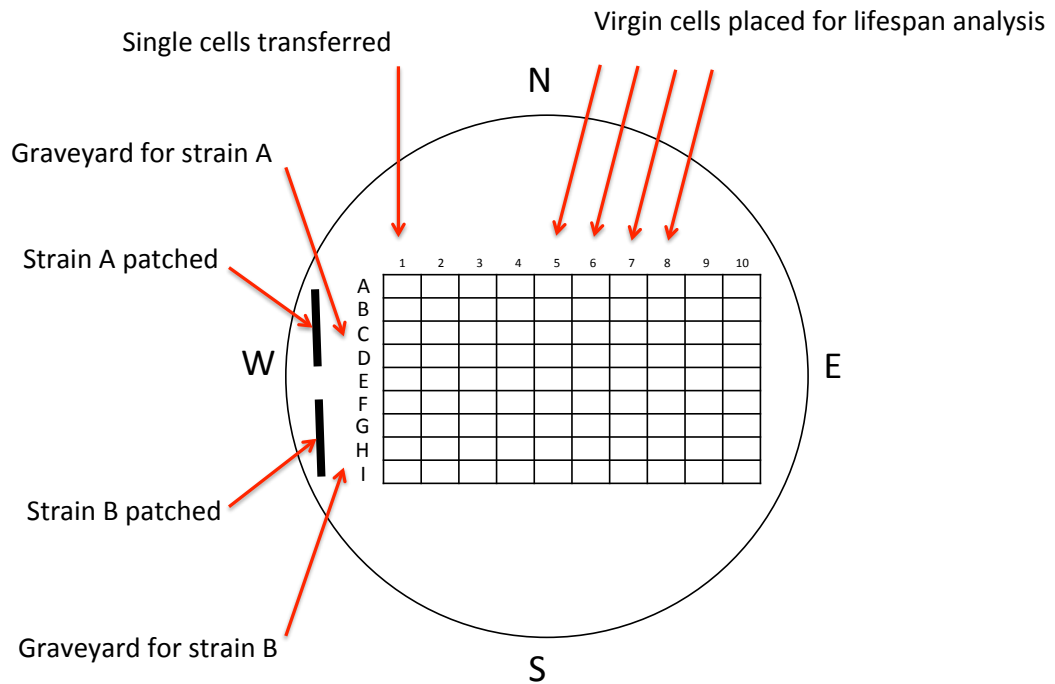


Figure 2.9. Schematic showing a typical replicative lifespan agar plate. The yeast strains are lightly patched along a vertical line on the left hand side of the experimental agar plate. Single colonies are transferred from the patch to a coordinate on the plate, e.g. A1. The plate is incubated at 30 °C for 2 hours to allow cells to divide, then virgin daughter cells are detached from mother cells using the needle and transferred to another coordinate on the plate, e.g. A5. The virgin daughter cells are now referred to as the 'mother cells' and their RLS will be determined. Plates are incubated at 30 °C for 2 hours, the number of daughter cells produced by the mother cells are recorded and the new daughter cells are detached and transferred to 'the graveyard'. This is repeated until the mother cell has stopped dividing.

2.8 FLUORESCENT MICROSCOPY FOR DETERMINATION OF VACUOLAR ACIDIFICATION.

Yeast strains from frozen stocks were streaked for single colonies onto YPD agar plates supplemented with the appropriate amount of glucose and incubated at 30 °C. After 2 days incubation, one colony of each yeast strain was inoculated into 5 ml YPD broth supplemented with the appropriate concentration of glucose and grown overnight at 30 °C, 200 rpm. 10 µl of the overnight starter culture was inoculated into 10 ml YPD broth and grown overnight at 30 °C, 200 rpm. The following morning, 500 µl of an overnight culture was inoculated into 10 ml YPD broth supplemented with the appropriate amount of glucose and grown to an OD₆₀₀ 0.6. Cells were transferred to 50 ml Falcon tubes and centrifuged at 1750 rpm for 5 minutes and harvested in 1 ml fresh YPD broth. Cells were incubated at room temperature for 1 hour and treated with either 500 nM Folimycin (Folimycin, *Streptomyces sp*, Merck Chemicals, Nottingham, UK) to inhibit the V-ATPase (Hughes and Gottschling, 2012) or CCCP (carbonyl cyanide *m*-chlorophenyl hydrazine, Sigma) to inhibit oxidative phosphorylation. After treatment, cells were centrifuged at 1750 rpm for 5 minutes and washed twice in 1.5 ml uptake buffer (YPD buffered to pH 7.6 with 100 mM HEPES). Cells were then stained with either 200 µM quinacrine (Quinacrine dihydrochloride, Sigma) or 175 nM DiOC₆ (3,3'-dihexyloxycarbocyanine iodide, Sigma) fluorescent dyes. For quinacrine staining, tubes were incubated at 30 °C for 10 minutes and incubated on ice for 5 minutes. For DiOC₆ staining tubes were incubated at room temperature for 15 minutes. Cells were washed 3 times in ice-cold wash buffer (100 mM HEPES, pH 7.6, 2% glucose) with 5 minutes centrifugation at 1750 rpm. Cell pellets were resuspended in 50 µl of CyGEL™ (abcam®, Cambridge, UK) and kept on ice. 10 µl of cells were loaded onto a labelled microscope slide and incubated at room temperature. After 2-3 minutes a coverslip was placed on top of the cells and the microscope slide placed on ice to allow the cell suspension to spread. Cells were imaged within 1 hour of staining.

Visualisation of folimycin treated cells was achieved using a Nikon Diaphot microscope with an excitation 440-480 nm and emission at 510-550 nm. Images were taken using a Nikon D90 camera. All other cells were visualised using a Nikon

Eclipse Ti S microscope using a 60X 1.40 Plan Apo objective lens and images were taken with a Nikon Digital Sight DS Fi1 camera. For visualisation of GFP-tagged strains, a GFP-B filter was used. The GFP-B filter allows 460-500 nm light through, and emitted GFP fluorescence is reflected off a 505 nm Long Pass Dichroic mirror and is detected by the camera after passing through a 510-560 nm Band Pass filter.

3.1 INTRODUCTION.

3.1.1 Using *Saccharomyces cerevisiae* to study ageing.

The budding yeast, *Saccharomyces cerevisiae*, is a well-established model organism that can be used to study ageing. In yeast there are two models of ageing; replicative lifespan (RLS) and chronological lifespan (CLS) (Kaeberlein, 2010). The replicative model of ageing defines the lifespan of a yeast cell by the number of daughter cells produced before senescence and was first described in the 1950s by Bob Mortimer (Mortimer and Johnston, 1959). Chronological lifespan is a post-mitotic model of ageing and defines the lifespan of a yeast cell as the length of time it can survive in a non-dividing state (Kaeberlein, 2010).

Interestingly, reducing the glucose concentration of the growth medium, a phenomenon known as dietary restriction (DR) or caloric restriction (CR) can extend both RLS and CLS in yeast (Kaeberlein, 2010). In fact, DR has been shown to extend the lifespan of *Caenorhabditis elegans*, *Drosophila melanogaster*, rodents and higher eukaryotes such as the primates, indicating that the pathways in which DR act are evolutionarily conserved (Kemnitz, 2011). Furthermore, primate studies have revealed DR to have a positive effect on healthspan, and animals subjected to DR are reported to have a reduced incidence of age-associated pathologies, notably, cancer, cardiovascular disease and diabetes (Colman and Anderson, 2011). Importantly, the exact mechanisms underlying lifespan extension by DR and how DR improves healthspan are still unknown.

3.1.2 Small heat shock protein expression during ageing.

One of the important players in the ageing process of yeast, worms, flies and mammals are the molecular chaperones which prevent protein misfolding and the accumulation of damaged proteins, both of which are features of ageing (Wadhwa et al., 2010). Heat shock protein (Hsp) expression is induced in response to stresses that cause proteins to denature and also reportedly induced during ageing (Fleming et al., 1988, Tower, 2011, Morrow and Tanguay, 2003). Yet other groups have reported a decrease in Hsp transcription and heat shock factor 1 (HSF-1) expression during ageing specifically in the neurons and the muscles (Nagai et al., 2010, Zhang et al., 2011). Hsps bind to misfolded proteins and can alter their folded structure,

and can also transfer misfolded proteins to the proteasome for degradation (Mymrikov et al., 2011). Through these actions the Hsps function to prevent the accumulation of damaged proteins and the formation of protein aggregates and therefore play a key role in quality control of proteostasis (Mymrikov et al., 2011).

Gene expression changes in response to ageing and there is an extensive down-regulation of mitochondrial and electron transport chain genes (Landis et al., 2012). The subsequent mitochondrial malfunction and production of reactive oxygen species (ROS) may be responsible for creating the accumulation of abnormal proteins, which in turn activate the expression of Hsp genes (Tower, 2011). Since the Hsps play a major part in the cell-defense mechanisms, it is likely that they may be vital regulators of the ageing process (Morrow and Tanguay, 2003). Research in invertebrate models has in fact shown a role for members of the Hsp and sHsp family in ageing. In *Drosophila*, Hsp70, Hsp22 and Hsp23 are up-regulated during normal ageing (Kurapati et al., 2000, Tower, 2011). Furthermore, Hsp22 overexpression results in lifespan extension (32%), increased locomotor activity and significantly increased resistance of flies to oxidative and thermal stress (Morrow et al., 2004b), whereas, flies that are not expressing Hsp22 have a decreased lifespan (40%), decreased locomotor activity and are more sensitive to mild stresses (Morrow et al., 2004a). These data therefore suggest that Hsp22 plays a pivotal role in *Drosophila* ageing.

In *C. elegans*, lifespan can be doubled by loss of the insulin growth factor 1 (IGF-1) receptor, DAF-2, this lifespan extension requires activation of the FOXO transcription factor, DAF-16, which in turn is responsible for *Hsp-16* transcription (Dorman et al., 1995, Murshid et al., 2013). In *C. elegans* extra copies of the *HSP-16* gene results in lifespan extension and increased tolerance to heat (Walker and Lithgow, 2003). Consistent with a role for the sHsps in *C. elegans* ageing, lifespan can be extended by overexpression of the transcription factor HSF-1, while reducing *hsf-1* activity accelerates ageing and shortens lifespan (Hsu et al., 2003).

In *S. cerevisiae* there are seven members of the small heat shock protein family (Table 3.1), most of which have chaperone activity with the exception of Hsp10 and Hsp12 (Gong et al., 2009, Welker et al., 2010). Hsp12 has been shown to have negligible anti-aggregation properties; instead Hsp12 functions as a

membrane stabilising 'lipid chaperone'. Hsp12 is natively unfolded but adopts a helical structure in the presence of lipids to stabilise the membrane in times of stress by modulating its fluidity (Welker et al., 2010). Unlike Hsp12, Hsp26 is a temperature-regulated chaperone (Haslbeck et al., 1999). The Hsp26 transcript is undetectable in unstressed cells and like Hsp12 is up-regulated in response to stresses such as heat, osmotic stress, carbon and nitrogen starvation, oxidative stress and low pH (Carmelo and Sa-Correia, 1997, Amoros and Estruch, 2001).

Name	Molecular weight	Molecules per cell	Localisation	Stress
HSP10	10 kD	149	–	Heat shock
HSP12	12 kD	4490	Cytoplasm, nucleus	Heat shock, oxidative stress, osmostress, stationary phase entry, alcohol and glucose depletion
HSP26	26 kD	19300	Ambiguous	Not expressed in unstressed cells, strongly induced by heat shock, salt shock, cell cycle arrest, nitrogen starvation, oxidative stress, low pH and glucose depletion
HSP30	30 kD	7800	Ambiguous	Heat shock, ethanol treatment, weak organic acid and entry into stationary phase
HSP31	31 kD	358	Cytoplasm, nucleus	Heat shock
HSP32	32 kD	Not visualised	Not visualised	Heat shock
HSP33	33 kD	Not visualised	Not visualised	Heat shock
HSP34	34 kD	Low signal	–	Mild heat shock and copper deprivation
HSP35 HSP36	–	169000	Cytoplasm, nucleus	Heat shock
HSP40	40 kD	119000	Cytoplasm, nucleus	Heat shock
HSP42	42 kD	1470	Cytoplasm	Heat shock

Table 3.1. The family of sHsps in *Saccharomyces cerevisiae*. Data from the *Saccharomyces* genome database showing up-regulation of sHsp mRNA in response to different stresses. Hsp12 and Hsp26 mRNA expression is induced in response to glucose depletion.

The existing literature reports the up-regulation of Hsp transcription in response to stresses such as heat, ethanol exposure, anoxia, and glucose deprivation (Lindquist, 1986, Lanks, 1986). I therefore set out to investigate if Hsp12 and Hsp26 are up-regulated at the protein level in response to 0.5% and 0.05% (w/v) glucose i.e. glucose depletion. If these proteins are induced in response to glucose depletion, this may suggest they play a role in lifespan extension by DR, especially since sHsps have been implicated in ageing in other model organisms. An alternative theory is that glucose depletion activates transcription of the Hsps genes, but this may be independent of any longevity roles. RLS analysis was therefore performed on *hsp26Δ* single and *hsp12/hsp26Δ* double mutants, with the aim of establishing if deletion of these two sHsps has any detrimental effects to the cell thereby reducing lifespan.

3.2 METHODS.

Methods were generally performed as described in Chapter 2; additional details are described below.

3.2.1. Construction of deletion strains.

The deletion strains were constructed by PCR-mediated gene disruption. A BY4741 *hsp26Δ* single knockout was constructed first by PCR amplifying a KANMX4 deletion cassette from an *hsp26Δ* deletion strain using *HSP26* ORF primers, which anneal either side of the ORF (Hsp26 original primers (forward 5'-CTCGTAACAGTAAGGTATTCGCACT-3', reverse 5'-GACAACCTCCATAGAGATACCTACA-3') or Hsp26 new primers, (forward, 5'-CCTGTCAAGGTGCATTGTTGG-3', reverse 5'-GTCGAGGTTTAACGCTTATTACC-3'). The resultant PCR product was used to transform competent BY4741 wildtype cells using the PCR-based disruption strategy (Wach, 1996, Gietz and Woods, 2002). Successful G418-resistant colonies were confirmed by PCR using the *HSP26* ORF primers.

To construct the *hsp12/hsp26Δ* double mutant, a HIS3MX6 deletion cassette was PCR amplified from an *hsp12Δ* deletion strain (constructed previously by Dr Michèle Riesen) using *HSP12* ORF primers, which anneal either side of the ORF (Hsp12 primers forward 5'-GTATACGCAAGCATTAAACAACCC-3', reverse 5'-ACACACGCATAACAAGATAAAGTGA-3'). The resultant PCR product was used to transform competent BY4741 *hsp26Δ* cells, as before. Colonies growing on media lacking histidine and containing G418 were confirmed as double knockouts by PCR using *HSP26* ORF and *HSP12* ORF primers.

3.2.2 Western blotting against Hsp12 and Hsp26.

SDS-PAGE and transfer to nitrocellulose was performed as described in Chapter 2. For detection of Hsp12 and Hsp26, the nitrocellulose membrane was incubated for 1 hour with the primary antibody (anti-Hsp26 C-terminal and anti-Hsp12 N-terminal) diluted 1:1000 in 1X PBS. Both antibodies were custom made years previously by Genosphere biotechnologies. The Hsp26 anti-peptide antibody was raised in rabbit against the peptide sequence corresponding to MSDAGRKGFGEKASC. The Hsp12 anti-peptide antibody was also raised in rabbit against the peptide sequence corresponding to CVKKIEVSSQESWGN. The purified

IgG fractions were used as specific antibodies against yeast Hsp26 and Hsp12 respectively. A horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody was used at 1:1000 in 3% (w/v) milk solution with 0.5% (w/v) Tween20. The membrane was visualised and imaged as described in Chapter 2.

3.3 RESULTS.

3.3.1 Expression and purification of recombinant His-SUMO-Hsp12 protein.

Microarray data has shown the expression of both *HSP12* and *HSP26* genes to be up-regulated in response to glucose depletion (Table 3.1). Furthermore, 2-D gel electrophoresis and mass spectrometry previously performed in the Morgan laboratory has identified induced expression of Hsp12 in response to 0.5% (w/v) glucose (Herbert et al., 2012). Studies have shown that correlations between RNA and protein levels are substantially stronger for some gene categories than others (Gry et al., 2009). It was therefore important to determine if the protein levels of Hsp12 and Hsp26 are also induced in response to glucose depletion. In order to determine the protein expression of Hsp12 and Hsp26 by Western blotting, anti-peptide antibodies against Hsp12 and Hsp26 were used. Both antibodies were custom made years previously by Genosphere biotechnologies.

To test the antigenicity of the custom made anti-Hsp12 antibody recombinant Hsp12 protein was made. Recombinant His-SUMO-Hsp12 protein was purified using affinity chromatography with a His-trap column (GE Healthcare) (Figure 3.1, B). Ion exchange chromatography (IEC) was then performed as a final purification step on fraction 20 eluted from the affinity chromatography purification. The His-SUMO-Hsp12 was eluted in fractions 15-18 from IEC. Fractions were analysed by gel electrophoresis and showed the presence of two bands, which may represent another protein contaminant or protein degradation probably due to the presence of proteases (Figure 3.1, C). A small fraction of the His-SUMO-Hsp12 purified by affinity chromatography was incubated with SUMO protease to cleave off the His-SUMO tag. The cleaved Hsp12 was then passed through a His-trap column; the protein was eluted in the first few fractions, as it no longer binds to the column (Figure 3.1, D).

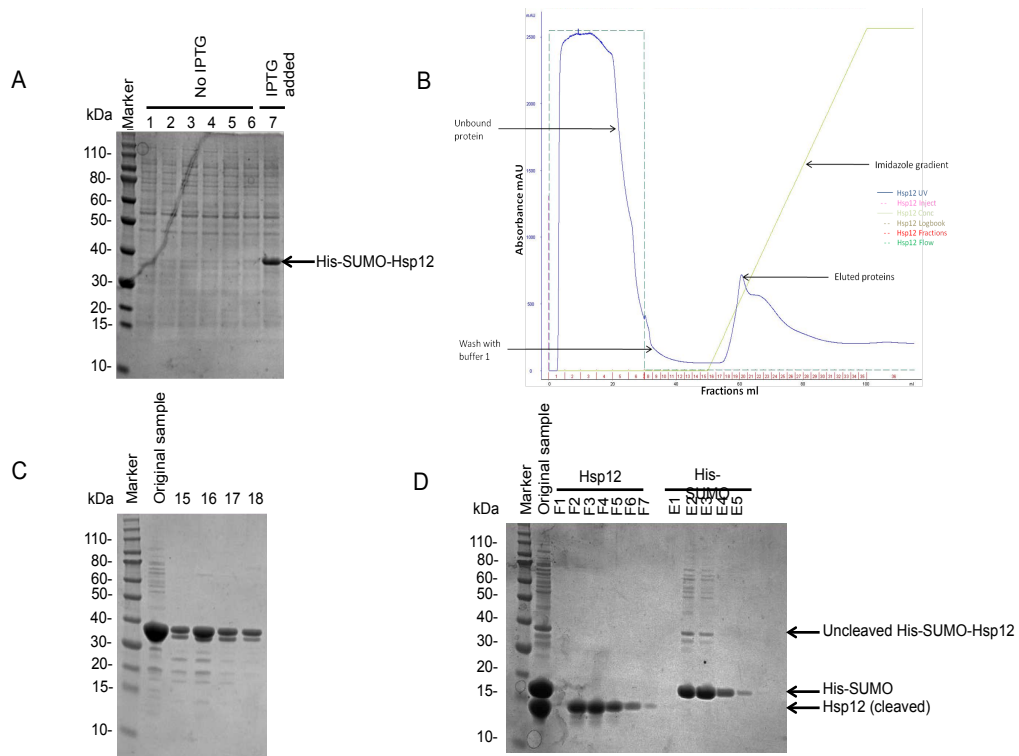


Figure 3.1. Purified recombinant His-SUMO-Hsp12 protein. A) Gel with Coomassie staining showing non-induced samples (lanes 1-6) and protein induction in lane 7 with the addition of IPTG (1 mM). B) Chromatogram of affinity chromatography. Unbound proteins are eluted during the washing phase. His-SUMO-Hsp12 is eluted with an imidazole gradient. Fraction 20 was subjected to a final purification step by ion exchange chromatography. C) Gel showing purified His-SUMO-Hsp12 after ion exchange chromatography. The presence of two bands or more may represent protease degradation or another protein contaminant. D). Gel showing Sumo protease-cleaved Hsp12 in flow-through fractions F1-F6. The His-SUMO tag is eluted in fractions E1-E5 with the addition of imidazole.

3.3.2 Determining the reactivity of Hsp12 and Hsp26 custom made antibodies.

An aliquot of the Hsp12 (N-terminal) antibody was affinity purified to reduce non-specific binding and improve reactivity; this was then used in Western blotting to probe the newly made recombinant Hsp12 protein. Further Western blots were performed using an aliquot of the unbound Hsp12 antibody fraction from affinity purification and an aliquot of unpurified Hsp12 antibody. The unpurified Hsp12 antiserum detected Hsp12 recombinant protein the best and was utilised in further Western blotting experiments to detect the presence of the yeast endogenous Hsp12 protein (Figure 3.2A).

The reactivity of the Hsp26 (C-terminal) antibody was determined by its ability to detect recombinant Hsp26 expressed in a high copy vector under the control of the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter (p425-GPD-*HSP26*) (Mumberg et al., 1995). The Hsp26 antibody detected the presence of a strong band at the predicted molecular weight of Hsp26 (~24 kDa) (Figure 3.2B). Both antibodies were next used for Western blotting experiments to determine the presence of the yeast endogenous proteins.

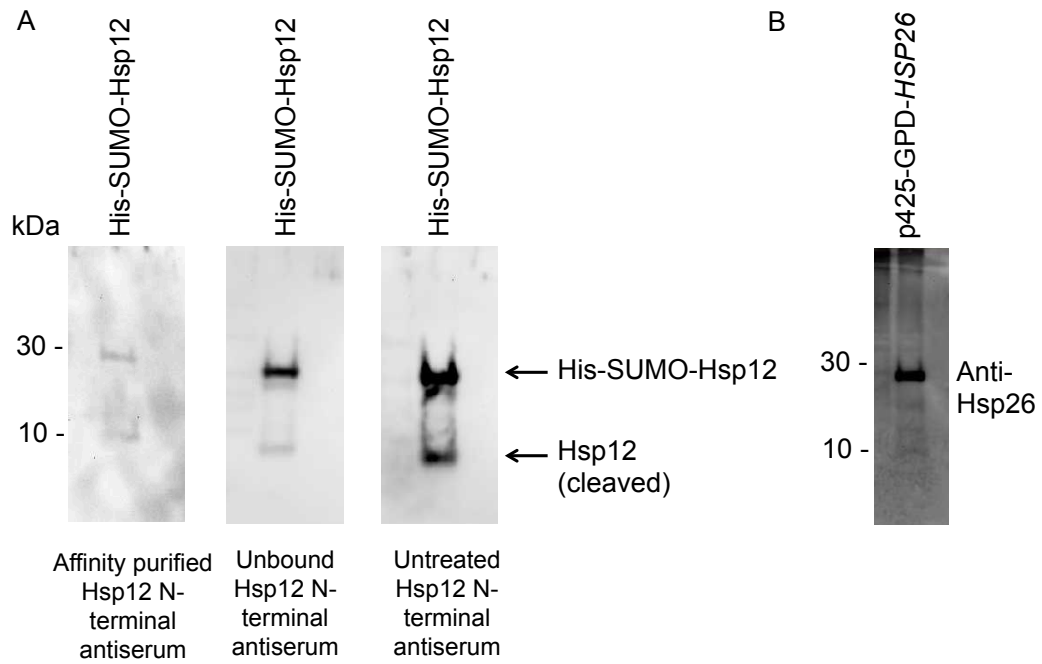
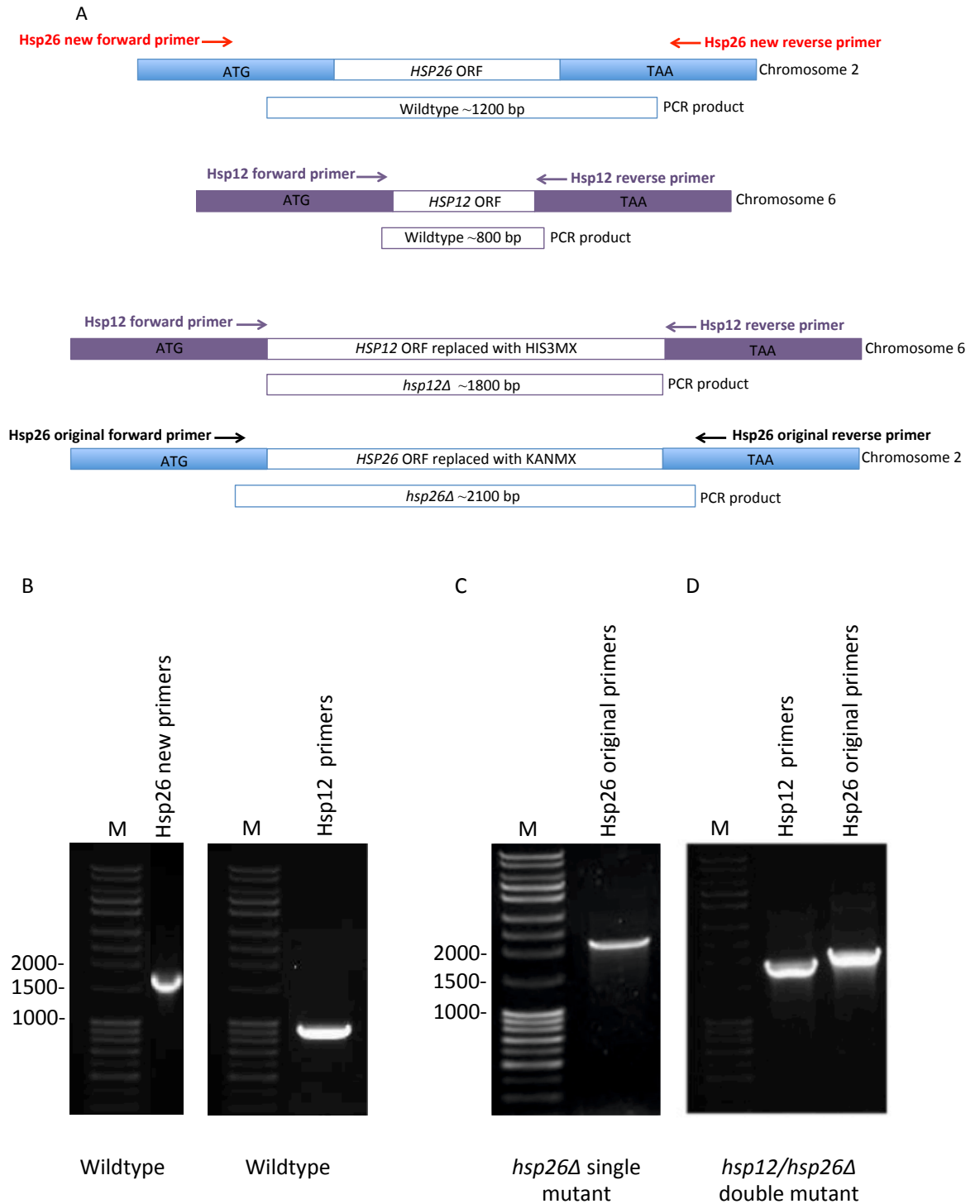


Figure 3.2 Western blotting with custom made anti-Hsp12 and anti-Hsp26 antibodies. A) Western blotting against cleaved recombinant Hsp12 protein using affinity purified Hsp12 (N-terminal) antiserum 1:1000 (left panel), the unbound Hsp12 antiserum fraction 1:1000 (middle panel) and raw Hsp12 (N-terminal) antiserum 1:1000 (right panel). Untreated Hsp12 (N-terminal) antiserum gave the most intense band detecting Hsp12 protein and was utilised in subsequent Western blotting experiments. B) Western blotting with anti-Hsp26 (C-terminal) antibody showing the presence of a strong band of the predicted size for Hsp26 protein.

3.3.3 Heat shock protein expression during glucose depletion.

As mentioned earlier, the mRNA levels of Hsp12 and Hsp26 are induced in response to glucose depletion (Table 3.1). From this it may be assumed that the corresponding protein levels of Hsp12 and Hsp26 will also be up-regulated. To investigate this hypothesis by Western blotting, single *hsp26Δ* and double *hsp12/hsp26Δ* mutants were constructed in the BY4741 yeast genetic background. These mutants would be used as negative controls and confirm the specificity of the custom made antibodies.

To construct the *hsp26Δ* single mutant by PCR-mediated gene disruption, the *HSP26* ORF was replaced with a KANMX4 deletion cassette, which confers resistance to G418. The *hsp12/hsp26Δ* double mutant was then constructed by replacing the *HSP12* ORF with a HIS3MX6 deletion cassette, which allows growth on media lacking histidine. Successfully transformed colonies were confirmed by PCR where the ORF was amplified by gene specific primers (Hsp26 or Hsp12) which anneal either side of the ORF and show a difference in band size when the gene has been replaced with the deletion cassette (Figure 3.3).



To determine the protein expression of Hsp12 and Hsp26 during DR, yeast were grown in YPD broth supplemented with 2%, 0.5% and 0.05% (w/v) glucose to an OD_{600} 0.6. Coomassie stained gels determined protein loading and the corresponding nitrocellulose membranes were probed with anti-Hsp12 and anti-Hsp26 antibodies (1:1000). Protein levels of Hsp12 and Hsp26 were induced in response to 0.5% and 0.05% (w/v) glucose, consistent with that reported for their corresponding mRNA levels (Figure 3.4). Strikingly, growth in media supplemented with 0.05% (w/v) glucose induced the greatest expression of both Hsp12 and Hsp26 proteins, suggesting a correlation between sHsps and DR.

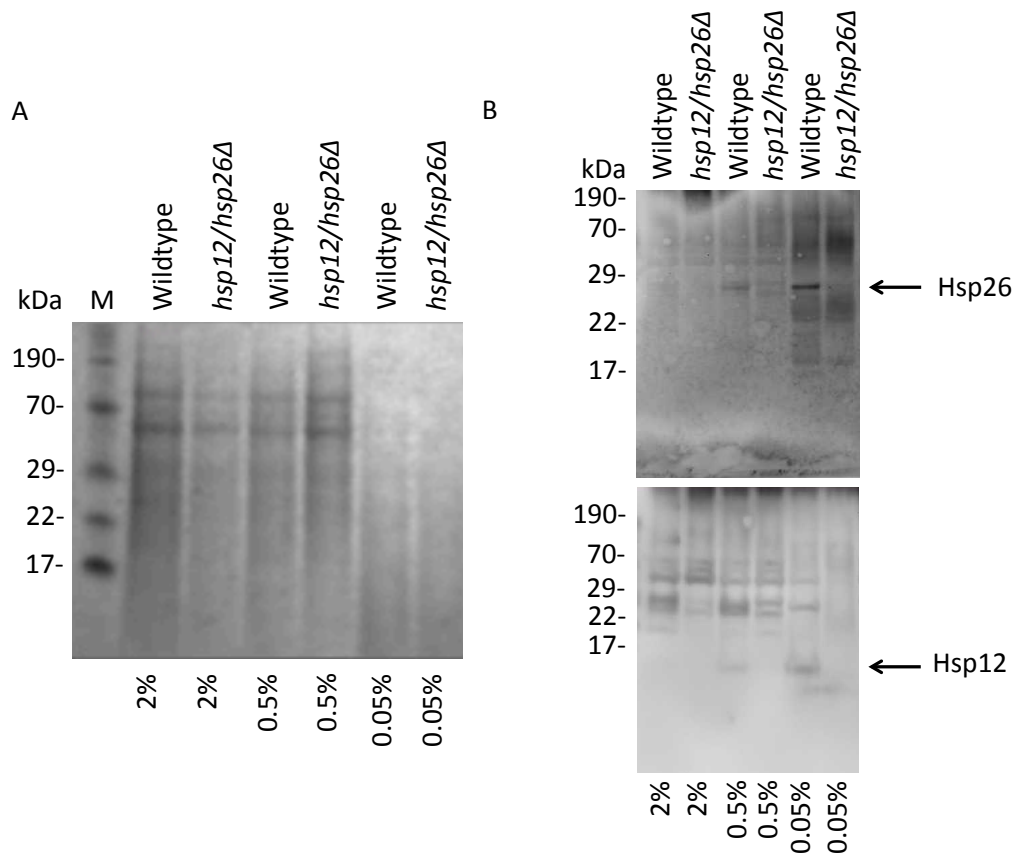


Figure 3.4 Up-regulation of Hsp12 and Hsp26 protein expression during glucose depletion. Yeast strains were grown overnight in YPD broth supplemented with 2%, 0.5% and 0.05% (w/v) glucose respectively to an OD_{600} 0.6. A) Gel with Coomassie staining showing that increased Hsp26 and Hsp12 protein levels at 0.05% (w/v) glucose are not due to increased protein loading. B) Western blot with anti-Hsp26 (C-terminal) and anti-Hsp12 (N-terminal) showing the presence of a band representing Hsp26 (Top panel) and Hsp12 (Bottom panel) in the wildtype strain which increases in intensity in response to growth in 0.5% and 0.05% (w/v) glucose.

3.3.4 Replicative lifespan analysis.

Published work from the Morgan laboratory has shown a role for Hsp12 in yeast longevity; lifespan extension by moderate DR is abolished in a BY4741 *hsp12Δ* knockout strain, indicating an essential role for Hsp12 in DR mediated lifespan extension (Herbert et al., 2012) (Figure 3.5). As Hsp12 and Hsp26 are up-regulated during glucose depletion, lifespan analysis was performed on an BY4741 *hsp26Δ* single and BY4741 *hsp12/hsp26Δ* double mutant to determine if they too have a longevity phenotype. Statistics were calculated using OASIS Online Application for Survival Analysis (Yang et al., 2011). Statistical significance was assessed using the Wilcoxon Rank-Sum test and deemed significant at $p < 0.05$. With standard conditions of growth at 30 °C and 2% (w/v) glucose, there was no significant difference in mean replicative lifespan between the BY4741 wildtype and *hsp12/hsp26Δ* double mutant (p -value 0.146). The *hsp26Δ* single mutant had the longest lifespan with a mean RLS of 18.7, which was statistically greater than the wildtype (p -value 0.0206) and *hsp12/hsp26Δ* double mutant (p -value 0.0292) (Figure 3.6).

Lifespan analysis was next performed on media supplemented with 0.05% (w/v) glucose, since previous results indicated a role for *HSP12* in DR mediated lifespan extension. Under DR conditions there was no difference in mean and maximum replicative lifespan between the BY4741 *hsp26Δ* single mutant and its wildtype strain (p -value 0.4165). Similar to that reported for the *hsp12Δ* single mutant, DR was also unable to extend the replicative lifespan of the BY4741 *hsp26Δ* single mutant (CTL RLS, 18.7, DR RLS, 21.1, p -value 0.5977) (Figure 3.6C). However, double deletion of *HSP12* and *HSP26* caused a significant reduction in replicative lifespan, with the BY4741 *hsp12/hsp26Δ* double mutant showing a reduced mean RLS of 15.1 compared to the wildtype, mean RLS 21.6, and *hsp26Δ* single mutant mean RLS of 21.1 (Figure 3.6B, D). The *hsp12/hsp26Δ* double mutant also showed a reduced maximum lifespan of 37, compared to the wildtype, 45 and *hsp26Δ* single mutant, 49. Further analysis of data from collective lifespan experiments shows no significant difference in RLS for the wildtype strain and *hsp12/hsp26Δ* double mutant with 2% glucose (p -value 0.1462). However, with 0.05% (w/v) glucose, a

significant difference in mean RLS is seen for the wildtype strain compared to the *hsp12/hsp26Δ* double mutant (p -value 0.000026) (Figure 3.7).

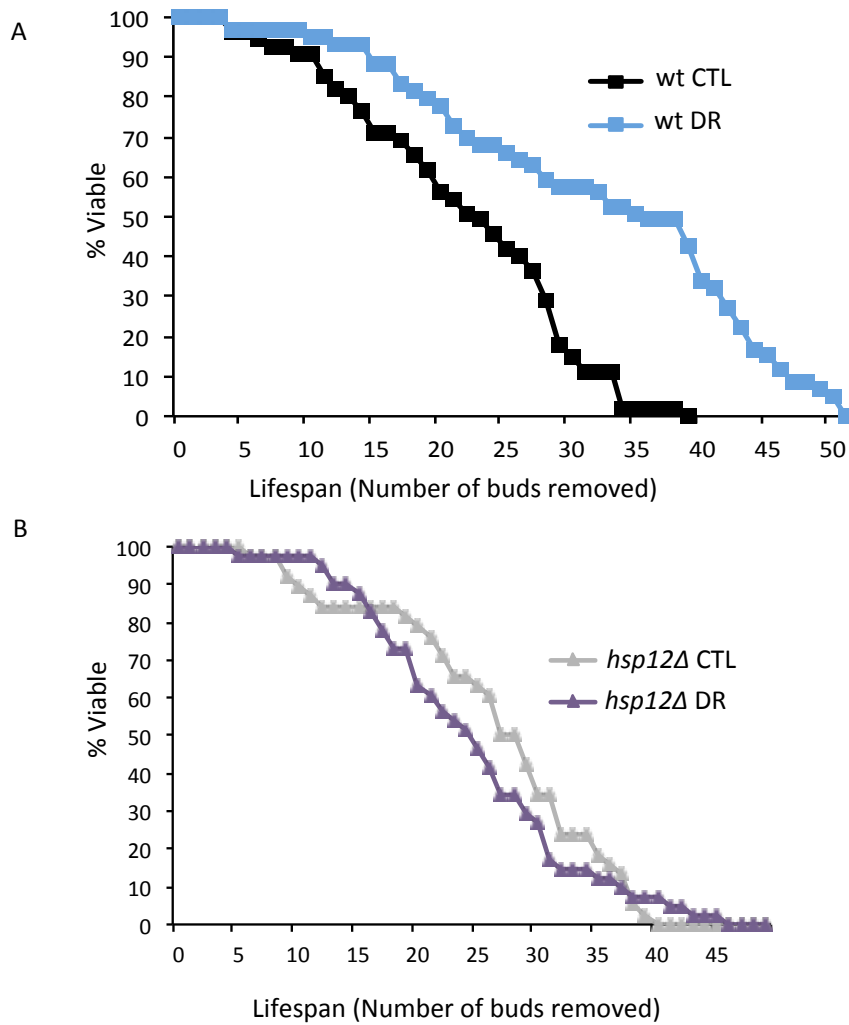


Figure 3.5 *HSP12* is required for DR mediated lifespan extension. RLS analysis on YPD agar using manual micromanipulation, collective data from two independent experiments. A) Graph showing lifespan extension of the BY4741 wildtype strain when grown on media supplemented with 0.5% (w/v) glucose (n=59, mean RLS 31) compared to growth with standard media supplemented with 2% (w/v) glucose (n=55, mean RLS 21). B) RLS analysis with 0.5% (w/v) glucose is unable to extend lifespan in the BY4741 *hsp12Δ* deletion strain (0.5% (w/v) glucose n=42, mean RLS 25, 2% (w/v) glucose n=39, mean RLS 26). Lifespan analysis performed by Alan Morgan and Effie Kosmidou (Herbert et al., 2012).

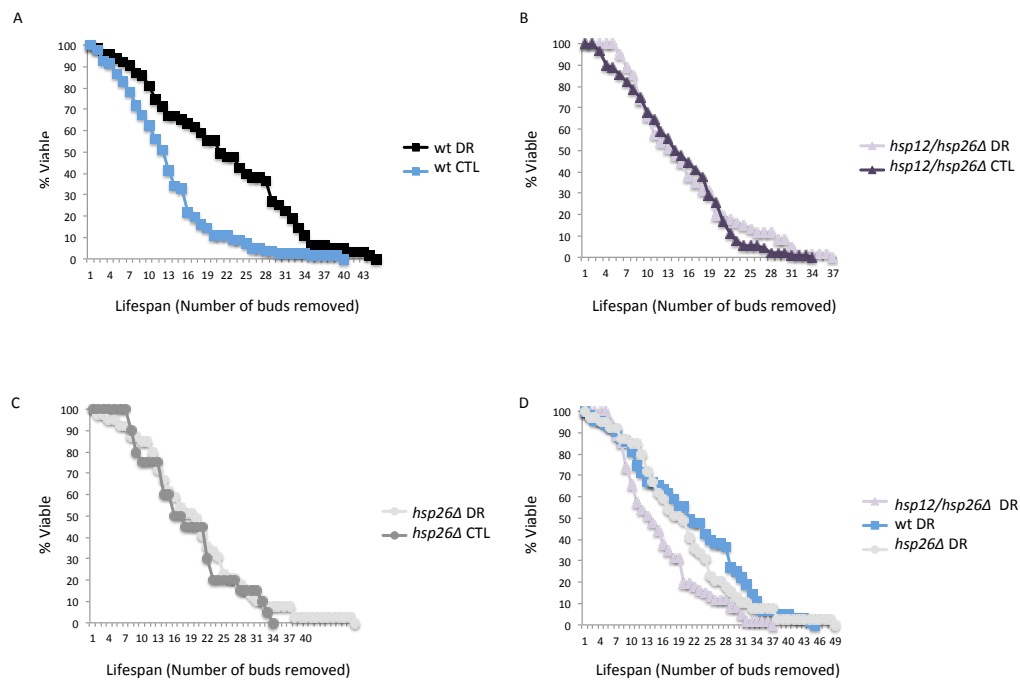


Figure 3.6 The *hsp12/hsp26Δ* double mutant has a reduced RLS. A) RLS analysis of the BY4741 wildtype strain showing lifespan extension when grown on media supplemented with 0.05% (w/v) glucose (CTL n=82, RLS 13.11 DR n=63 RLS 21.62). B) RLS analysis showing a reduction in mean and maximum lifespan of the BY4741 *hsp12/hsp26Δ* double mutant when grown on 0.05% (w/v) glucose (CTL n=90 RLS 14.86, DR n=61 RLS 15.1). C) RLS analysis, showing no extension in lifespan of BY4741 *hsp26Δ* single mutant when grown on 0.05% (w/v) glucose compared to 2% (w/v) glucose. (CTL n=20 RLS 18.7, DR n=39, RLS 21.1 *p*-value 0.5977). D) RLS analysis with 0.05% (w/v) glucose showing the reduced mean and maximum lifespan of the BY4741 *hsp12/hsp26Δ* double mutant (RLS 15.1) compared to the wildtype (RLS 21.62) and *hsp26Δ* single mutant (RLS 20.08).

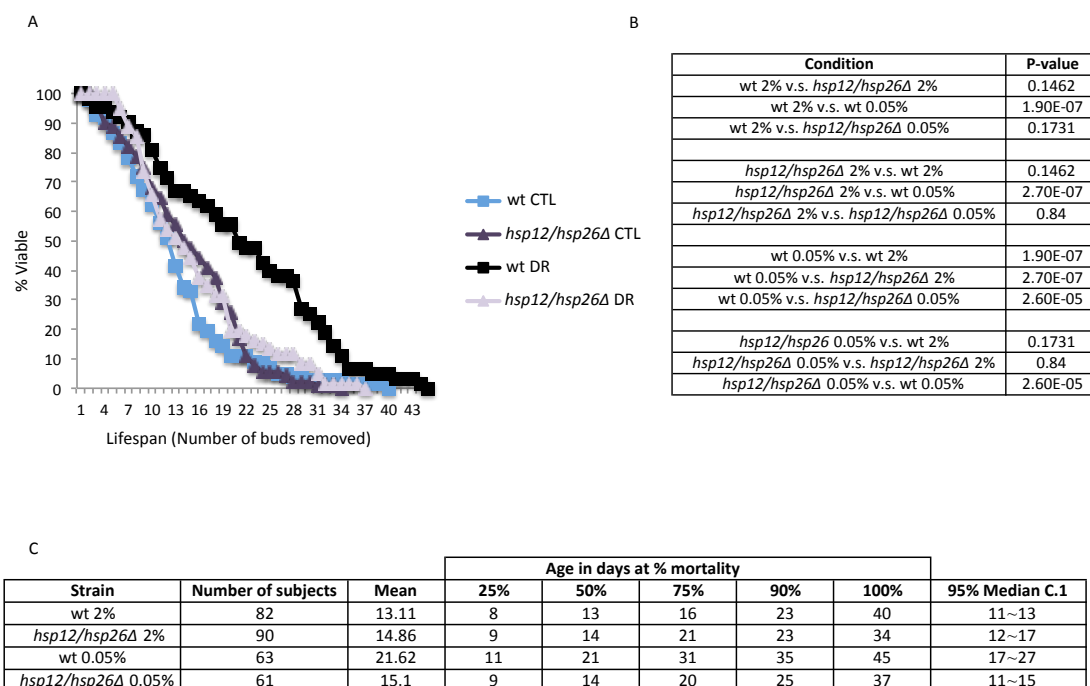


Figure 3.7 RLS analysis of the BY4741 wildtype and *hsp12/hsp26Δ* double mutant, 2% (w/v) glucose vs 0.05% (w/v) glucose, collated data. A) Graph showing lifespan extension of the wildtype strain by growth on media supplemented with 0.05% (w/v) glucose. In contrast, the *hsp12/hsp26Δ* double mutant has a reduced mean and maximum lifespan when grown on 0.05% (w/v) glucose. B) C) Tables showing a statistically significant reduction in RLS for the *hsp12/hsp26Δ* double mutant compared to the wildtype, statistical significance was assessed using the Wilcoxon Rank-Sum test and deemed significant at $p < 0.05$ using OASIS Online Application for Survival Analysis (Yang et al., 2011).

Since DR is unable to extend lifespan of the *hsp12Δ* single mutant, lifespan analysis was performed on BY4741 wildtype strains transformed with the empty vector, YCP:LEU, and the expression vector driving *HSP12* under the control of its endogenous promoter, YCP:LEU::pHSP12::Hsp12. The aim of this experiment was to determine if *HSP12* overexpression has any effect on longevity. Lifespan analysis was performed on synthetic complete media lacking leucine (SC-LEU) to select for yeast cells carrying the YCP:LEU plasmid. With 2% (w/v) glucose the strain overexpressing *HSP12* (WT:YCP:LEU::pHSP12:Hsp12) showed an increase in RLS (mean RLS 21.8) similar to that recorded for the WT:YCP:LEU strain (mean RLS 22.80) grown on media supplemented with 0.05% (w/v) glucose (Figure 3.8). Consistent with the findings that *HSP12* is required for DR to mediate lifespan extension, DR was unable to further extend the RLS of the WT:YCP:LEU::pHSP12:Hsp12 strain overexpressing *HSP12* (mean RLS 16.7), and there was no statistical difference between RLS on standard media and with severe DR (p -value 0.1557).

RLS analysis has shown that growth on 0.05% (w/v) glucose is unable to extend the lifespan of an *hsp12Δ* knockout strain and an *hsp26Δ* knockout strain. This suggests that *HSP12* and *HSP26* are required to mediate lifespan extension by DR. Interestingly overexpression of *HSP12* with 2% (w/v) glucose causes lifespan extension, implying a novel role for *HSP12* in ageing. Furthermore, double deletion of *HSP12* and *HSP26* causes a reduction in mean and maximum lifespan on media supplemented with 0.05% (w/v) glucose. Collectively these results suggest an important role for Hsp12 and Hsp26 in processes associated with ageing.

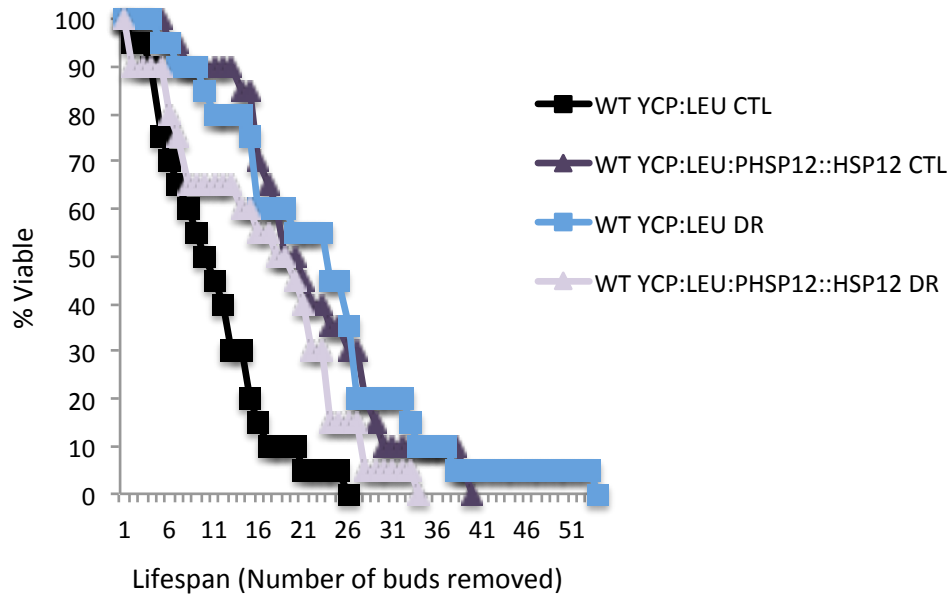


Figure 3.8 *HSP12* overexpression increases replicative lifespan. Graph showing plotted lifespan analysis data of BY4741 wildtype strains transformed with YCP:LEU and YCP:LEU::pHsp12:Hsp12 plasmid DNA. Yeast strains were grown on SC-LEU media. Growth on media supplemented with 0.05% (w/v) glucose extends the RLS of the BY4741 WT:YCP:LEU strain (n=20, mean RLS 22.8 buds) compared to that on 2% (w/v) glucose (n=20, mean RLS 11 buds). *HSP12* overexpression increases the RLS with growth on 2% (w/v) glucose (n=20, mean RLS 21.8 buds). There is no significant difference in RLS between the BY4741 WT:YCP:LEU:pHsp12:Hsp12 strain when grown on media supplemented with 2% (w/v) glucose or 0.05% (w/v) glucose (n=20, mean RLS 16.7 buds).

3.4 DISCUSSION.

3.4.1 Hsp12 and Hsp26 are up-regulated in response to glucose depletion.

When exposed to severe environmental conditions, such as high or low temperature, limitations of substrates, increased levels of metabolites, extreme pH or oxidative stress, *S. cerevisiae* demonstrates a stress response (Nisamedtinov et al., 2008). This stress response consists of inhibition of general protein synthesis and enhanced synthesis of a set of proteins, termed the Hsps (Lanks, 1986). The Hsps are found in virtually all organisms and act to resist proteotoxicity and maintain proteostasis by binding to misfolded proteins and preventing the formation of protein aggregates, both of which are fundamental components of the 'normal' ageing process (Nonaka and Hasegawa, 2011, Murshid et al., 2013). DR has been shown to significantly increase yeast lifespan and can be mimicked by genetic mutations of nutrient-sensing pathways or by reducing the glucose concentration of the growth medium to 0.5% or below (Riesen and Morgan, 2009).

Western blotting revealed the up-regulation of Hsp12 at the protein level in response to reduced concentrations of glucose, with the greatest up-regulation at 0.05% (w/v) glucose. Consistent with this finding, Hsp transcription is reportedly induced in response to glucose depletion and Hsp12 in particular, induced in response to 0.5% (w/v) glucose (Kasambalides and Lanks, 1983, Herbert et al., 2012). *HSP12* was first isolated in 1990 and regarded as a general stress responsive gene as it was shown to be induced several hundred-fold by heat shock, during osmostress, oxidative stress, high concentrations of ethanol, during nutrient limitation and entry into stationary phase (Praekelt and Meacock, 1990, Varela et al., 1995, de Groot et al., 2000). Despite this huge up-regulation, deletion of *HSP12* is not reported to cause a reduced tolerance to stresses (Petko and Lindquist, 1986) (Praekelt and Meacock, 1990).

Like Hsp12, Western blotting also revealed Hsp26 up-regulation in response to glucose depletion, with the strongest band present with 0.05% (w/v) glucose. This finding may explain why previous 2-D gel electrophoresis did not identify the up-regulation of Hsp26 protein in response to 0.5% (w/v) glucose. In fact, Northern blotting has shown that the Hsp26 transcript is not detectable in unstressed cells

and like Hsp12, is induced by heat shock, salt shock, nitrogen starvation and entry into stationary phase (Susek and Lindquist, 1990) (Carmelo and Sa-Correia, 1997). Similar to that reported for Hsp12, disruption of *HSP26* has no detectable effect on growth rates at various temperatures, in fermentative or respiratory metabolism, in rich or minimal media, in thermotolerance, resistance to ethanol, spore development or stationary phase growth (Petko and Lindquist, 1986).

As cultures of *S. cerevisiae* grow they generally alter their extracellular nutrient conditions. To achieve balanced growth the yeast cell must respond, and does so by making metabolic adjustments (Brauer et al., 2008, De Wever et al., 2005). In the presence of sufficient nutrients, such as glucose, yeast cells will grow exponentially until glucose levels become depleted and the cell reaches stationary phase (De Wever et al., 2005). This change in nutritional conditions is detrimental to growth and associated with a global change in gene expression (Gasch et al., 2000, Brauer et al., 2005). Notably, genes involved in glycolysis and protein synthesis are down-regulated and those associated with stress protective mechanisms such as the Hsp genes as well genes associated with gluconeogenesis and the tricarboxylic acid cycle are up-regulated (Gasch et al., 2000, Haurie et al., 2003, De Wever et al., 2005). Interestingly, cultures of *S. cerevisiae* growing on a non-fermentable carbon source express a high level of *HSP12* mRNA, which is rapidly repressed upon the addition of fermentable glucose (de Groot et al., 2000). Basal *HSP26* transcription is repressed in yeast cells growing under normal conditions and de-repressed upon heat shock, entry into stationary phase and during sporulation (Susek and Lindquist, 1990). The expression of *HSP12* and *HSP26* is therefore controlled by both stress-responsive signalling and by glucose-dependent mechanisms (de Groot et al., 2000). *HSP26* gene induction in response to stresses can occur via two regulatory systems: either by the heat shock transcription factor (HSF), which binds to heat shock elements (HSEs) or by Msn2 and Msn4 transcription factors which bind to stress response elements (STRE) and induce transcription (Amoros and Estruch, 2001, Susek and Lindquist, 1990, Rep et al., 1999). The STRE are found in the promoters of the *HSP12* gene and in response to stresses Msn2 and Msn4 translocate to the nucleus and induce transcription of *HSP12* (Rep et al., 1999). Activation of Hsp transcription in *Saccharomyces pombe* (*S. pombe*) requires HSF, which has been

shown to have a domain structure similar to that of *Drosophila* and humans (Gallo et al., 1993). In *S. pombe*, the binding of HSF is fully induced only under stress conditions, this is similar to that reported for *Drosophila* and humans but differs from the constitutive binding of HSF in *S. cerevisiae* (Gallo et al., 1993). In *Candida albicans* (*C. albicans*) heat shock transcription factor 1 (HSF1) becomes hyperphosphorylated in response to heat shock and activates transcription by binding to HSEs in the promoters of the *HSP* genes (Nicholls et al., 2011). Msn2 and Msn4 are also able to induce transcription of heat responsive genes in *C. albicans* (Sheth et al., 2008). It is thought that the yeasts' ability to quickly adapt global gene expression in response to environmental conditions allows for growth when the milieu becomes favourable but suppresses growth under stress conditions (De Wever et al., 2005, de Groot et al., 2000, Brauer et al., 2005).

In addition to up-regulation of *HSP12* and *HSP26* in response to stress and during development, studies have also reported the induction of the Hsps during yeast ageing, in particular *HSP12* and *HSP26*. Transcriptome analysis of old yeast cells isolated by elutriation, have shown the up-regulation of *HSP12* and *HSP26* (Lesur and Campbell, 2004, Lin et al., 2001). During the production of wines, yeast cells are subjected to osmotic and oxidative stress, exposure to ethanol and variations in temperature, stresses similar to those found during biological ageing (Aranda et al., 2002). Furthermore, the growth of yeast cells in the presence of a high concentration of osmolytes has been shown to increase yeast lifespan by a mechanism requiring SIR2, which is reportedly similar to DR mediated lifespan extension (Kaeberlein et al., 2002). Some groups suggest that DR presents a mild stress to the cell, provoking a stress response, which increases the organisms' defenses and survival chances (Sinclair, 2005). The induction of *HSP12* and *HSP26* during such stresses correlates with stress resistance and longevity (Aranda et al., 2002, Marchal et al., 2011, Jazwinski, 1999). This correlation is further supported by studies that show that exposure to a low dose of toxicant or radiation provides protection against damage from a larger dose (Hoffmann et al., 2013). Consistent with this idea, yeast lifespan is increased by exposure to a variety of mild stresses including increased salt and low amino acids or low glucose (Sinclair, 2005). Other

studies have shown exposure of yeast cells to chronic sub-lethal heat shock throughout their lifetime allows survival during acute lethal heat shock (Jazwinski, 1999). Several studies have demonstrated mitochondrial hormesis by exposure to a low non-lethal concentration of H₂O₂, which then protects against a higher lethal dose (Collinson and Dawes, 1992, Jamieson, 1992, Davies et al., 1995). In addition other studies have shown exposure to low doses of ionizing radiation, heavy metals, alkylating agents and thermal stress to induce protection and modulate lifespan (Crawford and Davies, 1994, Le Bourg, 2007, Moskalev, 2007, Rattan, 2005).

3.4.2 The roles of the Hsps in longevity.

Since both the mRNA and protein levels of Hsp12 and Hsp26 are up-regulated in response to glucose depletion this may indicate they play a role in DR mediated lifespan extension. Reducing the glucose concentration to 0.05% to mimic severe DR extended the mean RLS of the wildtype strain to 21.6 from 13.1 seen with 2% (w/v) glucose. Interestingly, like that reported for *HSP12*, DR was unable to extend the lifespan of the *hsp26Δ* single mutant, as there was no statistical difference between the mean RLS at 2% (w/v) glucose, 18.7, compared with severe DR, 21.1 (*p*-value 0.5977). One explanation for this longevity effect may be that in the absence of *HSP26*, DR-induced stress causes the aggregation of a variety of cellular proteins, thereby limiting lifespan.

RLS analysis with 0.05% (w/v) glucose identified a striking reduction in RLS of the *hsp12/hsp26Δ* double mutant in comparison to the wildtype and *hsp26Δ* single mutant. Since there was no statistical difference between mean RLS of the wildtype and *hsp12/hsp26Δ* double mutant with 2% (w/v) glucose this may suggest a novel role for *HSP12* and *HSP26* in DR mediated lifespan extension. In yeast, *HSP104* is critical for tolerance to extreme heat, and required for tolerance to ethanol, arsenite and long-term cold storage (Sanchez et al., 1992). The ability of Hsp104 to prevent protein aggregation requires the presence of *SSD1* (Mir et al., 2009). Hsp104 has also been shown to act in a chaperone system with Hsp70 encoded by *SSA* and Hsp40 encoded by *YDJ1* to reactivate aggregated proteins, in fact Hsp104 controls the aggregation state of yeast Sup35-based [*psi*⁺] prion-like factor (Glover and Lindquist, 1998, Abbas-Terki et al., 2001). Furthermore, overexpression of

Hsp104 cures yeast of these prions (Abbas-Terki et al., 2001). In addition to Hsp104, Hsp70 is also essential for yeast cell viability and its overexpression is able to cure the [URE3] prion state in yeast cells, an effect which is further benefited by the presence of the Hsp40 cochaperone Ydj1p (Xu et al., 2013). The chaperones therefore work together and have overlapping functions; this is further supported by a study that shows during reactivation of proteins they are transferred from Hsp26 to Hsp104 and Hsp70 (Haslbeck et al., 2005). Furthermore, Hsp26 and Hsp42 have also been shown to prevent prionogenesis by the [PSI⁺] Sup35 prion protein (Duennwald et al., 2012). Overexpression of Hsp26 and Hsp42 prevents induction and cures [PSI⁺] and also enhances Hsp104 [PSI⁺] curing (Duennwald et al., 2012, Cashikar et al., 2005). Yeast has two almost identical Hsp90 isoforms, Hsc82 and Hsp82, of which at least one has to be maintained for cell viability (Abbas-Terki et al., 2001).

There is further evidence for a role of the Hsps in ageing in other model organisms. In a *Drosophila* model of Parkinson's disease, the chaperone Hsp70 has been shown to protect cells against the deleterious effects of α -synuclein. Further studies examining the molecular mechanisms of geldanamycin show this drug enhances the stress response and chaperone activity thereby protecting neurons against α -synuclein neurotoxicity (Auluck et al., 2005). In tissue culture models, overexpression of Hsps, such as Hsp40 and Hsp70, has been shown to rescue cell death caused by proteins with abnormal polyglutamine expansions (Vacher et al., 2005). In *Drosophila*, deletion of Hsp70 leads to enhanced sensitivity to neurodegenerative effects (Gong and Golic, 2006), whereas in a mice model of Huntington's disease, overexpression of Hsp104 reduced the formation of aggregates and prolonged lifespan by 20% (Vacher et al., 2005). In *Drosophila*, Hsp22 and Hsp70 are preferentially up-regulated with ageing and Hsp22 overexpression results in lifespan extension (Wadhwa et al., 2010). Overexpression of Hsp26 and Hsp27 also results in a 30% extension in *Drosophila* lifespan (Wang et al., 2004). In *C. elegans* increased activity of the sHsps, notably HSP-16.1, HSP-16.49 and HSP-12.6 has been linked with lifespan extension and a delay in protein aggregation (Hsu et al., 2003). The sHsps may extend lifespan by preventing

aggregation of damaged and oxidised proteins, which accumulate with increased age.

Ten sHsps are expressed in different human tissues and denoted HSPB1-HSPB10 (Datskevich et al., 2012). Like in yeast, in humans the HSPBs play an important role in survival of the cell during exposure to stresses (Datskevich et al., 2012). The HSPBs contain α -crystallin domain and share similar sequence homology to yeast Hsp26 (Datskevich et al., 2012, White et al., 2006). In human disease, mutations in the HSPBs result in congenital cataracts, desmin-related myopathy and dilated cardiomyopathy (Kampinga and Garrido, 2012). Hsp mediated protection against intracellular damage is advantageous in diseases that are caused by proteotoxicity (Kampinga and Garrido, 2012). However, the cytoprotective property of HSPBs has also been reported to be detrimental in tumour progression and in conferring resistance to chemotherapy (Wu and Tanguay, 2006).

Recent work has shown that unlike other studied yeast sHsps, Hsp12 does not form large oligomeric structures, has limited sequence homology outside the kingdom of fungi and negligible anti-aggregation activity (Welker et al., 2010, Herbert et al., 2012). Yet deletion of *HSP12* abolishes lifespan extension by DR in yeast (Herbert et al., 2012). Since it has been shown that Hsp12 is not a 'holdase' chaperone like its Hsp26 and Hsp42 family members, it has been proposed that Hsp12's underlying effect on lifespan is due to its ability to function as a membrane stabilising 'lipid' chaperone (Herbert et al., 2012, Welker et al., 2010). This is consistent with a study reported by Kaeberlein et al., where Ssd1 was shown to increase RLS by increasing plasma membrane stability (Kaeberlein et al., 2004a). Hsp12 is natively unfolded in solution however adopts a 4 α -helical structure in the presence of SDS. Each α -helix has been shown to move independently and this is thought to allow Hsp12 to link with several distinct membrane sub-domains thereby stabilising the membrane (Herbert et al., 2012).

S. cerevisiae *HSP12* shows significant homology to *S. pombe* Hsp9 (Taricani et al., 2001). In *S. pombe* *hsp9Δ* mutants show increased susceptibility to heat stress, while *HSP9* overexpression results in heat tolerance (Ahn et al., 2012). The function of Hsp9 in *S. pombe* differs from that of *S. cerevisiae* Hsp12, as evidence suggests Hsp9 has dual functions in both stress response and regulating the G2-M

checkpoint (Ahn et al., 2012). *C. albicans* contains two *HSP12* genes termed CaHSP12a and CaHSP12b, evidence suggest both copies are expressed and the proteins encoded by them differ in only two amino acids (Fu et al., 2012). *C. albicans HSP12* gene expression has been shown to be regulated by pH in a mechanism that requires *RIM101*, this differs from the regulation of *S. cerevisiae HSP12* (Sheth et al., 2008). Furthermore, regulation of *HSP12* in response to changes in environmental CO₂ has shown a requirement for cAMP signalling (Sheth et al., 2008). Regulation of *S. cerevisiae HSP12* also requires cAMP signalling (Reinders et al., 1998). Outside the kingdom of fungi, Hsp12 has been shown to have limited homology with mammalian ageing-associated protein 2 (Herbert et al., 2012). In addition, Hsp12 shares 20% homology with α -synuclein, which is also is natively unfolded like Hsp12 (Welker et al., 2010). Despite an unknown conserved amino acid sequence for Hsp12, it is still reasonable that the functions of Hsp12 are evolutionally conserved perhaps by a pattern of side-chain properties (Welker et al., 2010).

Unlike Hsp12, Hsp26 forms large oligomeric complexes, which dissociate into dimers upon heat shock and are able to bind to non-native proteins and prevent their aggregation (Haslbeck et al., 1999). Hsp26 acts as a molecular chaperone and is able to suppress aggregation of variety of substrate proteins suggesting that the protein has non-specific binding properties (White et al., 2006). The target substrates of Hsp26 overlap 90% with the other cytosolic yeast heat shock protein, Hsp42 (Haslbeck et al., 2004). Hsp26 has 100-fold more anti-aggregation activity than that of Hsp12 (Franzmann et al., 2008, Herbert et al., 2012). It is therefore likely that Hsp12 and Hsp26 affect longevity in different ways.

Hsp26 contains a highly conserved tetrapeptide sequence Gly-Val-Leu-Thr, and shows significant homology with a range of eukaryotic sHsps and with vertebrate α -crystallins (Bossier et al., 1989). The α -crystallin domain is a hydrophobic stretch of 80-100 amino acids and located in the C-terminus (Taricani et al., 2001). The α -crystallin of *S. cerevisiae Hsp26* shows similar homology to wheat Hsp16.9, archaeal Hsp16.5, *Mycobacterium tuberculosis Hsp16.3* and human α A crystallin and α B crystallin (White et al., 2006).

Considering the existing literature, it is reasonable to assume that the

absence of *HSP12* and *HSP26* during DR-induced stress leads to improper proteostasis and membrane instability, ultimately having detrimental effects to the cell and affecting lifespan of the yeast.

3.4.3 Conclusion.

The results extracted from these initial experiments show that both Hsp12 and Hsp26 are up-regulated in response to growth on 0.5% and 0.05% (w/v) glucose. Both *HSP12* and *HSP26* show a longevity phenotype, in which both genes appear to be essential for DR to mediate lifespan extension. Furthermore, the *hsp12/hsp26Δ* double knockouts are characterised by a reduced RLS with DR, suggesting that *HSP12* and *HSP26* interact genetically. To understand why the double mutant shows a reduced RLS it was next important to investigate various processes associated with yeast ageing, such as stress resistance, rDNA silencing and protein aggregation. If the double mutant is defective in any of these processes this may shed light on the mechanisms by which Hsp12 and Hsp26 affect longevity.

4.1 INTRODUCTION.

4.1.1 Theories of yeast ageing.

Studies in yeast ageing have implicated a role for the nucleolus, a region of the nucleus containing components of the ribosomal assembly and the ribosomal DNA (rDNA), which is a tandem array of 100-200 copies of a 9.1 kb repeat (Kaeberlein and Powers, 2007, Sinclair and Guarente, 1997). As yeast cells age, the structure of the nucleolus becomes enlarged and fragmented. Recombination events between the rDNA repeats occur leading to the excision of toxic, self-replicating extrachromosomal rDNA circles (ERCs), which accumulate and contribute to ageing (Sinclair et al., 1998, Bishop and Guarente, 2007). In support of this hypothesis, deletion of the gene encoding the rDNA replication fork block protein, Fob1, increases lifespan in many yeast strains and reduces rDNA recombination and the accumulation of ERCs (Kaeberlein and Powers, 2007).

Sir2 is a nicotinamide adenine dinucleotide (NAD⁺)-dependent histone deacetylase (Anderson et al., 2003) and a member of a complex of proteins that mediate transcriptional silencing at selected loci in the yeast genome: the rDNA, telomeres and mating locus (Guarente, 2011). Silencing of the rDNA requires Sir2 and is mediated by the regulator of nucleolar silencing and telophase exit (RENT) complex (Tanny et al., 2004). rDNA silencing is a characteristic feature of yeast ageing, yet homologues of yeast Sir2 have been found in nematodes, fruit flies and mammals (Guarente, 2011). There are seven sirtuins in mammals which share significant sequence homology to the *S. cerevisiae* gene, *SIR2*, in addition to NAD-dependent protein deacetylation activity (Yu and Auwerx, 2009). SIRT1 is the mammalian orthologue of Sir2 (Guarente, 2011). In yeast, deletion of the *SIR2* gene is known to shorten lifespan possibly by increasing recombination at the rDNA locus and is characterised by reduced silencing in the rDNA (Smith et al., 1999). In contrast, cells with an extra copy of *SIR2* live 30% longer than wildtype strains and exhibit increased silencing activity at the rDNA repeats (Lin et al., 2002, Anderson et al., 2003, Guarente, 2011). One theory suggests that Sir2 is activated upon the shift from anaerobic fermentation to aerobic respiration during moderate DR, as this increased respiration raises the cellular NAD⁺/NADH ratio (Bishop and Guarente,

2007). Another theory suggests that DR induces the expression of pyranzinamidase/nicotinamidase (Pnc1) which catabolises nicotinamide, an inhibitor of Sir2, thereby increasing activity of Sir2 (Anderson et al., 2003). It is therefore possible that activation of Sir2 occurs either by depleting levels of its inhibitor nicotinamide or by increasing the availability of its co-substrate NAD⁺ (Anderson et al., 2003).

Interestingly DR has also been shown to extend lifespan in some *SIR2* knockout strains leading to the proposal of an alternative theory, which is independent of Sir2 (Kaeberlein et al., 2004b). Deletion of either *SCH9*, an ortholog of mammalian S6 kinase, or *TOR1*, the target of rapamycin pathway, increases yeast lifespan independent of Sir2, additionally DR is unable to further increase the lifespan of *sch9Δ* and *tor1Δ* mutants (Kaeberlein et al., 2005). The target of rapamycin (TOR) pathway was originally shown to mediate the effects of DR in flies and yeast and has since become a major player in the field of ageing and DR (Katewa and Kapahi, 2010). Every eukaryote genome examined contains a *TOR* gene; TORs are large proteins that belong to a group of kinases known as phosphatidylinositol kinase-related kinase (PIKK) family (Wullschleger et al., 2006).

TOR signalling is known to regulate cell growth and metabolism in response to environmental factors (Wullschleger et al., 2006). In *S. cerevisiae*, TOR promotes cell growth and proliferation when activated by nutrients in the form of amino acids or carbohydrates (McCormick et al., 2011). In higher eukaryotes activation of TOR occurs via the insulin/insulin growth factor-1 (IGF-1) signalling pathway (IIS) in response to amino acids and glucose (McCormick et al., 2011). Notably, exposure of mice to the mTOR inhibitor, rapamycin, or deletion of the mTOR substrate, S6K1, results in lifespan extension (McCormick et al., 2011). Consistently, yeast and worm studies have linked this lifespan extension of reduced TOR signalling to that of DR (McCormick et al., 2011). The molecular mechanisms underlying the effects of TOR signalling on longevity are still not clear, yet one hypothesis suggests the effects of TOR signalling are related to reduced ribosomal protein biogenesis (Kaeberlein et al., 2005).

4.1.2 Protein aggregation, autophagy and mitochondrial dysfunction.

Protein aggregation as a result of exposure to stresses poses a major threat to all organisms and is managed at the cellular level by a 'protein quality control' network consisting of molecular chaperones and proteases (Basha et al., 2012). The heat shock family of proteins play a major role in the maintenance of protein homeostasis or proteostasis and assists in the correct folding of proteins and in the degradation of aggregated proteins by the proteasome or the lysosome (Lopez-Otin et al., 2013). With increasing age there is a progressive decline in the maintenance and repair pathways important for 'protein quality control' and as a consequence there is a gradual accumulation of oxidized, mis-folded and aggregated molecules (Gelino and Hansen, 2012). These accumulations have deleterious effects on tissue and organ integrity which leads to disease and ultimately death (Gelino and Hansen, 2012). Like that described for human ageing, yeast ageing is also characterised by the gradual accumulation of cellular and molecular damage (Lionaki et al., 2013). As the mother cell ages, there is a progressive accumulation of oxidized proteins, similar to senescence seen in worms, flies and mammals (Erjavec et al., 2007, Aguilaniu et al., 2003). Oxidized proteins accumulate within the replicatively aged mother cell, and are not inherited by the newborn daughter cells (Aguilaniu et al., 2003). Notably, the retention of oxidized proteins in the mother cell is a process which is Sir2 dependent and requires the activity of chaperones such as Hsp104. Mother cells with *SIR2* deletions do not retain oxidatively damaged proteins during cytokinesis, hence retention of oxidized proteins requires the presence of Sir2 (Aguilaniu et al., 2003, Erjavec et al., 2007). Furthermore, *HSP104* overexpression suppresses accelerated ageing in *sir2Δ* mutants (Erjavec et al., 2007).

Autophagy is evolutionarily conserved and plays a major role in proteostasis by providing a cytoprotective mechanism for the recycling of damaged proteins during exposure to stresses such as extreme temperatures, hypoxia and nutrient deprivation (Gelino and Hansen, 2012, Morselli et al., 2010, Tsuchiyama and Kennedy, 2012). Autophagy is induced in response to DR and linked with lifespan extension (Morselli et al., 2010). In nutrient rich conditions autophagy is inhibited in yeast by activated TORC1 (Target of Rapamycin Complex 1), however during DR, TORC1 is inactivated and autophagy induced (Alers et al., 2012). In yeast the

vacuole is often described as the counterpart of the mammalian lysosome since it is the destination for proteins targeted for degradation by autophagy (Tsuchiyama and Kennedy, 2012). A recent study has implicated the importance of the vacuole in mitochondrial dysfunction, a characteristic of both yeast and human ageing (Wang et al., 2013). With increased age, mitochondrial function declines and there is an increase in the levels of ROS which initiates an oxidative response and changes in gene expression, including stress responsive genes such as the *HSP* genes (Wang et al., 2013). It has recently been shown that DR can suppress mitochondrial dysfunction by maintaining an acidic pH in the vacuoles (Hughes and Gottschling, 2012). Since the vacuole, autophagy and mitochondria are evolutionarily conserved and DR has been shown to extend lifespan in many model organisms, this finding may be relevant to lifespan in other eukaryotes.

A common theory in yeast ageing implicates the activity of the Sir2 protein, which is required for transcriptional silencing at the rDNA, telomeres and mating locus (Kaeberlein et al., 1999). The molecular chaperones play a huge role in the maintenance of protein homeostasis and prevent the accumulation of protein aggregates (Morimoto and Cuervo, 2009). Yeast ageing like ageing in worms, flies and mammals is characterised by the accumulation of oxidized and aggregated proteins, and segregation of such aggregates to yeast mother cells is Sir2-dependent (Aguilaniu et al., 2003). The *hsp12/hsp26Δ* double mutant was characterised in order to determine the presence of any defects in processes known to affect yeast ageing such as Sir2 activity, silencing, stress resistance, protein aggregation and mitochondrial and vacuolar dysfunction.

4.2 METHODS.

Methods were generally performed as described in chapter 2, additional details are described below.

4.2.1. Yeast strains.

Yeast strains from multiple genetic backgrounds were utilised in this chapter. The JS128 *hsp26Δ*, JS128 *hsp12/hsp26Δ*, BY4741 *hsp12/hsp26Δ* and AEY1017 *hsp12/hsp26Δ* knockout strains were constructed by PCR-mediated gene disruption. In these strains the *HSP26* ORF was replaced with a KANMX4 deletion cassette. For *hsp12/hsp26Δ* double mutants the *HSP12* ORF was replaced with a HIS3MX6 deletion cassette. A second JS128 *hsp12/hsp26Δ* double mutant was also constructed by PCR-mediated gene disruption, however in this strain the *HSP12* ORF was replaced with a KANMX4 deletion cassette and the *HSP26* ORF replaced with a NATMX deletion cassette.

For rDNA silencing assays the rDNA reporter strain, JS128, was used. The JS128 strain has a *URA3* marker integrated into the nontranscribed spacer region 1 of the rDNA (Smith and Boeke, 1997). Partial silencing of the *URA3* reporter strain enables growth on media lacking uracil and media containing FOA. A loss of silencing at the rDNA locus results in increased expression of the *URA3* gene enabling growth on media lacking uracil, but prevents growth on media containing FOA due to the production of 5-fluorouracil, which is a toxic.

For telomeric silencing assays, the well-established AEY1017 reporter strain was used. The AEY1017 strain has a *URA3* marker strain integrated at the subtelomeric region of chromosome VII (Meijsing and Ehrenhofer-Murray, 2001). Like that described for the rDNA reporter strain, partial silencing of the *URA3* marker enables growth on media lacking uracil and media containing FOA. A loss of silencing at the telomeric locus results in increased expression of the *URA3* gene enabling growth on media lacking uracil but preventing growth on media containing FOA.

4.3 RESULTS

4.3.1 Deletion of *HSP12* and *HSP26* does not cause a general sickness phenotype to the cell.

The Hsps are up-regulated in response to many stresses and act to protect the cell against proteotoxicity. Both Hsp12 and Hsp26 are undetectable in non-stressed conditions, yet in times of stress, notably heat stress, their transcript is up-regulated several hundred-fold (Praekelt and Meacock, 1990, Petko and Lindquist, 1986). It is therefore interesting that disruption of *HSP12* or disruption of *HSP26* results in no observable susceptibility to thermostress (Praekelt and Meacock, 1990, Petko and Lindquist, 1986). In chapter 3, Western blotting revealed the up-regulation of Hsp12 and Hsp26 in response to glucose depletion and RLS analysis suggests a role for *HSP12* and *HSP26* in DR mediated lifespan. Notably, the *hsp12/hsp26Δ* double mutant shows a reduced RLS in response to DR; furthermore, DR is unable to extend the lifespan of *hsp12Δ* and *hsp26Δ* single knockouts. One tempting explanation for this is that deletion of both *HSP12* and *HSP26* results in a general sickness, as the cell is unable to cope with DR-induced stress.

In order to investigate this hypothesis, BY4741 single and double mutants were exposed to oxidative and environmental stresses, including exposure to ethanol, H₂O₂, caffeine, NEM, rapamycin, MMS, NaCl, glucose starvation and growth at different temperatures. These stresses were chosen as most have been reported to induce the expression of *HSP* genes (Lanks, 1986, Lindquist, 1986, Carmelo and Sa-Correia, 1997). In particular, yeast mutants with *HSP12* gene disruptions have been reported to show an increased susceptibility to 12 mM caffeine and 5 mM H₂O₂ (Motshwene et al., 2004, Welker et al., 2010). The *hsp12/hsp26Δ* double mutant showed a slight reduction in growth on media supplemented with 13% ethanol; however for all other stresses the *hsp12/hsp26Δ* strain showed no difference in growth to the wildtype. These results suggest that the BY4741 *hsp12/hsp26Δ* double mutant does not have an increased susceptibility to stimuli that cause stress to the cell (Figure 4.1).

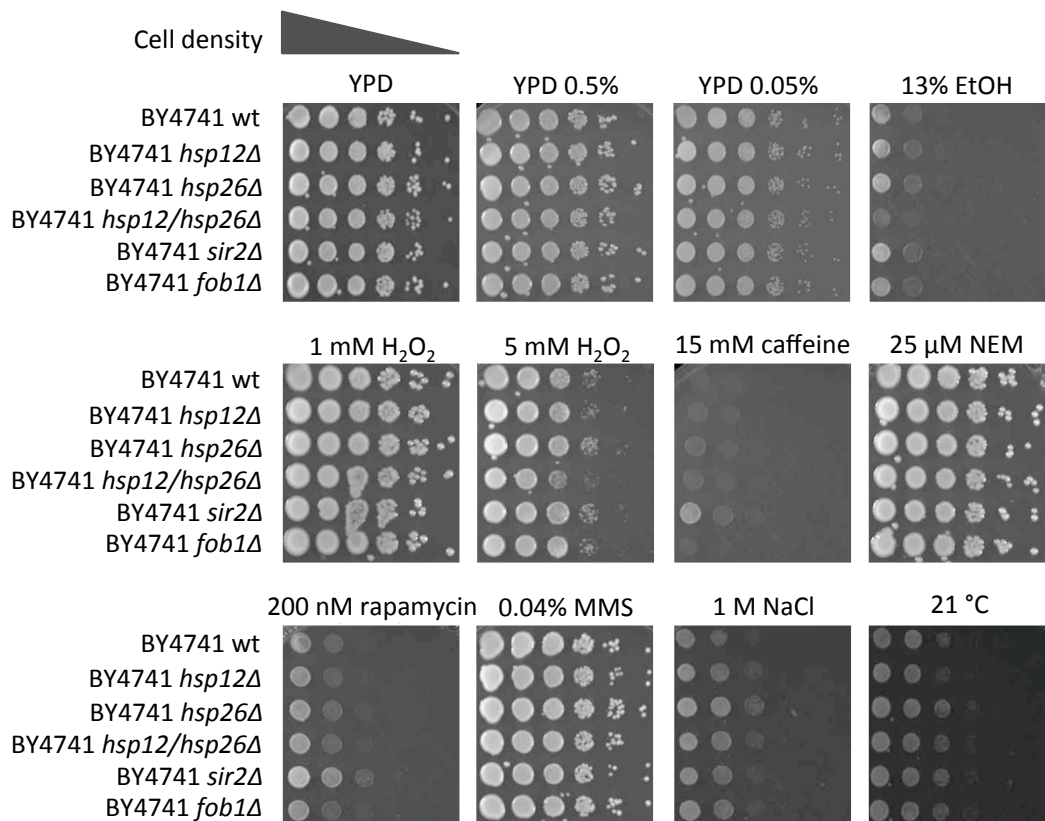


Figure 4.1 *Hsp12/hsp26Δ* double mutants are not stress sensitive. Yeast strains were serially diluted and replica plated onto selective media, 0.5% and 0.05% (w/v) represent glucose concentrations. Drug stress assay imaged after incubation for 3 days showing sensitivity of BY4741 *hsp12/hsp26Δ* strain to 13% ethanol visualised by reduced growth in comparison to the wildtype and single knockout strains. The *hsp12/hsp26Δ* strain shows no difference to the wildtype strain when grown on media containing H₂O₂, caffeine, NEM, rapamycin, MMS, NaCl, 0.5% and 0.05% (w/v) glucose or at a reduced temperature (21 °C).

4.3.2 Deletion of *HSP12* and *HSP26* does not increase susceptibility to stresses or affect silencing at the telomeres.

Since DR is thought to increase Sir2 activity and the *hsp12/hsp26Δ* double mutant shows a reduced RLS like that reported for *sir2Δ* mutants (Kaeberlein et al., 2004b), it was next important to determine if double deletion of *HSP12* and *HSP26* has any effect on Sir2 activity. Since Sir2 is required for silencing at the telomeres, rDNA and mating locus, one way to analyse Sir2 activity is to examine telomeric silencing (Kaeberlein et al., 1999). Deletion of *SIR2* inhibits telomeric silencing which is visualised by increased growth on media lacking uracil and reduced growth on media containing FOA (Kaeberlein et al., 1999). We next asked does the *hsp12/hsp26Δ* double mutant have any defect in telomeric silencing? Telomeric silencing assays were performed on wildtype, *hsp12Δ*, *hsp26Δ*, *fob1Δ* and *sir2Δ* strains. The wildtype strain was used as a negative control and the *sir2Δ* strain as a positive control since deletion of *SIR2* inhibits telomeric silencing (Riesen and Morgan, 2009). The *fob1Δ* strain was also included as a control, as deletion of *FOB1* has been shown to specifically inhibit rDNA silencing but not telomeric silencing (Riesen and Morgan, 2009). Telomeric silencing assays with 2% (w/v) glucose (Figure 4.2A) and with 0.5% and 0.05% (w/v) glucose (data not shown) revealed no difference in growth on selective media between the wildtype strain, single and *hsp12/hsp26Δ* double mutants. As expected, the *sir2Δ* positive control showed increased growth on media lacking uracil and no growth on media containing FOA, indicating a loss of telomeric silencing (Figure 4.2A).

To further confirm that the *hsp12/hsp26Δ* double mutant does not show an increased susceptibility to stresses, and that this finding is not BY4741 strain-specific, stress assays were performed on single and double mutants in the AEY1017 yeast genetic background. Like that reported for the BY4741 genetic background, *hsp12Δ* and *hsp26Δ* single and double knockouts showed no increase in susceptibility compared with the wildtype strain on media supplemented with caffeine, NEM, rapamycin, MMS, NaCl, paraquat, glucose starvation or growth at different temperatures. The ethanol sensitivity seen in the BY4741 double mutant was not seen in the AEY1017 double mutant, suggesting that this sensitivity is not significant (Figure 4.2B). These results exclude general sickness (a reduction in

growth in comparison to the wildtype) as a possible explanation for the reduced RLS of the *hsp12/hsp26Δ* double mutant.

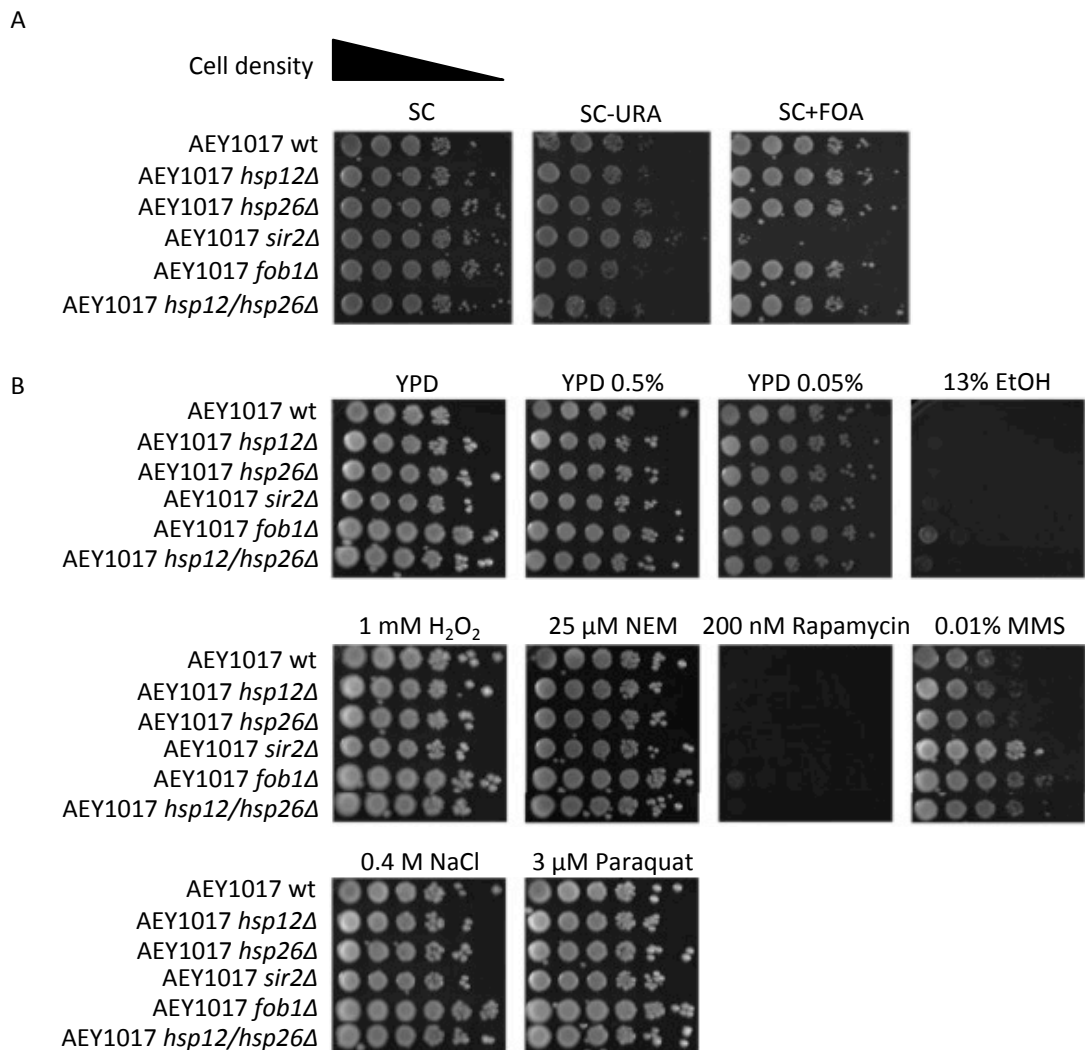


Figure 4.2 Deletion of *HSP12* and *HSP26* in the AEY1017 strain has no effect on telomeric silencing or on susceptibility to stresses. A) Telomeric silencing assays, plates were imaged after incubation for 2 days. As expected, the *sir2Δ* control strain inhibits telomeric silencing which is visualised by increased growth on media lacking uracil (SC-URA) and no growth on media containing FOA (SC+FOA). The *hsp12/hsp26Δ* double mutant shows no telomeric silencing defects. B) Stress assay. Yeast strains were serially diluted and replica plated onto selective media, 0.5% and 0.05% represent glucose concentration, plates were imaged after incubation for 3 days. The AEY1017 single and double *HSP12* and *HSP26* gene knockouts show no increase in susceptibility to media containing NaCl, rapamycin, MMS, paraquat, H₂O₂, NEM, EtOH and glucose depletion when compared to the wildtype.

4.3.3 Double deletion of *HSP12* and *HSP26* does not affect silencing at the rDNA.

So far results have shown that the *hsp12/hsp26Δ* double mutant is not characterised by a general sickness phenotype or any defects in telomeric silencing. It was important to investigate other avenues that may explain the reduced lifespan of the *hsp12/hsp26Δ* double mutant. Although telomeric assays show that the *hsp12/hsp26Δ* double mutant is not characterised by a major change in overall Sir2 activity, it may still be possible that the *hsp12/hsp26Δ* double mutant shows an effect on Sir2 activity specifically at the rDNA. This hypothesis is supported by the fact that Sir2 activity at the telomeres and mating loci is mediated by the Sir2/Sir4 complex, while Sir2 silencing at the rDNA is mediated by the RENT complex (Tanny et al., 2004). As mentioned earlier, Sir2 has been implicated in DR mediated lifespan extension in yeast, as deletion of the *SIR2* gene is known to shorten lifespan by increasing recombination at the rDNA locus and is characterised by reduced silencing in the rDNA (Smith et al., 1999). It may be that the *hsp12/hsp26Δ* double mutant also shortens lifespan by affecting rDNA silencing specifically. To investigate this, rDNA silencing assays were performed using the JS128 rDNA reporter strain. As in the telomeric silencing assays, the wildtype strain was used as a negative control, and the *sir2Δ* strain as a positive control for loss of silencing. Two different *hsp12/hsp26Δ* double mutants were included: in one strain the *HSP12* ORF was replaced with a HIS3MX6 cassette and the *HSP26* ORF replaced with a KANMX4 cassette. The remaining *hsp12/hsp26Δ* double mutant was constructed slightly differently, as the *HSP12* ORF was replaced with a KANMX4 deletion cassette and the *HSP26* ORF with an NATMX cassette. Ultimately, these strains are the same and should give reproducible results.

Single gene knockouts of *HSP12* and *HSP26* did not show any rDNA silencing phenotypes. As expected the *sir2Δ* mutant was characterised by a loss of rDNA silencing visualised by increased growth on media lacking uracil and reduced growth on media containing FOA. A loss of rDNA silencing phenotype was also seen for the *fob1Δ* positive control, as expected (Figure 4.3A). The *hsp12/hsp26Δ* (*his/kan*) double mutant also showed a similar growth pattern to the *sir2Δ* mutant, with increased growth on media lacking uracil and reduced growth on media containing FOA (Figure 4.3B). However, it was concluded that the *hsp12/hsp26Δ* double

mutant shows no loss of rDNA silencing as this result was not reproduced by the *hsp12/hsp26Δ* (kan/nat) double mutant (Figure 4.3C). Like that described for the BY4741 and AEY1017 genetic backgrounds, the JS128 *hsp12/hsp26Δ* double mutant did not show any increased susceptibility to caffeine, NaCl or MMS.

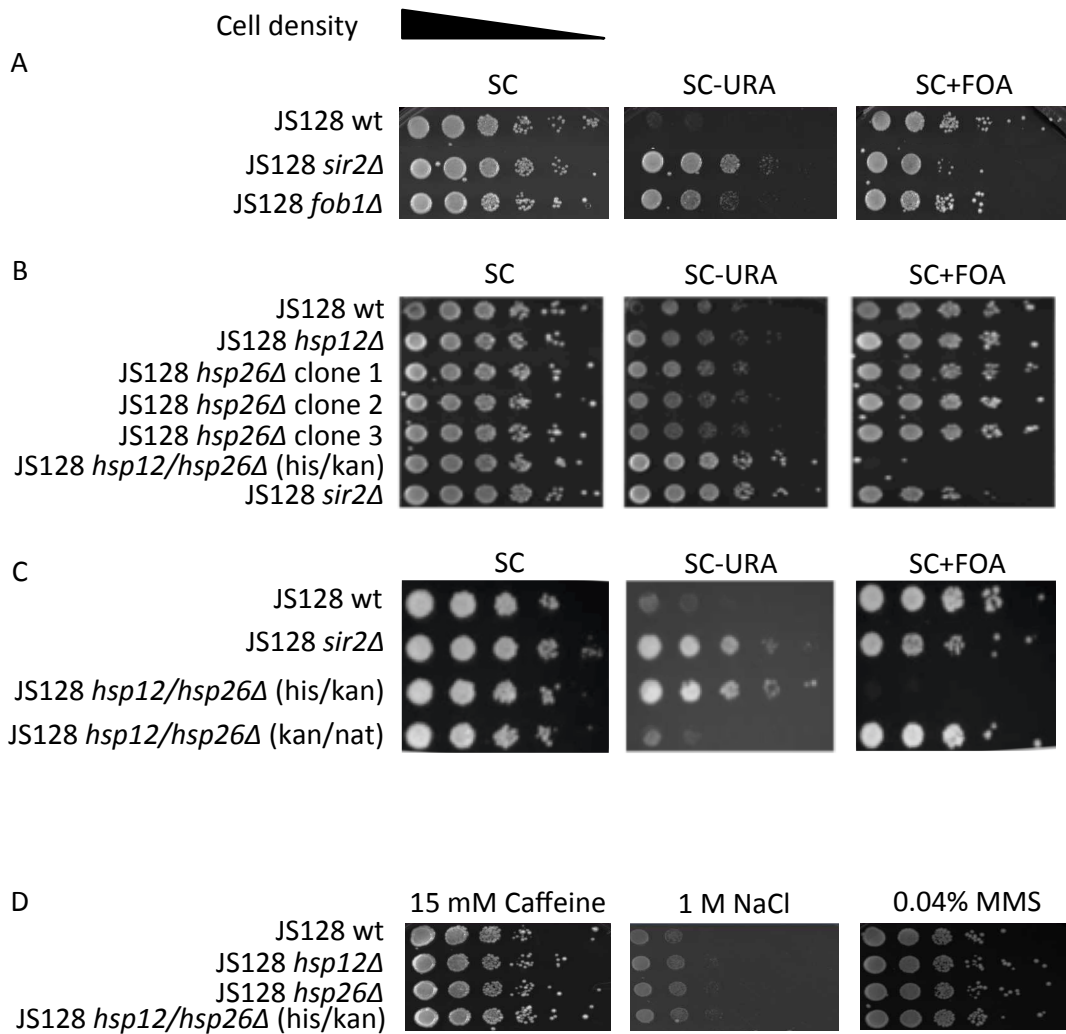


Figure 4.3 The JS128 *hsp12/hsp26Δ* double mutant shows no rDNA silencing defects. Yeast strains were serially diluted and replica plated onto selective media, plates were imaged after incubation for 2 days. A) Silencing assays with wildtype negative control, and *sir2Δ* and *fob1Δ* positive controls for loss of rDNA silencing. B) rDNA silencing assays. The *hsp12/hsp26Δ* double mutant shows increased growth on media lacking uracil and a lack of growth on media containing FOA, similar to the *sir2Δ* mutant. Single mutants show no difference in growth in comparison to the wildtype. C) The *hsp12/hsp26Δ* (kan/nat) mutant shows no difference in growth to the wildtype. The positive *sir2Δ* control shows a loss of rDNA silencing. D) Drug stress assay showing no increase in susceptibility of the JS128 *hsp12/hsp26Δ* double mutant to caffeine, NaCl or MMS.

4.3.4 Glucose depletion did not cause an observable increase in protein aggregation in the *hsp12/hsp26Δ* double mutant.

Double deletion of *HSP12* and *HSP26* does not cause increased susceptibility to stresses nor any observable defects in silencing at the telomeres or the rDNA. Since Hsp26 is a well-established protein chaperone and recent reports suggest Hsp12 functions as a membrane stabilising ‘lipid chaperone’, it may be that in the absence of *HSP12* and *HSP26*, aggregated proteins accumulate in the cell, having detrimental effects (Herbert et al., 2012, Welker et al., 2010, Haslbeck et al., 1999). A study by Haslbeck et al., has in fact shown that *hsp26Δ* single mutants are characterised by increased protein aggregation compared to the wildtype when heat shocked at 43 °C for 1 hour. Furthermore, increased protein aggregation is more apparent in an *hsp26/42Δ* double mutant after incubation at 25 °C for 1 hour (Haslbeck et al., 2004).

Aggregation assays were performed as detailed in figure 4.4 with 2% and 0.05% (w/v) glucose to determine if the *hsp12/hsp26Δ* double mutant shows increased protein aggregation in comparison to the wildtype. Yeast strains were grown to an OD₆₀₀ 0.6 and lysed in non-denaturing lysis buffer. After centrifugation at 5,000 g the supernatant was divided and incubated at either room temperature or 43 °C for 1 hour. After incubation the supernatant samples were centrifuged at 13,523 g to pellet any aggregated material. The supernatant and pellet samples were then boiled in laemmli and proteins separated by SDS-PAGE. Two independent aggregation experiments were analysed by densitometry using Image-Studio Lite Software (LI-COR). The total amount of protein in each lane was calculated summarizing the protein content of all visible bands. With 2% glucose the *hsp12/hsp26Δ* double mutant showed increased protein aggregation when compared to the wildtype (Figure 4.5B). With 0.05% (w/v) glucose the wildtype showed increased protein aggregation indicating that glucose depletion causes proteotoxicity (Figure 4.5D). Interestingly the *hsp12/hsp26Δ* double mutant showed less protein aggregation with 0.05% (w/v) glucose when compared to the wildtype, suggesting that the *hsp12/hsp26Δ* double mutant is able to cope with DR-induced protein aggregation (Figure 4.5D). It was therefore concluded that the reduced lifespan of the *hsp12/hsp26Δ* double mutant is not due to increased proteotoxicity.

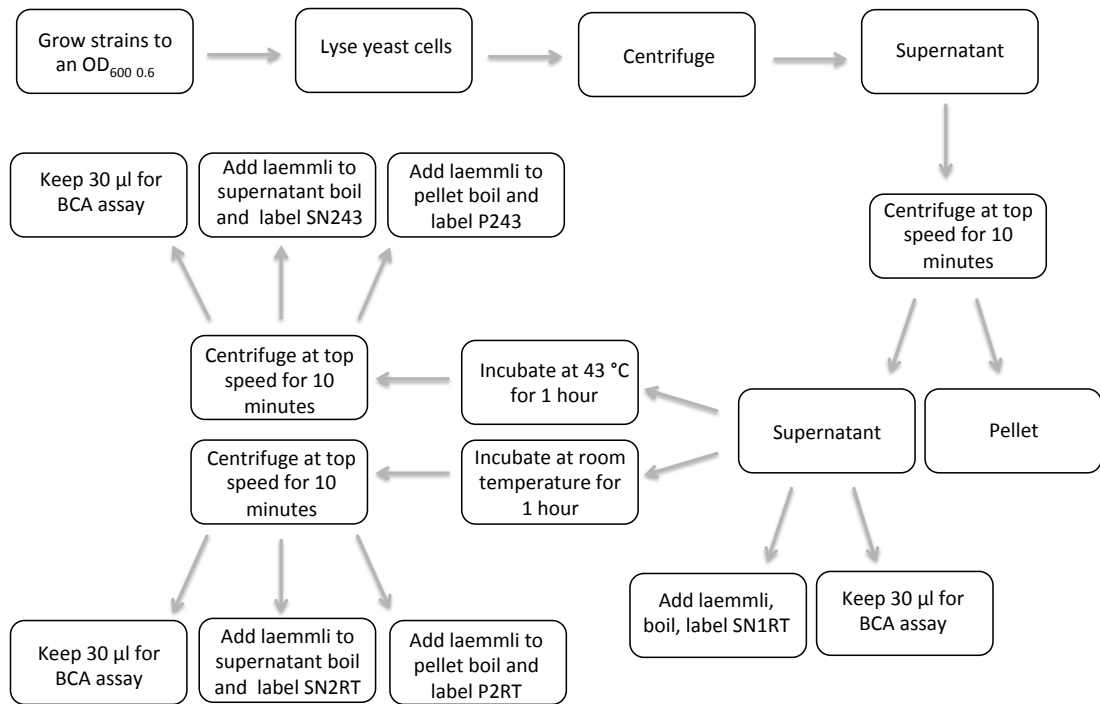


Figure 4.4 Flow diagram of aggregation assay methodology. Yeast strains were grown in liquid media to an OD_{600} 0.6, lysed in NDLB and centrifuged at 5,000 g. After another centrifugation step at top speed (13,523 g) the supernatant was incubated at either room temperature or heat shocked at 43 °C. Proteins were separated by SDS-PAGE, protein loading was normalized so that all concentrations were the same as SN1RT.

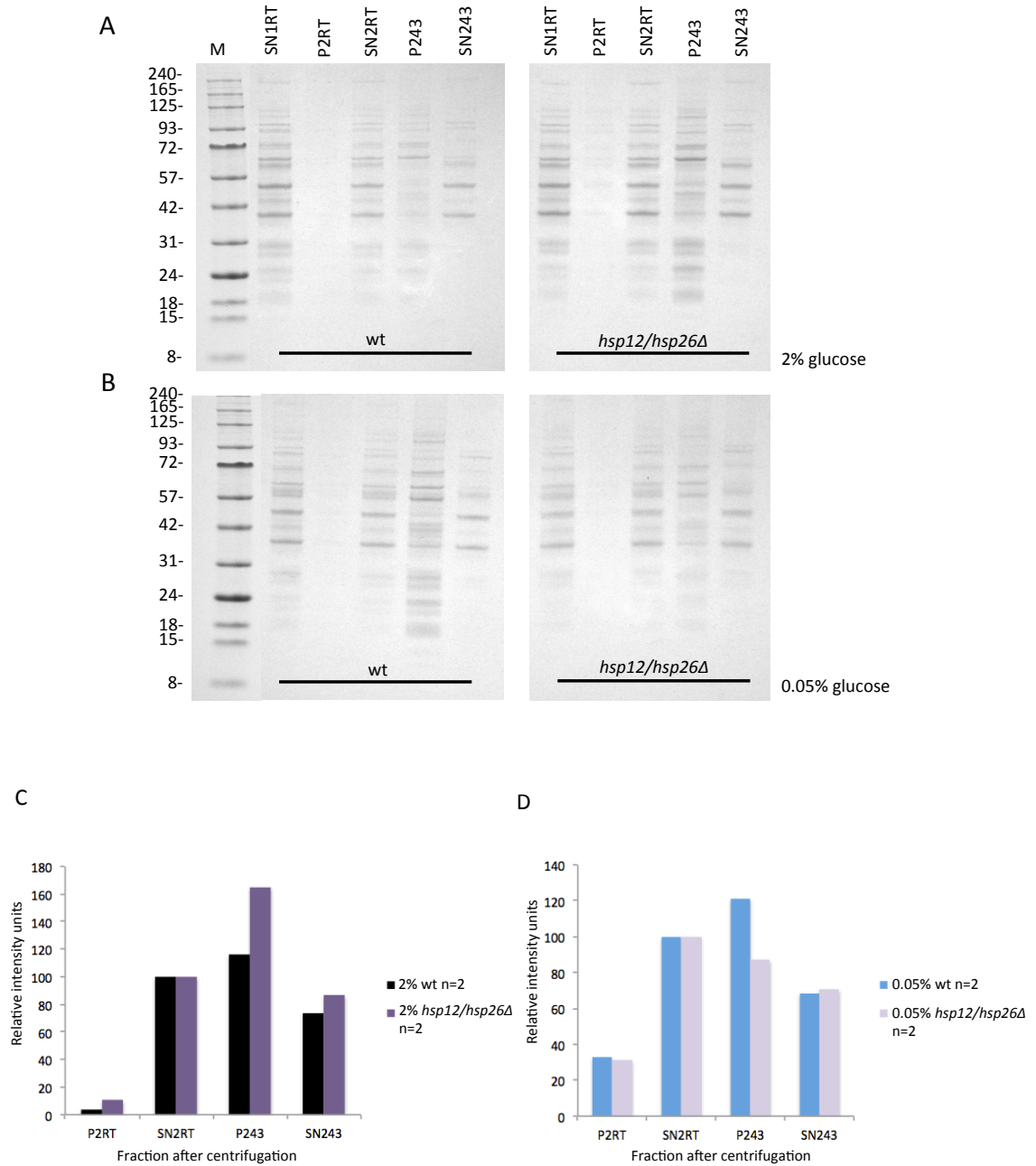


Figure 4.5 The *hsp12/hsp26Δ* double mutant does not show increased protein aggregation. A,B) Coomassie-stained SDS-PAGE gels of aggregated proteins in the pellet and supernatant fractions from wildtype and sHsp deletion strains. Protein loading was normalized to SN1RT. Yeast strains were grown in broth supplemented with 2% or 0.05% (w/v) glucose to an OD₆₀₀ 0.6. Insoluble protein was separated by centrifugation at 13,523 g for 10 minutes. C) Plotted densitometry data from two independent aggregation experiments with 2% (w/v) glucose. The total amount of protein in each lane was calculated by summarizing the protein content of all visible bands using Image-Studio Lite Software (LiCor) and normalized to SN2RT. D) Plotted densitometry data from two independent aggregation experiments with 0.05% (w/v) glucose, normalized to SN2RT.

4.3.5 Double deletion of *HSP12* and *HSP26* does not increase mitochondrial dysfunction or alter vacuolar acidity.

A role for mitochondria in the ageing process has long been suspected (Lopez-Otin et al., 2013). This correlation is supported by the fact that the structure and functions of mitochondria are altered during the ageing process of many eukaryotes (Wang et al., 2013). A recent study in replicatively aged yeast cells has shown that mitochondrial dysfunction arises from altered vacuolar pH and that acidic vacuoles are regenerated in newborn daughters but reduced in aged mother cells (Hughes and Gottschling, 2012). The authors report that preventing a decline in vacuolar acidity suppresses mitochondrial dysfunction and that DR can mediate lifespan extension at least in part by increasing vacuolar acidity (Hughes and Gottschling, 2012).

Our investigations so far have provided no possible explanations as to why the *hsp12/hsp26Δ* double mutant has a reduced lifespan. Since mitochondrial dysfunction is a common feature of yeast ageing it was important to establish if the *hsp12/hsp26Δ* double mutant shows alterations in mitochondrial structure and function in comparison to the wildtype. With the new finding that vacuolar pH is a critical regulator of ageing, it was also important to determine if the *hsp12/hsp26Δ* double mutant shows a decline in vacuolar acidification in comparison to the wildtype.

Vacuolar acidity can be visualised by quinacrine staining, which diffuses across membranes and concentrates in acidic compartments (Hughes and Gottschling, 2012, Weisman et al., 1987). Initial experiments were focused on comparing the quinacrine accumulation in the wildtype to that of the *hsp12/hsp26Δ* double mutant when grown in broth supplemented with 2% (w/v) glucose. An aliquot of wildtype cells were treated with Folimycin to inhibit the V-ATPase and used as a control for quinacrine staining. Wildtype cells treated with folimycin showed a reduction in quinacrine accumulation, confirming specific vacuolar staining (Figure 4.6A & B). Fluorescent microscopy of a Vma2-GFP yeast strain, labelling a subunit of the vacuolar H⁺-ATPase, looked similar to quinacrine staining and further confirmed quinacrine staining specificity (Figure 4.6C). The vacuolar acidification of the mother cells and daughter cells was next compared. Several

hundred mother and daughter pairs were analysed. In contrast to the findings reported by Hughes and Gottschling, the results in the study did not show enhanced quinacrine staining in the daughter cells when grown in DR conditions. Furthermore, there was no obvious observable difference in vacuolar acidification between the wildtype and *hsp12/hsp26Δ* double mutant. Wildtype and *hsp12/hsp26Δ* double mutant mother cells grown in 2% or 0.05% (w/v) glucose showed no reduction in vacuolar acidification when compared to their daughter cells. These results suggest the *hsp12/hsp26Δ* double mutant has no alterations in vacuolar acidification compared to that of the wildtype (Figure 4.7).

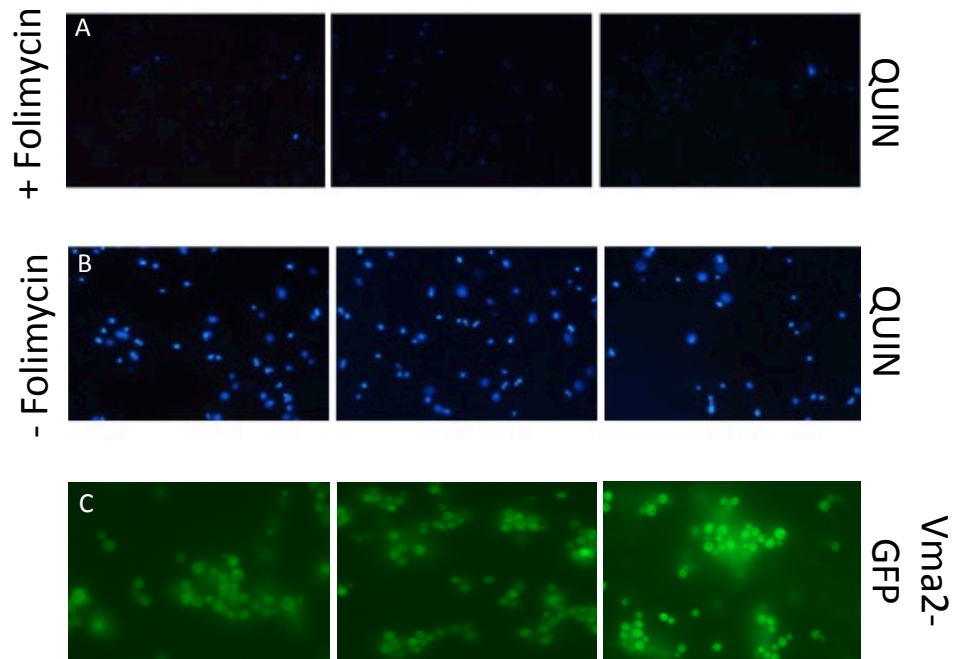
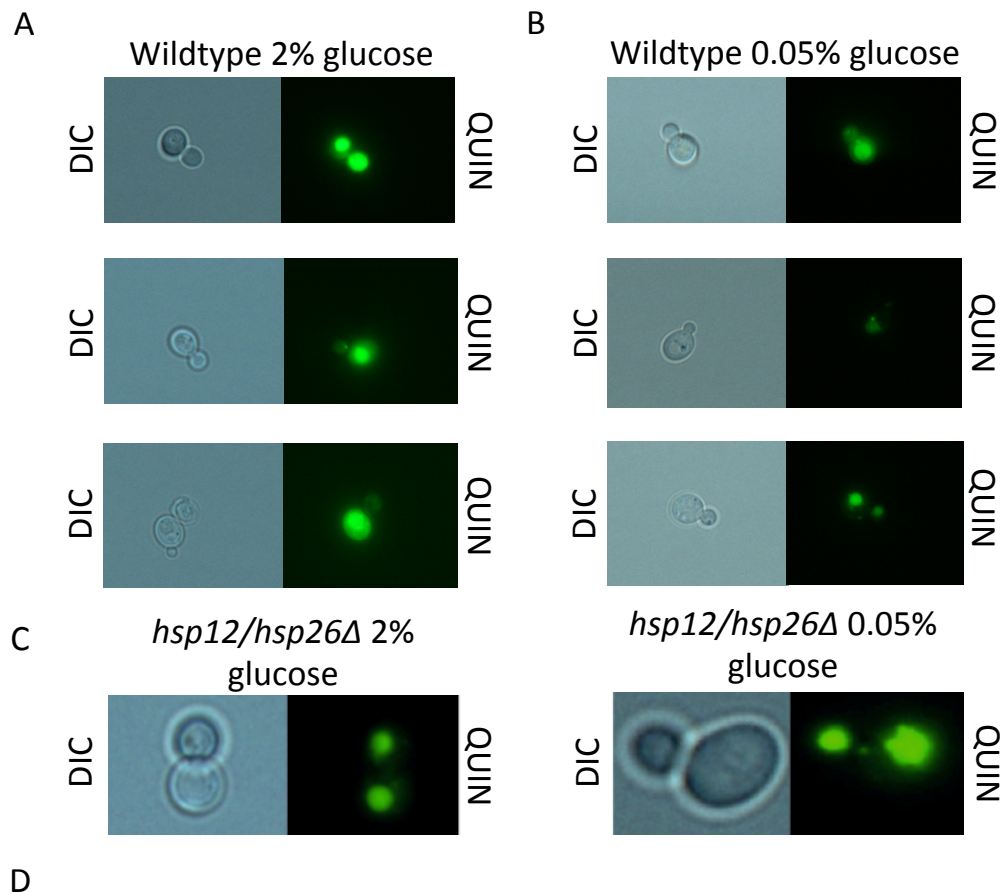


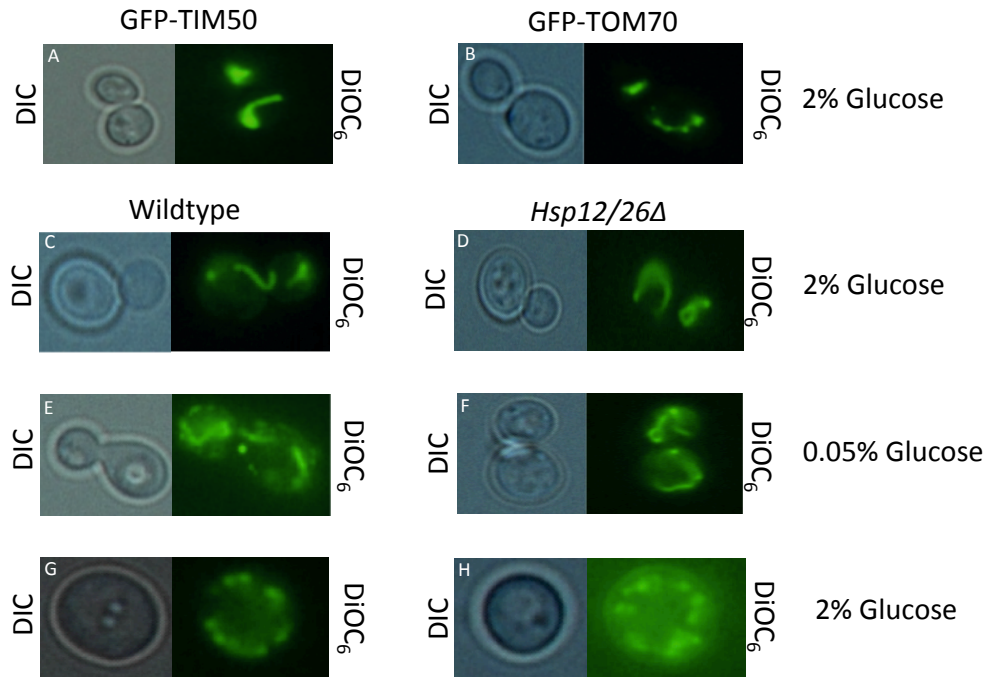
Figure 4.6 Treatment with Folimycin inhibits the V-ATPase. Yeast cells were grown in liquid broth supplemented with 2% (w/v) glucose to an OD_{600} 0.6, divided equally and one aliquot treated with 500 nM folimycin to inhibit the V-ATPase. Vacuolar acidity was determined by quinacrine staining and cells were visualised using a DAPI filter on a Nikon Diaphot microscope with a X60 magnification lens. A) Wildtype cell treated with 500 nM folimycin show a reduction in quinacrine staining confirming inhibition of the V-ATPase. B) Wildtype cells without folimycin treatment showing increased quinacrine staining in comparison to folimycin treated cells. C) Fluorescent microscopy of Vma2-GFP yeast strain using the GFP filter. The Vma2 protein is a subunit of the vacuolar H^+ -ATPase and similarity of staining confirmed specificity of quinacrine staining.



Yeast strain	Glucose concentration (w/v)	No. of daughter cells with greater quinacrine staining	No. of mother cells with greater quinacrine staining	No. of cells with equal quinacrine staining
Wildtype	2%	21	18	48
Wildtype	0.05%	8	21	19
<i>hsp12/hsp26Δ</i>	2%	9	18	39
<i>hsp12/hsp26Δ</i>	0.05%	4	1	5

Figure 4.7 Glucose depletion does not increase vacuolar acidity. Cells were visualised using a GFP filter on a Nikon Eclipse Ti S microscope using a X60 1.40 Plan Apo objective lens. A) 3 representative DIC images of wildtype mother and daughter cells grown in liquid broth supplemented with 2% (w/v) glucose. Quinacrine staining is equally dispersed between the mother and daughter cells and does not indicate an increased acidity in the daughter cell in comparison to the mother cell (Top panel). Quinacrine staining is greater in the daughter cell (Middle panel). Quinacrine staining is greater in the mother cell (Bottom panel) B) 3 representative DIC images of wildtype mother and daughter cells grown in liquid broth supplemented with 0.05% (w/v) glucose. Quinacrine staining is equal between mother and daughter cells (Top panel). Quinacrine staining is greater in the daughter cell (Middle panel). Quinacrine staining is greater in the mother cell (Bottom panel) C) DIC of *hsp12/hsp26Δ* mother and daughter cell grown in liquid broth supplemented with 2% (w/v) glucose (left panel) and 0.05% (w/v) glucose (right panel). Quinacrine staining is equally dispersed between the mother and daughter cells showing no difference in acidification D) Table showing analysis of quinacrine staining for wildtype and *hsp12/hsp26Δ* mother and daughter cells, results do not suggest that daughter cells have an increased vacuolar acidification with DR.

The mitochondrial structure of the *hsp12/hsp26Δ* double mutant was next compared to the wildtype. Mitochondrial structures were analysed by 3,3'-dihexyloxycarbocyanine iodide (DiOC₆) staining. DiOC₆ is a lipophilic dye and when used at a low concentration stains mitochondria in living yeast cells (Koning et al., 1993). The specificity of DiOC₆ staining was confirmed by fluorescent microscopy showing similarity to an outer mitochondrial protein (TOM70) TOM70-GFP and an inner mitochondrial protein (TIM50) TIM50-GFP yeast strains (Figure 4.8A & B). The *hsp12/hsp26Δ* double mutant showed no changes in DiOC₆ staining to that of the wildtype when grown in media supplemented with 2% and 0.05% (w/v) glucose (Figure 4.8C, D, E & F). Wildtype and *hsp12/hsp26Δ* double mutant cells were next grown at 30 °C, 200 rpm for 1 month in liquid broth supplemented with 2% (w/v) glucose so that cells entered stationary phase. The expression of *HSP12* is strongly induced upon entry into stationary phase so the rationale behind this approach was to determine if the *hsp12/hsp26Δ* double mutant shows a phenotype since the *HSP12* gene has been deleted in this strain. Analysis of multiple old wildtype and *hsp12/hsp26Δ* double mutant cells showed no obvious differences in the mitochondrial structure. The old wildtype cells showed puncta staining and the *hsp12/hsp26Δ* double mutant showed equal numbers of puncta and tubular staining of the mitochondria (Figure 4.8G, H & I).



I

Yeast strain and age (2% (w/v) glucose)	No. of cells with puncta morphology	No. of cells with tubular morphology	No. of cells with equal puncta and tubular morphology
Wildtype 1 month old	9	0	1
Wildtype log phase growth	0	21	0
<i>hsp12/hsp26Δ</i> 1 month old	5	5	1
<i>hsp12/hsp26Δ</i> log phase growth	0	5	0

Figure 4.8 The *hsp12/hsp26Δ* does not show any obvious differences in mitochondrial structure in comparison to the wildtype. Cells were visualised using a GFP filter on a Nikon Eclipse Ti S microscope using a X60 1.40 Plan Apo objective lens. A) B) GFP-TIM50 and GFP-TOM70 yeast strains were used as controls to confirm specific mitochondrial staining with DiOC₆. C) DIC of wildtype mother and daughter cells grown in 2% (w/v) glucose. DiOC₆ staining shows no apparent differences in mitochondrial structure to the *hsp12/hsp26Δ* double mutant (D). E) DIC of wildtype mother and daughter cells grown in 0.05% (w/v) glucose. DiOC₆ staining shows no apparent differences in mitochondrial structure in comparison to the *hsp12/hsp26Δ* double mutant (F). G) DIC of 1-month old wildtype cell, DiOC₆ staining shows no difference in mitochondrial structure to 1-month old *hsp12/hsp26Δ* double mutant (H). I) Table showing mitochondrial morphology determined as punctar (mitochondrial fragmentation) or tubular (normal mitochondria) for wildtype and *hsp12/hsp26Δ* double mutants collected at log phase and at 1 month old.

4.4 DISCUSSION

4.4.1 Double deletion of *HSP12* and *HSP26* does not increase susceptibility to stresses.

Under normal physiological conditions both Hsp12 and Hsp26 are undetectable in the cell, yet in response to a wide variety of stresses such as elevated temperature, anoxia and starvation, both Hsp12 and Hsp26 are strongly induced (Praekelt and Meacock, 1990, Carmelo and Sa-Correia, 1997). In chapter 3, Western blotting showed a strong induction of Hsp12 and Hsp26 in response to glucose depletion. From this it may be assumed that the induction of these sHsps provides protection against the toxic effects of stress, therefore, when *HSP12* and *HSP26* are deleted from the genome the yeast becomes susceptible (Petko and Lindquist, 1986). Contrary to this hypothesis many groups have reported no observable defects in tolerance to stresses when either the *HSP12* or *HSP26* gene is disrupted (Petko and Lindquist, 1986, Praekelt and Meacock, 1990, Fu et al., 2012). Indeed, in this chapter, I have shown that Hsp12 and Hsp26 single and double mutants are not sensitive to a range of stresses. *HSP26* mutations have no detectable effects on thermotolerance, on fermentative or respiratory metabolism, on growth rates in rich and minimal media, on log phase or stationary phase or during sporulation or resistance to ethanol (Petko and Lindquist, 1986). Similar results are also reported for *HSP12*, with the exception of Welker et al., and Motshwene et al., who reported a drastic loss of viability in an *hsp12Δ* strain when heat shocked at 58 °C and when exposed to 5 mM H₂O₂, 0.4 M NaCl, 12 mM caffeine or 0.43 M Congo Red (Welker et al., 2010, Praekelt and Meacock, 1990, Motshwene et al., 2004). Repeating similar heat shock experiments to that published by Welker et al., 2010, did not reveal an increased sensitivity of *hsp12Δ* mutants to 58 °C (data not shown). Both Congo Red and caffeine are known to affect cell wall integrity, it would therefore be reasonable to assume that cells lacking *HSP12* may show an increased sensitivity to both drugs since Hsp12 has recently been shown to play a role in stabilising membranes during times of stress (Motshwene et al., 2004, Welker et al., 2010). Exposing *hsp12Δ* single and *hsp12/hsp26Δ* double mutants to media supplemented with 15 mM caffeine however, did not reveal any sensitivity to this stress. It is important to remember

that these studies were focused on single *hsp12Δ* or single *hsp26Δ* mutants therefore, the novel finding that the *hsp12/hsp26Δ* double mutant has a reduced RLS may be explained by an increased susceptibility as a combined effect of double deletion of *HSP12* and *HSP26*.

Drug stress assays to investigate the effects of common stresses (e.g. ethanol and variations in temperature), environmental stresses (e.g. NaCl and caffeine) and oxidative stresses (e.g. H₂O₂ and paraquat) revealed a slight reduction in growth on media supplemented with 13% ethanol for the BY4741 *hsp12/hsp26Δ* double mutant when compared to the wildtype. Hsp12 is reportedly induced in response to ethanol and is thought to protect the cell against this stress (Pacheco et al., 2010). However, drug stress assays showed no increased susceptibility of the *hsp12Δ* single mutant to 13% ethanol. The results from the drug stress assay also disagree with those reported by Welker et al., as the *hsp12Δ* single mutants showed no increased susceptibility to 5 mM H₂O₂ or 1 M NaCl. This result was reproduced in both the BY4741 and AEY1017 genetic backgrounds. Consistent with that reported for *HSP26*, the *hsp26Δ* single mutant in both genetic backgrounds showed no increased susceptibility to multiple stresses. With the exception of 13% ethanol, the *hsp12/hsp26Δ* double mutant did not show a general sickness to a variety of stresses. There is no literature available for the stress phenotypes of *hsp12/hsp26Δ* double mutants, however with the exception of Welker et al., 2010 and Pacheco et al., 2010 the findings in this study are consistent with that reported for *hsp12Δ* and *hsp26Δ* single mutants.

4.4.2 Double deletion of *HSP12* and *HSP26* does not affect telomeric or rDNA silencing.

Telomere shortening is a hallmark of normal ageing in humans, but not a hallmark of yeast ageing; instead yeast sub-telomeric genes are transcriptionally silenced (Lopez-Otin et al., 2013, Kim et al., 1996). The transcriptional status of genes in the sub-telomeric region has been implicated as an important senescence factor for yeast and *SIR* mutations result in telomere shortening and loss of positional effect variegation (PEV) of gene expression (Kaeberlein et al., 1999, Kim et al., 1996). A common theory for how DR mediates lifespan extension implicates

the activity of the Sir2 protein, which is required for silencing at the telomeres in a complex with Sir3 and Sir4 (Gottschling et al., 1990, Lin et al., 2000). Deletion of *SIR2* has been shown to shorten yeast lifespan and inhibit telomeric silencing (Kaeberlein et al., 1999, Riesen and Morgan, 2009). Since the *hsp12/hsp26Δ* double mutant had a reduced RLS with severe DR and is not characterised by a general sickness when exposed to stresses known to retard ageing, it was important to investigate if the ability of DR to activate Sir2 is lost in the *hsp12/hsp26Δ* double mutants. One way to measure Sir2 activity indirectly is by analysing transcriptional silencing at the telomeres and rDNA. Silencing assays using the telomeric *URA3* marker strain AEY1017, detected no telomeric phenotype for the *hsp12/hsp26Δ* double mutant. As expected the positive control, *sir2Δ* strain, showed increased growth on media lacking uracil and did not grow on media containing FOA, consistent with an inhibition of telomeric silencing. The negative control strain, *fab1Δ*, showed no difference in growth to that of the wildtype, as expected.

In addition to the telomeres, Sir2 is also essential for transcriptional silencing at the silent mating loci and at the rDNA (Rine and Herskowitz, 1987, Sinclair and Guarente, 1997). DR is thought to increase Sir2 activity leading to increased silencing at the rDNA and reducing recombination between rDNA repeats (Sinclair and Guarente, 1997). The reduction in rDNA recombination subsequently reduces the formation of extra-chromosomal rDNA circles, which would otherwise accumulate in the nucleus of the mother cell and cause ageing (Sinclair and Guarente, 1997). Deletion of *SIR2* not only inhibits telomeric silencing but also inhibits silencing at the rDNA (Riesen and Morgan, 2009).

A genome scale genetic interaction map has shown a negative genetic interaction between *HSP26* and establishment of silent chromatin (*ESC2*) and loss of rDNA silencing (*LRS4*) genes (Costanzo et al., 2010). *ESC2* encodes a protein (Esc2), which is required for silencing, and its overexpression has been shown to restore silencing in mutants lacking Sir1 protein function (Dhillon and Kamakaka, 2000). Moreover, loss of Esc2 protein function leads to the derepression of silencing at the mating locus and telomeres (Dhillon and Kamakaka, 2000). There are 11 different *LRS* genes encoding proteins that are thought to be positive regulators of rDNA silencing (Smith and Boeke, 1997). *LRS4* encodes a protein, which has been shown

to reside within the nucleolus where it participates in rDNA silencing (Rabitsch et al., 2003). Since DR was unable to extend the RLS of the *hsp26Δ* single mutant and double deletion of *HSP12* and *HSP26* resulted in a reduced RLS, it was important to investigate any rDNA silencing defects. Silencing assays using the rDNA *URA3* marker strain, JS128, showed no loss of rDNA silencing for the *hsp26Δ* single and *hsp12/hsp26Δ* double mutant. The *sir2Δ* and *fob1Δ* mutants showed a loss of rDNA silencing phenotype characterised by increased growth on media lacking uracil and reduced growth on media containing FOA, as expected (Riesen and Morgan, 2009). These experiments indicate that deletion of *HSP12* and *HSP26* does not affect Sir2 activity at the telomeres or rDNA.

4.4.3 The *hsp12/hsp26Δ* double mutant does not show increased protein aggregation.

Ageing is a complex process characterised by the accumulation of oxidized, misfolded or aggregated proteins, which have deleterious effects on cellular homeostasis (Gelino and Hansen, 2012). With replicative age, there is an accumulation of oxidatively damaged proteins, which are retained within the yeast mother cell by a Sir2-dependent process (Erjavec et al., 2007). Oxidatively damaged proteins are not inherited by daughter cells during cytokinesis, however in *Sir2Δ* mutants, damaged proteins are no longer retained in the mother cell and are inherited by newborn daughters (Aguilaniu et al., 2003). This suggests an important mechanism of the yeast cell for dealing with oxidative damage, which is vital for the fitness of new daughter cells (Aguilaniu et al., 2003). It may be that DR extends lifespan by affecting this Sir2 dependent mechanism, however there is no data available on this hypothesis.

Hsp26, a member of the sHsp family acts as a molecular chaperone and is able to bind to non-native proteins during stress conditions preventing the formation of aggregates (Haslbeck et al., 2004). In contrast, Hsp12 another member of the sHsp family has negligible anti-aggregation activity but instead acts as a membrane chaperone, stabilising membranes under stress conditions (Welker et al., 2010, Herbert et al., 2012). With this in mind, it is reasonable to assume that the *hsp12/hsp26Δ* double mutant may be more susceptible to proteotoxicity and show

increased insoluble protein aggregates in comparison to the wildtype. Since glucose depletion is regarded as a stress and both proteins are strongly induced in response to severe DR it may be that the double mutant also shows enhanced protein aggregation with 0.05% (w/v) glucose in comparison to 2% (w/v) glucose. Contrary to this hypothesis, aggregation assays did not suggest that the *hsp12/hsp26Δ* double mutant has increased levels of insoluble protein aggregates when compared to the wildtype. This result may be explained by the presence of other sHsps and Hsps, which are able to compensate for the loss of *HSP12* and *HSP26*. Hsp42, for example, is another cytosolic sHsp, which has a 90% overlap in its substrate proteins to that of Hsp26 (Haslbeck et al., 2004). Unlike Hsp26, which is induced in response to stresses, Hsp42 is active under physiological conditions (Haslbeck et al., 2004). Studies have reported increased aggregation of insoluble proteins in single *hsp26Δ* and *hsp42Δ* mutants which increases further in an *hsp26/42Δ* double mutant, suggesting that these proteins can compensate for one another (Haslbeck et al., 2004). Since the *hsp12/hsp26Δ* double mutant did not show increased levels of protein aggregation it could be that the presence of other chaperones such as Hsp104, Hsp90, Hsp70 and Hsp40 may also compensate for the absence of *HSP12* and *HSP26* (Glover and Lindquist, 1998, Burnie et al., 2006). Hsp104 for example, is the most crucial Hsp of *S. cerevisiae*, which enhances survival when exposed to extreme temperatures and high concentrations of ethanol (Glover and Lindquist, 1998, Sanchez et al., 1992, Sanchez and Lindquist, 1990). Hsp104 is able to refold denatured or aggregated proteins with the help of additional chaperones - Hsp70 and Hsp40 (Glover and Lindquist, 1998). In addition, Hsp90 is required for correct folding of difficult-to-fold proteins such as Swe1 and has a critical role as a chaperone when the yeast is grown on maltose as an alternative carbon source (Burnie et al., 2006, Bali et al., 2003).

An alternative interpretation of the aggregation results may be that Hsp26 is a more specific chaperone and less promiscuous than its cytosolic family member, Hsp42 (Haslbeck et al., 2004). Hsp26 substrates are thought to range from 10 to 100 kDa in size and have a pI range between 4 to 7 (Haslbeck et al., 2004). It may be that these substrates do not aggregate during the experimental conditions tested and therefore we do not see any apparent increase in protein aggregation for the

hsp12/hsp26Δ double mutant. It may also be that the *in vitro* aggregation assay utilised in this study is not very sensitive. Much more precise indicators of protein aggregation can be achieved by using sophisticated *in vivo* chaperone assays such as protein firefly luciferase fused to green fluorescent protein (FFL-GFP) (Abrams and Morano, 2013). The FFL protein is extremely sensitive to stress-induced mis-folding and aggregation, from which the luciferase activity can be monitored by an enzymatic assay (Abrams and Morano, 2013). In addition, GFP labeling of the FFL protein allows visualization of aggregation or solubility by microscopy (Abrams and Morano, 2013). Therefore using the FFL-GFP method may show that the *hsp12/hsp26Δ* double mutant does have increased insoluble protein aggregates in comparison to the wildtype. In addition to performing FFL-GFP assays it would also be important to analyse the asymmetry of damaged proteins in the mother and daughter cells of the *hsp12/hsp26Δ* double mutants. It may be that in the *hsp12/hsp26Δ* double mutant damaged proteins are inherited by the daughter cells leading to a reduced RLS. To investigate this hypothesis, old yeast cells would need to be obtained; this could be achieved by biotin-streptavidin magnetic sorting (Wang et al., 1992). Oxidized proteins could then be analysed by *in situ* immunofluorescence of carbonylated proteins, by analysing mitochondrial structure by DiOC₆ staining and by detecting the presence of ROS by dihydroethidium (DHE) (Aguilaniu et al., 2003). Future work will include both of these experiments to help understand the roles of *HSP12* and *HSP26* in DR mediated lifespan extension.

4.4.4 The *hsp12/hsp26Δ* double mutant does not show a reduction in vacuolar acidity or any alterations in mitochondrial structure.

The *hsp12/hsp26Δ* double mutant is not defective in various processes associated with yeast ageing, including stress resistance, telomeric silencing, rDNA silencing or protein aggregation. It was therefore important to investigate other ageing theories to understand the longevity effect of *HSP12* and *HSP26*. The mitochondrial free radical theory of ageing (MFRTA) defined by Dr Harman, claims that ageing is the accumulation of free radicals and oxidative damage (Harman, 1956). This theory is supported by the fact that during ageing mutations accumulate in mtDNA and bioenergetic functions of mitochondria decline with age in humans

and animals (Wang et al., 2013). In yeast, mitochondria dysfunction is also exhibited with increased age and shares similar characteristics to that seen in metazoa, notably, mitochondrial fragmentation, loss of mtDNA and increased levels of ROS (Hughes and Gottschling, 2012). A recent study in yeast ageing has shown that mitochondrial dysfunction arises because of changes in vacuolar pH and that DR can promote lifespan extension by increasing vacuolar acidity (Hughes and Gottschling, 2012). Since mitochondrial dysfunction leads to increased ROS, which activates stress responsive genes to combat the oxidative cellular damage, it may be that the *hsp12/hsp26Δ* double mutant has increased levels of oxidative damage and/or a reduction in vacuolar acidity, which is contributing to its reduced lifespan (Merksamer et al., 2013).

Fluorescent microscopy revealed no obvious difference in vacuolar acidity between wildtype cells and *hsp12/hsp26Δ* double mutant cells grown in either 2% or 0.05% (w/v) glucose. These findings do not support the hypothesis that DR increases vacuolar acidification. In contrast to the findings by Hughes and Gottschling 2012, fluorescent microscopy did not show a difference in acidity between mother and daughter cells, therefore this study does not support the hypothesis that renewed lifespan of daughter cells is a result of the regeneration of acidic vacuoles. Furthermore, the *hsp12/hsp26Δ* double mutant did not show any alterations in mitochondrial structure from that of the wildtype. This study could not find any evidence to support the hypothesis that mitochondrial dysfunction can be suppressed by maintaining an acidic pH in the vacuoles. The vacuoles play an important role in protein homeostasis, as they are the destination of proteins targeted for degradation by autophagy (Tsuchiyama and Kennedy, 2012). DR has been shown to cause vacuolar fusion events, where the 4-5 vacuoles that are normally present when nutrients are abundant becomes one large vacuole (Tsuchiyama and Kennedy, 2012). Vacuolar fusion is thought to be important for yeast longevity as replicatively old yeast cells have alterations in vacuolar membranes. Furthermore, mutants defective in vacuolar fusion have shortened lifespans with DR (Tsuchiyama and Kennedy, 2012, Gebre et al., 2012). Fluorescent microscopy did not show any differences in vacuolar structure of the *hsp12/hsp26Δ* double mutant compared to the wildtype. This study could not find any observable

mitochondrial or vacuolar dysfunctions, which may help to explain the reduced RLS of the *hsp12/hsp26Δ* double mutant. The findings in this study may differ from those reported by Hughes and Gottschling 2012, as there were significant differences in the methods used. Notably, Hughes and Gottschling 2012 used the mother enrichment programme (MEP) to examine mother cells at different ages. Further work is therefore required using either the MEP or by magnetic sorting, so cells at different ages can be examined; this would allow a better comparison of results especially for mitochondrial dysfunction as this occurs later on in the yeast lifespan after a decline in vacuolar acidity (Sinclair et al., 1998, Hughes and Gottschling, 2012). In contrast, old yeast cells are not required for analysis of vacuolar acidification, as a decline in quinacrine staining is reported early on in the lifespan of the mother cell and seen after the production of only 2 daughter cells (Hughes and Gottschling, 2012).

4.4.5 Conclusion.

Experiments so far have revealed a genetic interaction between *HSP12* and *HSP26*, as *hsp12/hsp26Δ* double mutants have a strongly reduced mean and maximum RLS with severe DR. Despite this, the *hsp12/hsp26Δ* double mutant does not show an increased susceptibility to stresses or increased levels of insoluble protein aggregates. Furthermore, the double mutant does not show any defects in silencing at the telomeres or rDNA nor any observable defects in the structures of the mitochondria or vacuoles. To shed light on the mechanisms by which Hsp12 and Hsp26 affect longevity, synthetic genetic array (SGA) analysis was next performed to identify genetic interactions of *HSP12/HSP26* on a genome wide scale.

5.1 INTRODUCTION.

5.1.1 Findings so far.

In the previous chapters we have shown that Hsp12 and Hsp26 are up-regulated in response to DR at the protein level, consistent with published mRNA data. RLS analysis has shown that both *HSP12* and *HSP26* are essential for lifespan extension by DR, yet *hsp12/hsp26Δ* double mutants are not defective in various processes associated with yeast ageing including stress resistance, Sir2 activity or protein aggregation. In addition, fluorescent microscopy failed to reveal any differences in the vacuole acidity or mitochondrial structure of *hsp12/hsp26Δ* double mutants in comparison to the wildtype. To shed light on the mechanisms by which Hsp12 and Hsp26 affect longevity, we next employed an unbiased approach of synthetic genetic array (SGA) analysis to identify genetic interactions of *HSP12* and *HSP26* on a genome wide scale.

5.1.2 Introduction to SGA analysis.

SGA analysis was first developed by Tong et al., 2001 (Tong et al., 2001). For SGA analysis a query strain is crossed to an array of ~5000 deletion mutants and the resultant double mutants are analysed for synthetic sick/lethal phenotypes (Tong et al., 2001). A primary goal of SGA analysis is to understand the cellular gene function and biological pathway in molecular detail (Vizeacoumar et al., 2009). *Saccharomyces cerevisiae* is a simple and genetically tractable eukaryotic system, which allows synthetic genetic interactions to be identified by introducing a second-site mutation, which then suppresses or enhances an original mutant phenotype (Tong, 2007b, Vizeacoumar et al., 2009). A synthetic lethal phenotype occurs between two single mutants, which are otherwise viable however, when combined to form a double mutant result in a lethal phenotype (Hartman et al., 2001, Kaelin, 2005). Synthetic lethality often indicates that the gene products impact on the same essential function, and are able to compensate for one another (Tong, 2007b). In fact, of the ~6000 genes of the yeast genome only ~1000 are essential for life, indicating that many pathways of eukaryotic cells buffer one another, thereby enabling survival during environmental and genetic perturbations (Hartman et al.,

2001, Davierwala et al., 2005). Interestingly, mammalian genomes also contain roughly the same proportion of essential genes as yeast (Davierwala et al., 2005).

Genetic screening used previously in yeast has identified genes involved in morphogenesis and bud emergence, cell polarity, secretion, vacuole protein transport and genes involved in DNA repair and meiosis (Bender and Pringle, 1991, Wang and Bretscher, 1997, Chen and Graham, 1998, Mullen et al., 2001, Tong et al., 2001). More recently, SGA analysis has been used to construct an interaction map of 5.4 million gene-gene pairs generating quantitative genetic interaction profiles for ~75% of all genes in yeast (Costanzo et al., 2010). SGA analysis has also been combined with high content screening (HCS), which uses automated microscopes combined with digital imaging to evaluate biological changes in response to environmental and genetic perturbations (Vizeacoumar et al., 2009). Combining SGA analysis and HCS enables monitoring of pathways by a fluorescent reporter to be assessed quantitatively, and has been used to dissect the function of the kinetochore protein Mcm21 (Vizeacoumar et al., 2010). With relation to human disease, the ability to integrate genetic and chemical-genetic perturbation data allows identification of genetic interaction hubs during chemical perturbations (Costanzo et al., 2010). In addition, SGA analysis can be used to design synthetic lethal therapies for cancer treatment and for understanding drug synergy.

Large-scale genetic screening provides a powerful tool for understanding the link between genotype and phenotype (Costanzo et al., 2010). With this in mind, SGA analysis was employed to provide a global view of functional relationships for *HSP12* and *HSP26* between genes and pathways on a genome-wide level, thereby helping us to understand the longevity roles in which these proteins act.

5.2 METHODS.

SGA analysis was performed in collaboration with Professor David Lydall at Newcastle University. Construction of the *hsp12Δ* and *hsp26Δ* query strains and confirmation by PCR and RSA was performed at the University of Liverpool. Once confirmed, query strains were sent to Newcastle University. SGA experiments on the *hsp12Δ* and *hsp26Δ* query strains were performed at Newcastle University and the resulting data sets were sent to the University of Liverpool for analysis. Analysis of all the SGA results was performed at the University of Liverpool. Spot tests to re-confirm synthetic genetic interactions were performed at the University of Liverpool.

Methods were generally performed as described in chapter 2, additional details are described below.

5.2.1 Interpretation of SGA results.

Synthetic genetic interactions were identified using a Colonyzer image analysis tool, which allows automated quantification of microorganism growth characteristics on solid agar (Lawless et al., 2010). Differences in growth between the query strain (*hsp26Δ/double* mutants or *hsp12Δ/double* mutants) and control strain (single mutants) were determined by calculation of a genetic interaction (GI) score. The mean and standard deviation of growth was determined and a *t*-test calculated. Genes on the same chromosome as the query strain (chromosome 2 for *HSP26* (YBRO72W) and chromosome 6 for *HSP12* (YFL014W)) were excluded from the study to reduce the generation of false positive results. Statistically significant negative interactions were classified according to the following criteria; genes which fall outside one standard deviation of the mean GIS, and have a Q value of <0.05. For SGA with stress conditions two types of data were collected from two different experiments. The control experiment compared the fitness of the query strain (*hsp26Δ/double* mutant or *hsp12Δ/double* mutant) on media supplemented with different stresses to the fitness of the query strain (*hsp26Δ/double* mutant or *hsp12Δ/double* mutant) on control media (SGA final media) incubated with standard conditions (30 °C). The data collected from this experiment was termed

control data and allowed us to determine the effect of the different stresses on the fitness of the query strain. The second experiment compared the growth of the query strain to the corresponding single mutants (control strain) on identical media. This data was termed matched data and allowed us to determine when a synthetic sick/lethal interaction was a result of a single mutation. Statistically significant negative interactions were identified as those genes that fall outside one standard deviation of the mean GIS and have a Q value <0.05 for both the control data and matched data. The GO terms of the genes identified by SGA analysis were found on the *Saccharomyces* genome database using GO Slim Mapper.

5.2.2. Construction of knockout strains by replica plating.

Double knockout mutants were made by replica plating as described in chapter 2. Single mutants from the genome deletion collection carrying a gene deletion linked to a KANMX deletion cassette were obtained from Professor David Lydall. Single mutants from the genome deletion were mated with the query strains (*hsp26Δ* or *hsp12Δ*) where the gene deletion is linked to a NATMX deletion cassette. The mating culture was inoculated onto media containing both clonNAT and G418 to select for diploids. Diploids were then replica plated onto enriched sporulation medium to allow the formation of haploid meiotic progeny. Haploids of *MATa* were next selected by replica plating onto SD + MSG-HIS-ARG-LYS + canavanine + thialysine media and incubated at 30 °C for 2 days. To select for *MATa* meiotic progeny carrying the *kan* resistant marker, haploids were next replica plated onto SD + MSG-HIS-ARG-LYS + canavanine + thialysine + G418. The *MATa* meiotic progeny carrying the *kan* resistant marker were then pinned onto SD + MSG-HIS-ARG-LYS-LEU + canavanine + thialysine + G418 + Hygromycin B and incubated for 2 days at 30 °C. Double mutants were finally selected by replica plating onto SGA final media as follows; SD + MSG-HIS-ARG-LYS-LEU + canavanine + thialysine + G418 + Hygromycin B + clonNAT to select for *MATa* meiotic progeny carrying both *kan* resistant and *nat* resistant markers.

5.2.3 Spot tests.

To confirm synthetic sick phenotypes identified by SGA analysis, double mutants were constructed by replica plating. Once constructed double mutants were grown overnight and assays prepared as detailed for qualitative silencing assays and stress assays. Double mutants were replica plated onto SGA final media (SD + MSG-HIS-ARG-LYS-LEU + canavanine + thialysine + G418 + Hygromycin B + clonNAT), YPD and minimal media (1.7 g yeast nitrogen base without amino acids and ammonium sulphate, 5 g ammonium sulphate, 20 g dextrose, 2 g synthetic complete, 1 L ddH₂O) and grown at 30 °C and 37 °C.

5.3 RESULTS

5.3.1 Construction of query strains for synthetic genetic array analysis.

The *hsp26Δ:NATMX* and *hsp12Δ:NATMX* query strains were constructed by PCR-mediated gene disruption. PCR using primers designed to amplify the NATMX deletion cassette from the PLD1222 plasmid DNA also contained flanking sequences homologous to the regions outside the target gene ORF (Figure 5.1B & C). The PCR amplified NATMX deletion cassettes were then transformed into competent yeast cells and the target gene (*HSP12* or *HSP26*) was replaced by homologous recombination. DNA was purified from successfully transformed colonies growing on media containing clonNAT and knockout mutants were confirmed by PCR amplification giving a band of the correct size for target gene replacement with the deletion cassette (Figure 5.1D & E).

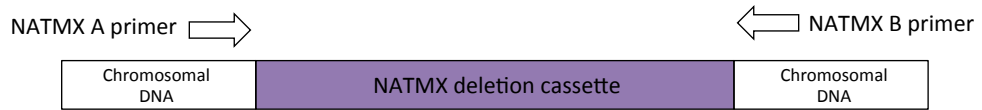
5.3.2 Confirmation of query strains by random spore analysis (RSA).

In addition to PCR confirmation of query strains, RSA was also performed as an additional confirmation that the NATMX deletion cassette had only integrated at the intended genome locus. The query strains were mated with 9 tester strains from the KANMX deletion collection. RSA is based on the theory of Mendelian segregation, therefore when the query strain is mated with unlinked genes on different chromosomes to the target gene, 25% will form double mutants. However, when the query strain is mated with the same target gene or linked ORFs close to the target gene no or very few double mutants will be formed. When the *hsp26Δ:NATMX* (chromosome 2, YBRO72W) query strain was mated with the *vps9Δ:KANMX* (chromosome 13, YML097C) unlinked gene, 29.6% double mutants were formed, as expected (Figure 5.2B). Similarly, when the *hsp12Δ:NATMX* (chromosome 6, YFL014W) query strain was mated with the unlinked gene, *rad9Δ:KANMX*, (chromosome 4, YDR217C) there were 30% double mutants formed (Figure 5.2C). However no double mutants were formed when the query strains were mated with the same genes from the KANMX deletion collection (Figure 5.2B & C). For example, when the *hsp26Δ:NATMX* (chromosome 2, YBRO72W) query strain was mated with the *hsp26Δ:KANMX* (chromosome 2, YBRO72W) linked gene no double mutants were formed. Similarly, when the *hsp12Δ:NATMX* (chromosome

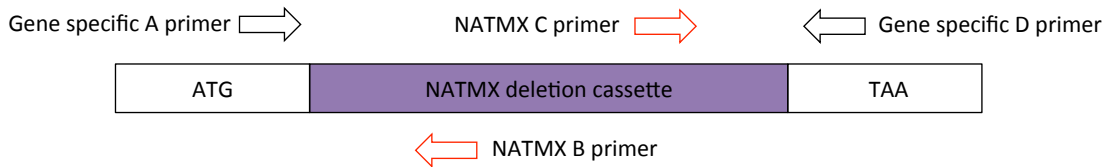
6, YFL014W) was mated with the *hsp12Δ*:KANMX (chromosome 6, YFL014W) linked gene no double mutants were formed. When the query strain was mated with linked ORFs close to the target gene no or very few double mutants were formed. For example, when the *hsp26Δ*:NATMX (chromosome 2, YBRO72W) query strain was mated with the YBRO74W (chromosome 2) strain, which is 2 genes away from *HSP26*, very few colonies were formed (6.9%). Likewise, when the *hsp12Δ*:NATMX (chromosome 6, YFL014W) query strain was mated with the YFL013C (chromosome 6) strain, which is 2 genes away from *HSP12*, very few colonies were formed (0.5%). RSA therefore confirmed both query strains had been constructed correctly.

A

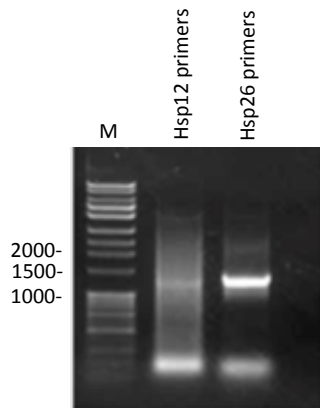
1. PCR amplification of the NATMX deletion cassette



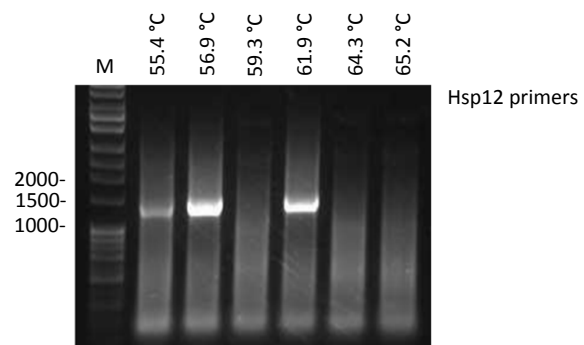
2. Confirmation of successful knockout using primers



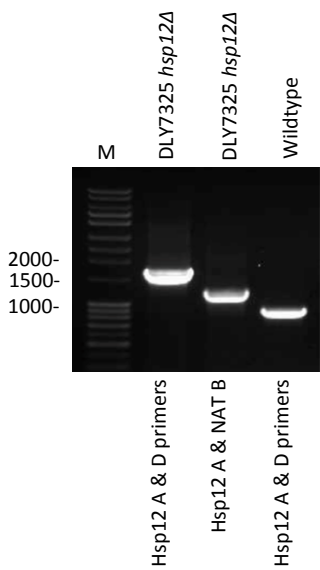
B



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D



E

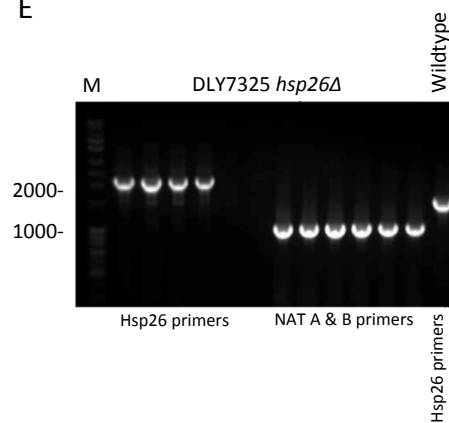


Figure 5.1 Amplification of NATMX deletion cassette from PDL1222 plasmid DNA. A) Schematic showing PCR amplification of the NATMX deletion cassette from PDL1222 plasmid DNA using NATMX forward (A) and reverse (D) primers, which anneal either side of the deletion cassette (step 1). The NATMX forward and reverse primers are also designed to have flanking sequences homologous to the sequences either side of the targeted ORF. Confirmation of successful knockout mutants is confirmed by PCR using gene specific primers (A and D) which anneal either side of the ORF or within the deletion cassette using NATMX B and C primers (step 2). B) PCR with Hsp12 primers (NATMX Hsp12 FWD CGATAATCTCAAACAAACAACACTCAAACAAAAAAAAAACTAATACAAC ACCAGCTGAAGCTTCGTACGC) (NATMX Hsp12 REV CATCATAAAGAAAAACCATGTAAC TACAAAG AGTTCCGAAAGATGCATAGGCCACTAGTGGATCTG) giving no band (lane 1). PCR with Hsp26 primers (NATMX Hsp26 FWD CAGGTATCCAAAAAGCAAACAACAACACTAAACAATTAACCCAGCTGAAGC TTCGTACGC) (NATMX Hsp26 REV CAAGCAACAATGGTCTCGCGAGAGGGACAACACTATGAGCCAGGTC ACTGCATAGGCCACTAGTGGATCTG) giving a band ~ 1300 bp (lane 2). C) PCR amplification (with temperature gradient) of NATMX deletion cassette from PDL1222 plasmid DNA using primers with flanking sequences for *HSP12*. D) PCR confirmation of *hsp12Δ:NATMX* query strain. The *hsp12Δ:NATMX* knockout mutant gives a band at ~ 1850 bps with Hsp12 A and D primers, which anneal outside of the NATMX deletion cassette in the genomic region (lane 1). The wildtype strain gives a band at ~ 800 bp (lane 3). E) PCR confirmation of *hsp26Δ:NATMX* query strain. The *hsp26Δ:NATMX* knockout mutant gives a band ~ 2 Kb with Hsp26 A and D new primers which anneal outside of the NATMX deletion cassette in the genomic region (lanes 1-4). The *hsp26Δ:NATMX* query strain gives a band ~ 1 Kb with NAT A and B primers, NAT A anneals at the beginning of the NATMX deletion cassette and NAT B anneals inside the NAT cassette (lanes 5-10). The wildtype strain gives a band at ~ 1600 bps (lane 11).

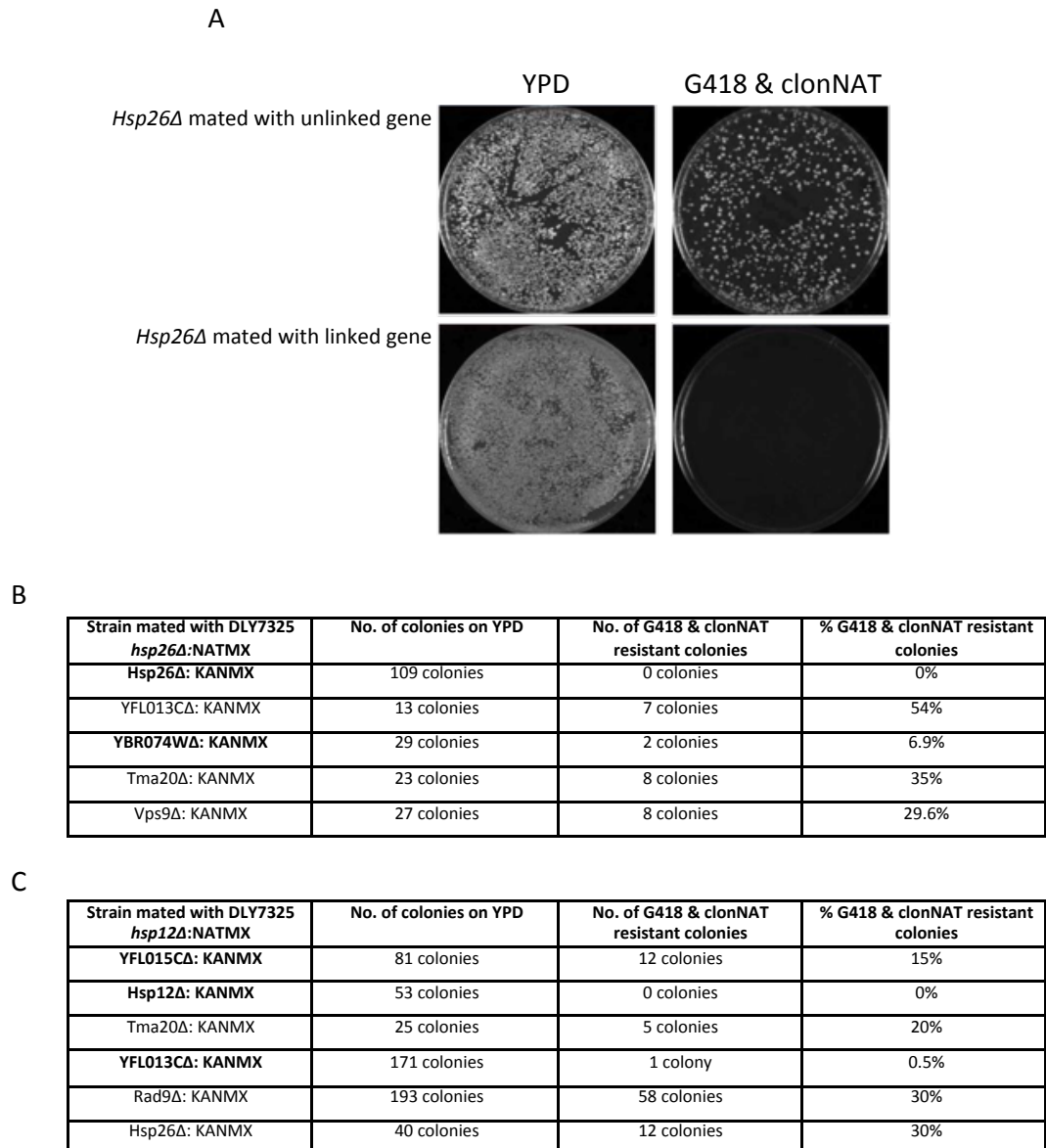


Figure 5.2 Random Spore Analysis of *hsp26Δ*:NATMX and *hsp12Δ*:NATMX query strain. A) Examples from RSA, when the *hsp26Δ*:NATMX query strain is mated with the *rad9Δ* tester strain from the KANMX deletion collection there are ~25% G418 and clonNAT resistant colonies compared to the corresponding YPD plate. When the *hsp26Δ*:NATMX query strain is mated with the *hsp26Δ* tester strain from the KANMX deletion collection there are no G418 and clonNAT resistant colonies. B) RSA results for the *hsp26Δ*:NATMX query strain, linked genes are highlighted in bold. C) RSA results for the *hsp12Δ*:NATMX query strain, linked genes are highlighted in bold.

5.3.3 Synthetic genetic array analysis of *HSP26* with standard conditions.

To shed light on the mechanisms by which Hsp12 and Hsp26 affect longevity, SGA analysis was performed to identify genetic interactions of *HSP12* and *HSP26* on a genome wide scale. SGA analysis of the *hsp26Δ* query strain was first performed with standard conditions of growth at 30°C and 2% (w/v) glucose. Synthetic genetic interactions were identified using a Colonyzer image analysis tool. The growth of each gene deletion mutant (control strain: DLY7388 *MATα lyp1::HPH::LEU2::NAT can1Δ::STE2pr-Sp_his5 his3Δ leu2Δ ura3Δ met15Δ LYS+* mated onto the KANMX deletion collection) was compared to the growth of each gene deletion in combination with *hsp26Δ* (query strain). The control strain contains a NATMX deletion cassette, which enables it to grow on media containing clonNAT. The control strain is then mated onto the KANMX deletion cassette and is therefore able to grow on media containing both clonNAT and G418. There were four independently generated replicates of each strain and differences in growth between the query and control strain were determined by calculation of a genetic interaction (GI) score. The mean and standard deviation of growth was determined and a *t*-test calculated to generate *p* values for each strain (n=4). The false discovery rate (FDR) correction was applied to the *p* values to generate Q values for each strain. An epistasis plot was then generated and the mean fitness of each gene deletion mutant was plotted against the mean fitness of each gene deletion in combination with *hsp26Δ*. A solid grey line represented a line of best fit of the expected fitness of the control strain and any genes that deviate from the solid grey line represent a genetic interaction. Genes that have passed a statistical test for significance were coloured green for enhancers or red for suppressors. Genes that were not statistically significant are coloured grey and have a Q value > 0.05 (Figure 5.3A). Statistically significant interactions of *HSP26* with standard conditions were classified according to the following criteria; a GI score of <-0.5, a Q value of <0.05 and a visual difference in growth compared to the control strain.

The agar plates of the control strain containing four replicas of each single mutant and the agar plates of the query strain containing four replicas of each double mutant were then analysed to confirm genetic interactions identified by statistical data. Significant genetic interactions were confirmed when the query

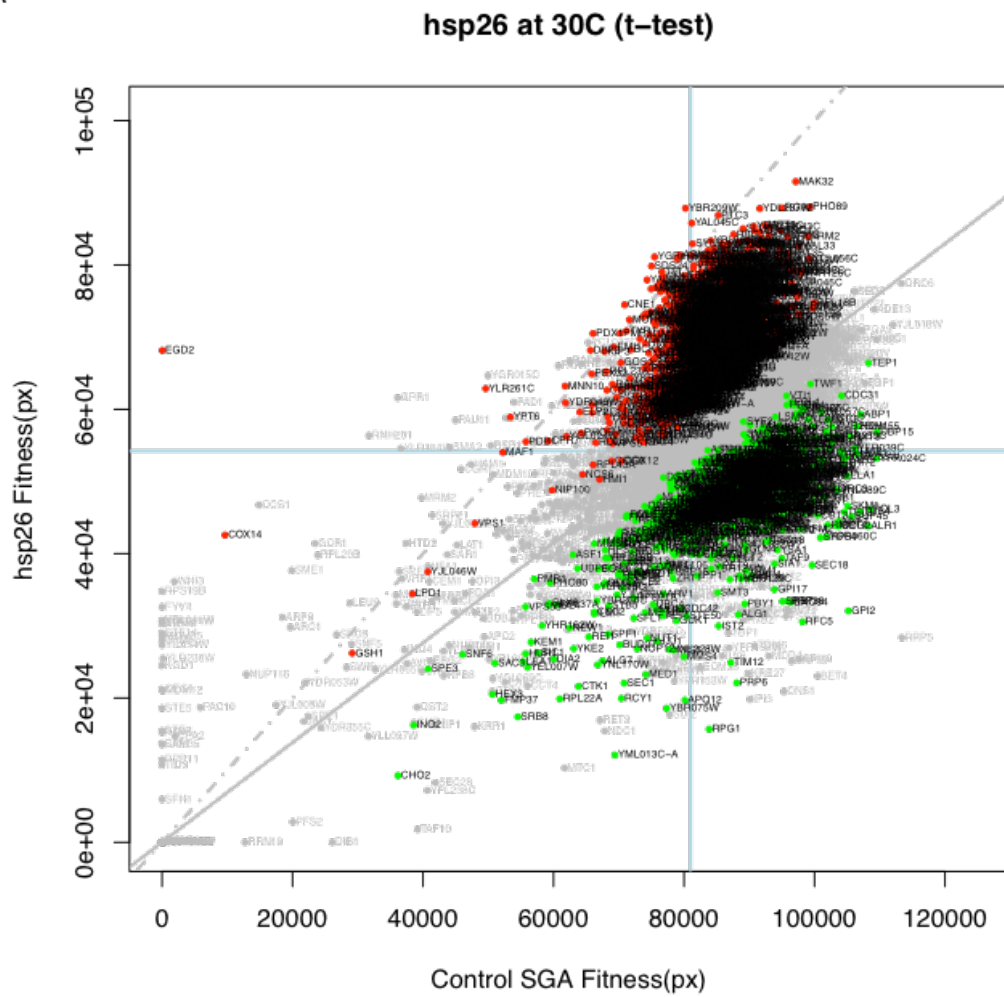
strain (double mutant) showed a reduction in growth in comparison to the control strain (single mutant). For example, in figure 5.3B, the red squares (red arrow) highlight a synthetic sick genetic interaction between *HSP26* and *RPL22A*. The query strain (*hsp26/rpl22aΔ* double mutant) shows a reduction in growth in comparison to the control strain (DLY7388 *rpl22aΔ* mutant). In contrast, genes highlighted by the black squares are non-significant genetic interactions as there is synthetic lethality for the control strain and the query strain. In figure 5.3b, the top black square (first arrow) highlights a lethal phenotype for the *esc2Δ* single mutant and is therefore not a result of double deletion with *HSP26*. The two remaining black squares (second and third arrows) highlight lethal phenotypes with the *ste11Δ* single mutant and *rsc2Δ* single mutants respectively. Like that seen for *esc2Δ*; lethality is not a result of double deletion with *HSP26*.

When analysing SGA data only negative genetic interactions were considered and analysed. Previous SGA analysis experiments have reported that virtually all (94%) of complexes characterised by negative genetic interactions contain at least one essential gene for a particular process (Baryshnikova et al., 2010). This observation suggests that essential complexes contain cellular buffering allowing the cell to survive a loss of a single non essential component of the complex (Baryshnikova et al., 2010). In contrast, only a relatively small fraction of complexes characterised by a positive genetic interaction contained an essential gene (Baryshnikova et al., 2010). In addition, negative genetic interactions are much easier to re-confirm by spot tests in comparison to positive genetic interactions.

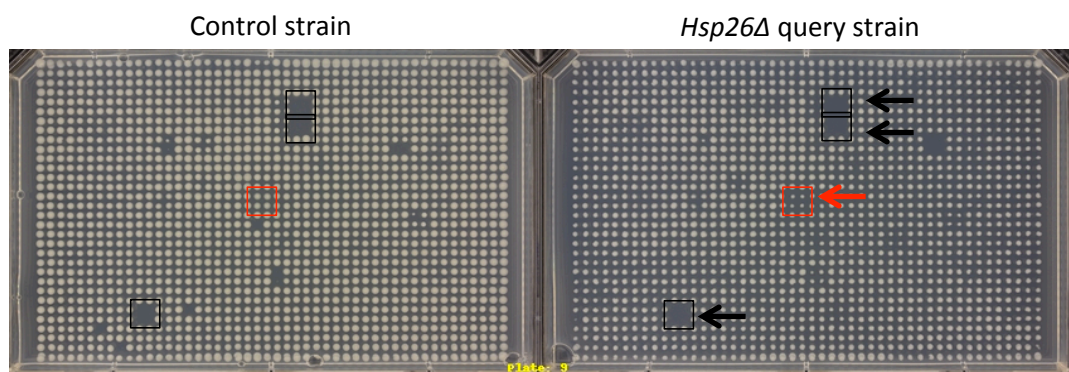
Under standard conditions (growth at 30 °C, 2% (w/v) glucose) 13 genes were identified as having a negative genetic interaction with *HSP26*, 3 of these; *NUT1*, *MED1* and *SRB8* are all associated with the Mediator complex (Figure 5.3 & table 5.4). In addition, comparing the frequency of GO terms for genes identified as significant interactors of *HSP26* with the corresponding genome frequency identified an over representation of genes involved in the regulation of transcription from RNA polymerase I and II. Consistent with this, there was a high frequency of genes involved in mRNA processing, DNA-dependent transcription elongation, regulation of translation and translation initiation. Not surprisingly, there was also a high frequency of genes associated with response to stresses such

as heat, osmotic stress, glucose limitation, response to chemical stimuli and pseudohyphal growth. There was an over representation of genes involved in endocytosis, endosomal transport, golgi vesicle transport and nuclear transport. The results also suggest Hsp26 may play a role in cytokinesis, mitosis and regulation of the cell cycle (Figure 5.5).

A



B



C

ORF	Gene	Q	GIS	QueryFitnessSummary	ControlFitnessSummary	Visual difference in growth
YCL032W	STE50	0.00104388	-27668.164	27559.47126	79222.92561	✓
YFLO23W	BUD27	0.00207843	-24141.897	25710.347	71512.03496	✓
YEL031W	SPF1	0.00455074	-21209.526	27434.08378	69778.27396	✓
YGL151W	NUT1	5.85E-05	-26394.84	26777.92575	76275.25649	✓
YPRO70W	MED1	0.0017998	-28401.782	25003.38395	76608.63068	✓
YCR081W	SRB8	0.00639938	-21650.83	15882.6616	53841.03383	✓
YML013C-A	YML013C-A	0.00087767	-37994.435	12866.94107	72959.61534	✓
YIL040W	APQ12	0.00114317	-36615.699	19015.25692	79801.48105	✓
YJL204C	RCY1	0.00054836	-31969.749	17727.95878	71290.35672	✓
YLR061W	RPL22A	1.58E-06	-24061.439	20745.23222	64274.26391	✓
YKL139W	CTK1	0.00168167	-24888.108	20797.54557	65535.14572	✓
YLR131C	ACE2	0.01958525	-13783.582	37843.52651	74058.04334	✓
YOR140W	SFL1	0.03528248	-14792.778	33576.87674	69385.29166	✓

Figure 5.3. SGA analysis of *hsp26Δ* with standard conditions. A) Epistasis plot, the mean fitness of each gene deletion mutant is plotted against the mean fitness of each gene deletion in combination with *hsp26Δ*. The solid grey line represents a line of best fit of the expected fitness of the control strain. Any genes that deviate from the solid grey line represents a genetic interaction, those genes that have passed a statistical test for significance are coloured green for enhancers or red for suppressors. B) Agar plates showing growth of control strain vs *hsp26Δ* query strain, a synthetic sick phenotype between *HSP26* and *RPL22A* is highlighted by the red square, there is a visual reduction in growth with the *hsp26/rpl22aΔ* double mutant in comparison to the control strain (*rpl22aΔ* single mutant). The black squares highlight *hsp26/esc2Δ*, *hsp26/ste11Δ* and *hsp26/rsc2Δ* double mutants, which are not significant as they show a lethal phenotype with the control strain. C) Table showing those genes identified as having a statistically significant genetic interaction with *HSP26*.

Gene	GO: Cellular	GO: Molecular	GO: Biological
STE50	Cytoplasm	Cellular response to heat, MAPK cascade, osmosensory signalling pathway, pheromone-dependent signal transduction	Protein kinase regulator activity, SAM domain binding
BUD27	Cytoplasm, colocalises with cytosolic ribosomes	Formation of translation preinitiation complex	Unknown
SPF1	Cis-Golgi network, endoplasmic reticulum membrane, integral to membrane, mitochondrion	Cellular calcium ion homeostasis, transmembrane transport	ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism
NUT1	Mediator complex, nucleus, mitochondrion	Regulation of transcription from RNA polymerase II promoter, transcription from RNA polymerase II promoter	Unknown
MED1	Core Mediator complex	Negative regulation of transcription from RNA polymerase II promoter, positive regulation of transcription from RNA polymerase II promoter	Unknown
SRB8	Mediator complex	Negative regulation of transcription from RNA polymerase II promoter, positive regulation of transcription by galactose, positive regulation of transcription from RNA polymerase II promoter	RNA polymerase II transcription coactivator activity
YML013C-A	Unknown	Unknown	Unknown
APQ12	Nuclear envelope, endoplasmic reticulum	Cellular lipid metabolic process, mRNA export from nucleus, nuclear envelope organisation	Unknown
RCY1	Endosome, Golgi apparatus, site of polarised growth	Early endosome to Golgi transport, endocytosis	SNARE binding
RPL22A	Cytosolic large ribosomal subunit	Cytoplasm translation	Structural constituent of ribosome
CTK1	Carboxy-terminal domain protein kinase complex, nucleolus, nucleoplasm, nucleus, colocalises with polysome	mRNA 3'-end processing, peptidyl-serine phosphorylation, phosphorylation of RNA polymerase II C-terminal domain, positive regulation of transcription elongation, DNA dependent, positive regulation of transcription from RNA polymerase I promoter, positive regulation of translational fidelity, protein phosphorylation	Cyclin-dependent protein kinase activity
ACE2	Cytosol, nucleus	Positive regulation of cytokinetic cell separation involved in cell cycle cytokinesis, regulation of transcription involved in G1 phase of mitotic cell cycle	Core promoter proximal region sequencing-specific DNA binding, RNA polymerase II core promoter proximal region sequence-specific DNA binding transcription factor activity, sequence-specific DNA binding
SFL1	Nucleus	Gene silencing, negative regulation of invasive growth in response to glucose limitation, negative regulation of pseudohyphal growth, negative regulation of transcription from RNA polymerase II promoter, positive regulation of transcription from RNA polymerase II promoter	RNA polymerase II core promoter proximal region sequence-specific DNA binding, RNA polymerase II repressing transcription factor binding, sequence specific transcription regulatory region DNA binding RNA polymerase II transcription factor recruiting transcription factor activity

Table 5.4. Table showing gene ontology (GO) terms for genes identified as having a significant genetic interaction with *HSP26*. Genes highlighted by red square are associated with the Mediator complex. Data from the *Saccharomyces* genome database.

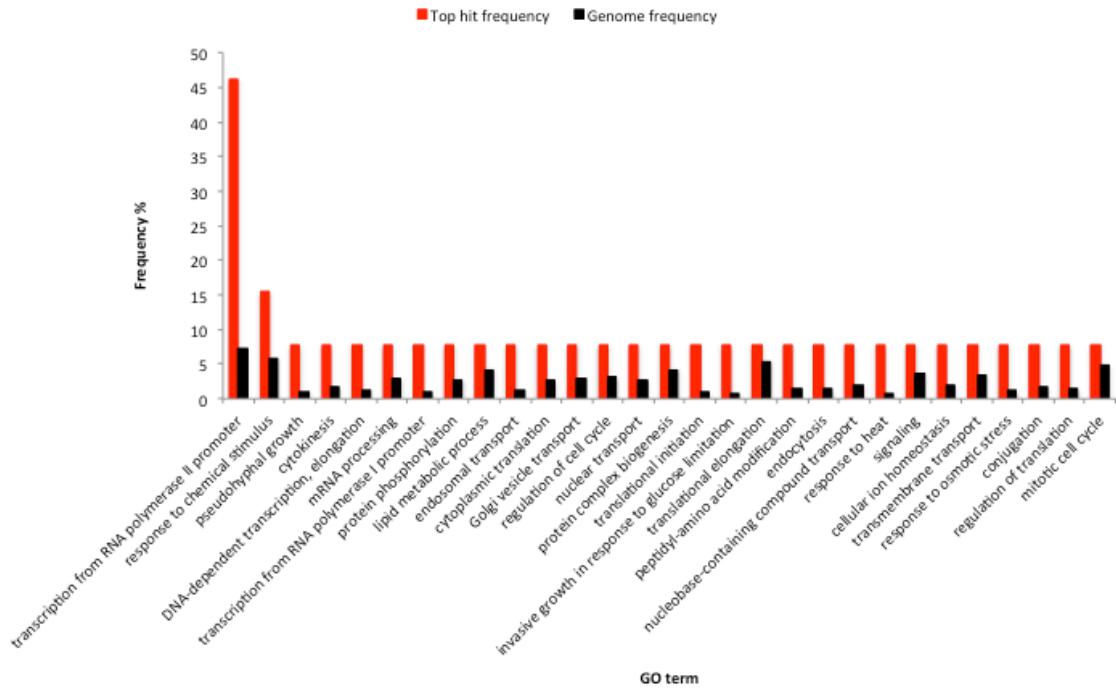


Figure 5.5 Histograms showing the GO terms of the genes identified by SGA analysis of *HSP26*. The GO term frequency of genes identified by *HSP26* SGA (red) was compared to that of the genome frequency (black). SGA analysis of *HSP26* with standard conditions showed an over representation of genes involved in transcription from RNA polymerase II and RNA polymerase I, mRNA processing, DNA dependent transcription elongation, regulation of translation and translation initiation. In addition, SGA analysis revealed an over representation of genes involved in cytokinesis, response to heat and osmotic stress and involved in invasive growth in response to glucose limitation and pseudohyphal growth. Data from the *Saccharomyces* genome database using GO Slim Mapper.

5.3.4 Confirmation of synthetic genetic interactions identified by SGA.

To confirm that those genes identified by SGA analysis were true negative genetic interactors of *HSP26*, 5 genes were selected and double mutants constructed by replica plating. *Rcy1Δ*, *bud27Δ*, *ctk1Δ*, *med1Δ* and *nut1Δ* single mutants from the KANMX deletion collection were obtained from David Lydall. Single mutants were mated with the *hsp26Δ* query strain and replica plating onto selective media, double mutants were finally selected on media lacking histidine and containing G418 and clonNAT. DNA was purified from double mutant colonies and mutants were confirmed by PCR with Hsp26 A and D original primers and gene specific primers (Appendix table 2 and table 3).

Spot tests were next performed on double mutants to reproduce the synthetic sick phenotypes identified by SGA analysis. The *hsp26/rad9Δ* double mutant made previously by RSA with the *hsp26Δ* query strain was used as a negative control as *RAD9* was not identified as having a significant genetic interaction with *HSP26*. Double mutants were plated onto SGA final media, YPD and minimal media and incubated at 30 °C and 37 °C. The fitness of the double mutants was compared to that of the corresponding single mutants, which were plated onto minimal media and incubated at the same temperatures. The single mutants were used as a control to confirm that synthetic sick phenotypes are a result of double mutation with *HSP26* and not a result of a single mutation. The *hsp26/rcy1Δ*, *hsp26/bud27Δ*, *hsp26/ctk1Δ* and *hsp26/med1Δ* double mutants show a slight reduction in growth in comparison to the *hsp26/rad9Δ* control on SGA final media when incubated at 30 °C (Figure 5.6B & C left panels). This reduction in growth becomes more obvious when incubated at 37 °C (Figure 5.6B right panel). However, the double mutants growing on YPD did not reproduce these findings (Figure 5.6C right panel). These results suggest that the genetic interaction for *HSP26* and *NUT1* identified by SGA analysis is not true. In addition, since the severe sickness phenotypes were specific to SGA final media and not reproduced on YPD media this suggests that the SGA final media, which is very selective, may be presenting an additional stress to the yeast.

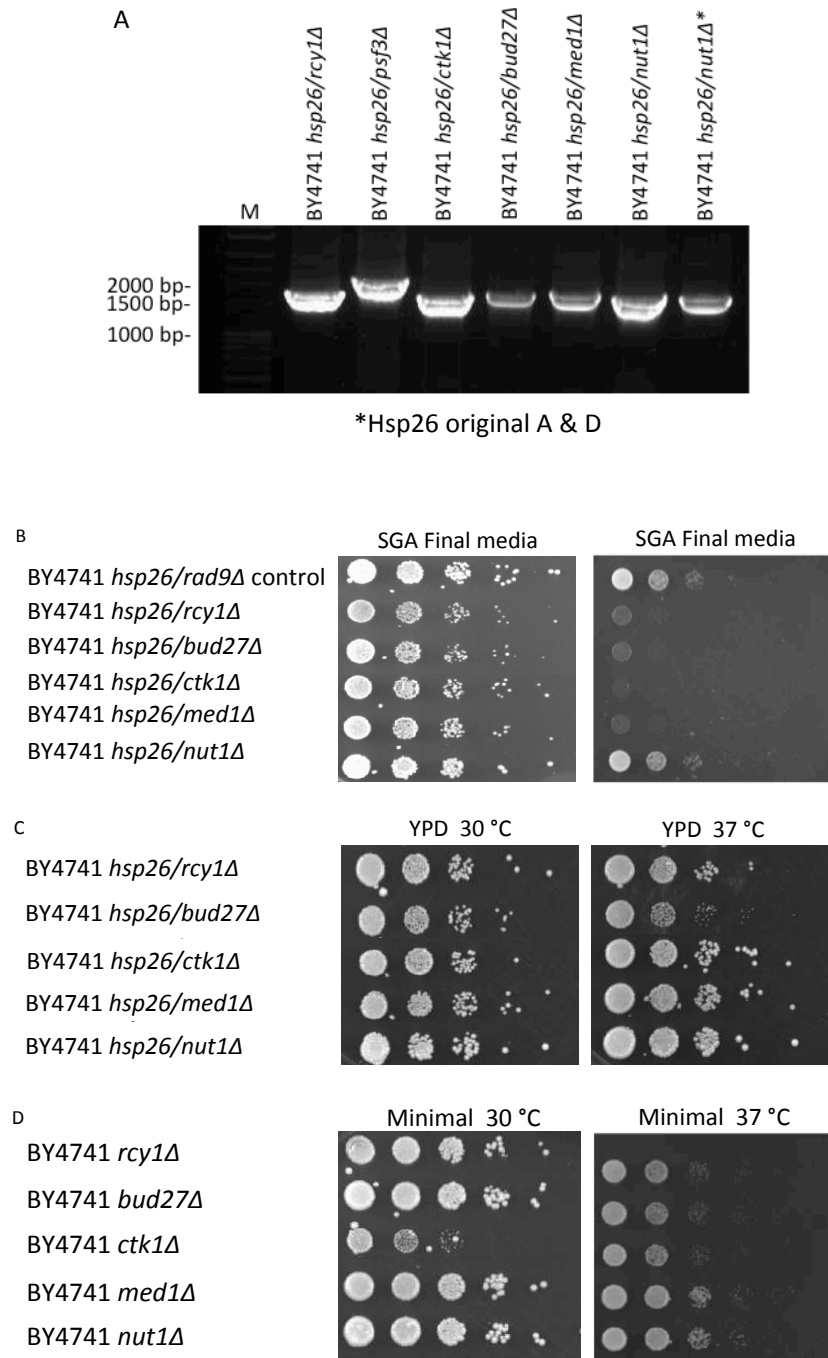


Figure 5.6 Spot tests to confirm synthetic genetic interactions identified by SGA analysis. A) PCR amplification with gene specific primers confirming double knockouts made by replica plating. Amplification of *RCY1Δ* ORF giving a band ~1688 bp (Lane 1), amplification of *BUD27Δ* ORF giving a band ~1730 bp (Lane 2), amplification of *CTK1Δ* ORF giving a band ~1500 bp (Lane 3), amplification of *MED1Δ* ORF giving a band ~1819 bp (Lane 4), amplification of *NUT1Δ* ORF giving a band ~1749 bp (Lane 5) and amplification of *HSP26Δ* ORF with Hsp26 A and D original primers giving a band ~1600 bp (Lane 6). B) Spot tests of double mutants grown on SGA final media, showing synthetic sick phenotypes for *hsp26/rcy1Δ*, *hsp26/bud27Δ*, *hsp26/ctk1Δ* and *hsp26/med1Δ* (right panel). C) Spot tests of double mutants on YPD media showing no synthetic sick phenotypes. D) Spot tests of single mutants on minimal media showing a slight reduction in growth for the *ctk1Δ* single mutant which is not seen when incubated at 37 °C.

5.3.5 Synthetic genetic array analysis of *HSP26* with glucose depletion.

One of the benefits of SGA analysis is that it can give insights into the cellular pathways in which a protein encoded by a particular gene may function. Since both Hsp12 and Hsp26 are not abundant in the cell under normal physiological conditions it was important to perform SGA analysis under stress conditions known to induce the expression of both proteins. Since microarray data and Western blotting has shown both Hsp12 and Hsp26 are up-regulated in response to glucose depletion, SGA analysis was next performed on media supplemented with 0.5% and 0.05% (w/v) glucose. For SGA with stress conditions two different types of experiments were performed. The first compared the fitness of *hsp26Δ/double* mutant strains (query strain) on each type of media (SGA final media plus stresses) to the fitness of the *hsp26Δ/double* mutant strains (query strain) on control media (SGA final media). The data collected from this experiment was termed control data and allowed us to determine the effect of the different stresses on the fitness of *hsp26Δ/double* mutants. The second experiment compared the growth of the *hsp26Δ/double* mutant strain (query strain) to the corresponding single mutant strains (control strain) grown on identical media. Data collected from this experiment was termed matched data and allowed us to identify when a synthetic sick/lethal phenotype was a result of a single mutation and not a genetic interaction with *HSP26*. The control data was analysed first, statistically significant genes were determined as those that fall outside one standard deviation of the mean GIS and have a Q value < 0.05. The matched data was next analysed for those genes identified from the control data, the final list of statistically significant genes was determined by those genes which fall outside one standard deviation of the mean GIS and have a Q value <0.05 in both the control and matched data sets. Since there was a huge amount of data to analyse it was impossible to confirm visual differences in growth for those genes identified as statistically significant.

SGA analysis with 0.5% (w/v) glucose showed similar results to that identified with standard conditions. When comparing the frequency of GO terms for genes identified by SGA to that of the corresponding genome frequency there was a high representation of genes associated with regulation of transcription from RNA polymerase II, DNA dependent transcription initiation and elongation and

regulation of translation. SGA analysis of *HSP26* with 0.5% (w/v) glucose also identified an over representation of genes associated with endocytosis, regulation of the cell cycle and genes involved in response to osmotic stress and starvation, similar to that identified with standard conditions (Figure 5.7).

With 0.05% (w/v) glucose, comparing the frequency of GO terms identified for *HSP26* to the genome frequency identified an over representation of genes associated with cytokinesis and chromosome segregation. SGA with 0.05% (w/v) glucose also revealed a high frequency of genes involved in mitochondrion organization, organelle inheritance and golgi vesicle transport, in addition to identifying a high frequency of genes involved in invasive growth in response to glucose limitation and pseudohyphal growth (Figure 5.8B & C).

A

ORF	Gene	P	Q	GIS	CONTROL DATA		MATCHED DATA	
					QueryFitnessSummary	ControlFitnessSummary	QueryFitnessSummary	ControlFitnessSummary
YIL031W	ULP2	3.45E-06	9.59E-05	-13130.924	42149.53073	99874.26052	42149.53073	55775.09949
YDR145W	TAF12	4.61E-08	4.86E-06	-12914.627	41685.45555	98645.04367	41685.45555	55187.08135
YFR044C	DUG1	3.65E-05	0.00048581	-11787.283	45578.99557	103642.6828	45578.99557	61235.81701
YFR032C	YFR032C	1.65E-09	4.74E-07	-11318.62	42207.51444	96704.76026	42207.51444	56612.35083
YGR134W	CAF130	2.80E-06	8.47E-05	-10968.871	41437.10975	94680.99685	41437.10975	55134.37524
YGR272C	YGR272C	0.00018237	0.00156069	-10642.342	40496.93774	92392.47076	40496.93774	54373.18666
YNL211C	YNL211C	5.46E-06	0.00013088	-10514.281	42598.92759	95958.73456	42598.92759	56519.11079
YGL195W	GCN1	7.75E-07	3.64E-05	-9962.1231	36035.39131	83102.92992	36035.39131	50760.65831
YPR075C	OPY2	1.10E-05	0.0002147	-9954.0835	41442.61737	92857.54861	41442.61737	57625.50347
YPR022C	YPR022C	0.00072672	0.00423908	-9566.4177	39831.45045	89246.29143	39831.45045	56322.48583
YDR444W	YDR444W	9.38E-05	0.00095399	-9536.9157	41510.65363	92226.77856	41510.65363	54991.66859
YFR022W	ROG3	2.08E-07	1.58E-05	-9239.1233	41374.67046	91443.08349	41374.67046	55397.89446
YGL108C	YGL108C	1.10E-05	0.0002147	-9118.1479	40737.30948	90073.00998	40737.30948	54473.95722
YNL328C	MDJ2	0.00050521	0.00327249	-8601.1387	43752.31074	94586.08997	43752.31074	58111.01888
YMR291W	YMR291W	0.00020187	0.00170377	-8550.3526	41354.09419	90161.51835	41354.09419	57039.24907
YPR079W	MRL1	1.13E-06	4.46E-05	-8437.9023	40805.1737	88966.63105	40805.1737	54233.7134
YDL056W	MBP1	0.00011404	0.00109446	-8340.8877	42276.25705	91449.13778	42276.25705	56150.60133
YOR128C	ADE2	0.00086705	0.00481913	-8129.6344	44779.80812	95590.59327	44779.80812	60348.91487

B

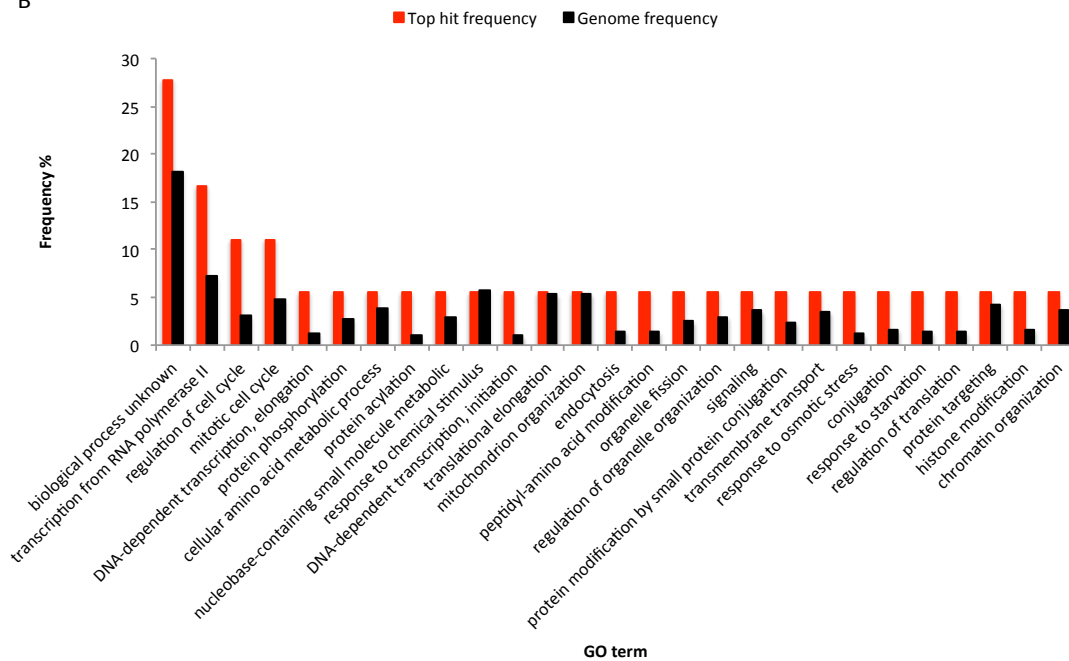


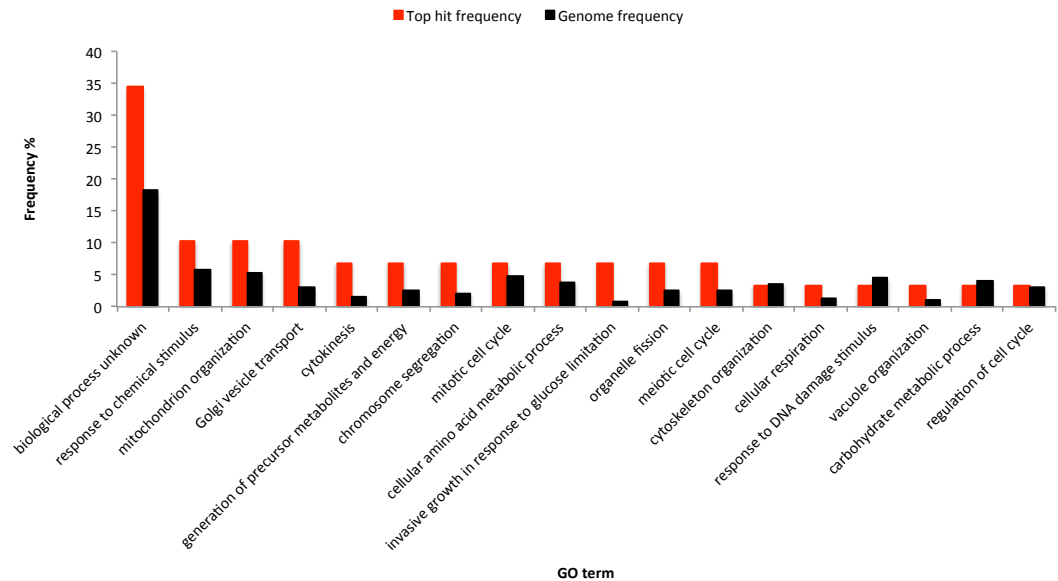
Figure 5.7 Significant genetic interactions of *HSP26* with 0.5% (w/v) glucose. A) Table of genes identified as statistically significant interactors of *HSP26*, falling outside one standard deviation of the mean GIS and with a Q value of < 0.05. B) Histogram showing the GO term frequency of genes identified by *HSP26* SGA analysis (from table 5.8a) with 0.5% (w/v) glucose (red) and the corresponding genome frequency (black). There is an over representation of genes identified by SGA analysis involved in regulation of transcription from RNA polymerase II, DNA dependent transcription initiation and elongation, regulation of translation, mitotic cell cycle, endocytosis and involved in response to starvation and osmotic stress. Data from the *Saccharomyces* genome database using GO Slim Mapper.

Chapter 5. An unbiased approach to identify genetic interactions of *HSP12/HSP26*

A

ORF	Gene	P	Q	GIS	CONTROL DATA		MATCHED DATA	
					QueryFitnessSummary	ControlFitnessSummary	QueryFitnessSummary	ControlFitnessSummary
YNR018W	YNR018W	1.18E-09	9.85E-08	-15855.521	9083.678876	88551.34109	9083.678876	19162.20815
YKL084W	HOT13	2.29E-07	3.85E-06	-13332.295	15912.81839	103840.3014	15912.81839	26895.53072
YGL141W	HUL5	1.26E-06	1.39E-05	-13172.898	14449.5968	98078.88723	14449.5968	23877.26676
YLL013C	PUF3	0.00305697	0.00711778	-13098.625	19143.76369	114482.6912	19143.76369	34523.21071
YER124C	DSE1	2.03E-08	6.65E-07	-12350.734	17253.85076	105116.6701	17253.85076	31251.82587
YOR351C	MEK1	7.13E-08	1.65E-06	-12291.674	15327.91599	98068.57166	15327.91599	28799.82121
YLR438W	CAR2	4.91E-13	4.23E-10	-12196.555	16272.24739	101083.8602	16272.24739	28061.89118
YGR209C	TRX2	4.12E-10	4.82E-08	-12143.351	14141.35502	93328.81383	14141.35502	24619.98565
YMR266W	RSN1	8.30E-05	0.00039305	-12142.598	14890.77945	95987.11235	14890.77945	26675.34191
YPL018W	CTF19	7.31E-06	5.67E-05	-12113.005	13299.32376	90231.2735	13299.32376	24712.48653
YJR135C	MCM22	1.58E-07	2.93E-06	-12098.948	17186.20714	103982.4767	17186.20714	27238.58531
YOR301W	RAY1	9.38E-10	8.08E-08	-12064.811	14289.0979	93574.53261	14289.0979	25892.74379
YER184C	YER184C	1.77E-09	1.34E-07	-12062.898	17828.00538	106133.3007	17828.00538	28633.73104
YLR436C	ECM30	4.79E-07	6.61E-06	-11988.821	13482.71927	90441.51497	13482.71927	23985.63567
YML056C	IMD4	1.04E-05	7.52E-05	-11854.306	14646.61386	94096.52336	14646.61386	24753.85549
YGR121C	MEP1	4.24E-07	6.07E-06	-11822.636	13752.89147	90810.74187	13752.89147	23404.1177
YFR044C	DUG1	6.77E-06	5.35E-05	-11753.14	17436.31776	103642.6828	17436.31776	26630.9833
YOR318C	YOR318C	1.45E-10	2.42E-08	-11732.21	16911.02572	101703.2197	16911.02572	28571.02459
YHR047C	AAP1	0.00016696	0.00067108	-11665.049	14166.91468	91721.26651	14166.91468	24856.81297
YNL044W	YIP3	5.71E-06	4.69E-05	-11659.082	13968.63037	90996.03581	13968.63037	25662.89197
YOR356W	YOR356W	2.08E-07	3.57E-06	-11386.17	16371.54231	98559.00035	16371.54231	27866.74274
YGL067W	NPY1	4.35E-05	0.00023425	-11212.483	15029.58735	93177.429	15029.58735	25208.78844
YMR132C	JLP2	1.50E-05	0.0001007	-11208.564	16685.09293	99041.69762	16685.09293	26946.32444
YPL229W	YPL229W	6.26E-05	0.00031604	-11116.056	14584.08281	91253.19912	14584.08281	26830.11143
YDR480W	DIG2	2.65E-08	7.82E-07	-10850.088	17480.64449	100593.6171	17480.64449	31293.01379
YPL108W	YPL108W	0.00084762	0.00246837	-10827.296	15923.41528	94983.45579	15923.41528	25722.40243
YMR158C-B	YMR158C-B	1.70E-06	1.75E-05	-10737.397	16532.04788	96825.31561	16532.04788	27399.84036
YPL121C	MEI5	1.73E-05	0.00011155	-10707.203	16496.7967	96592.93975	16496.7967	27506.2073
YPL199C	YPL199C	1.13E-08	4.48E-07	-10639.883	16207.58167	95326.99489	16207.58167	25883.18506

B



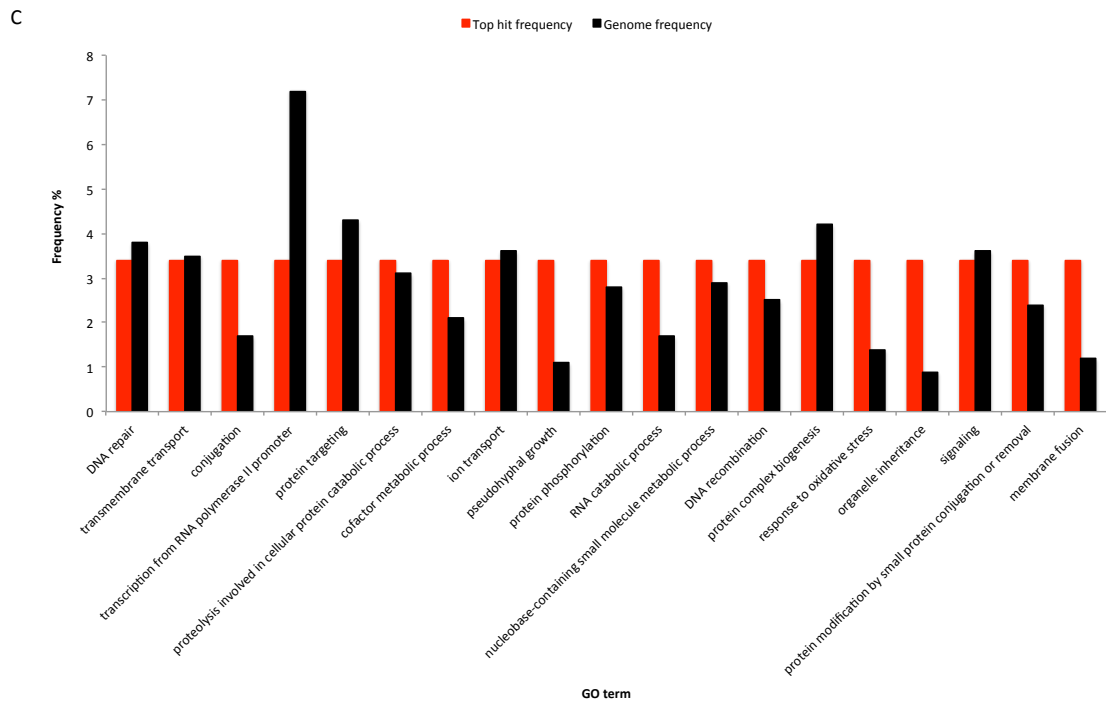


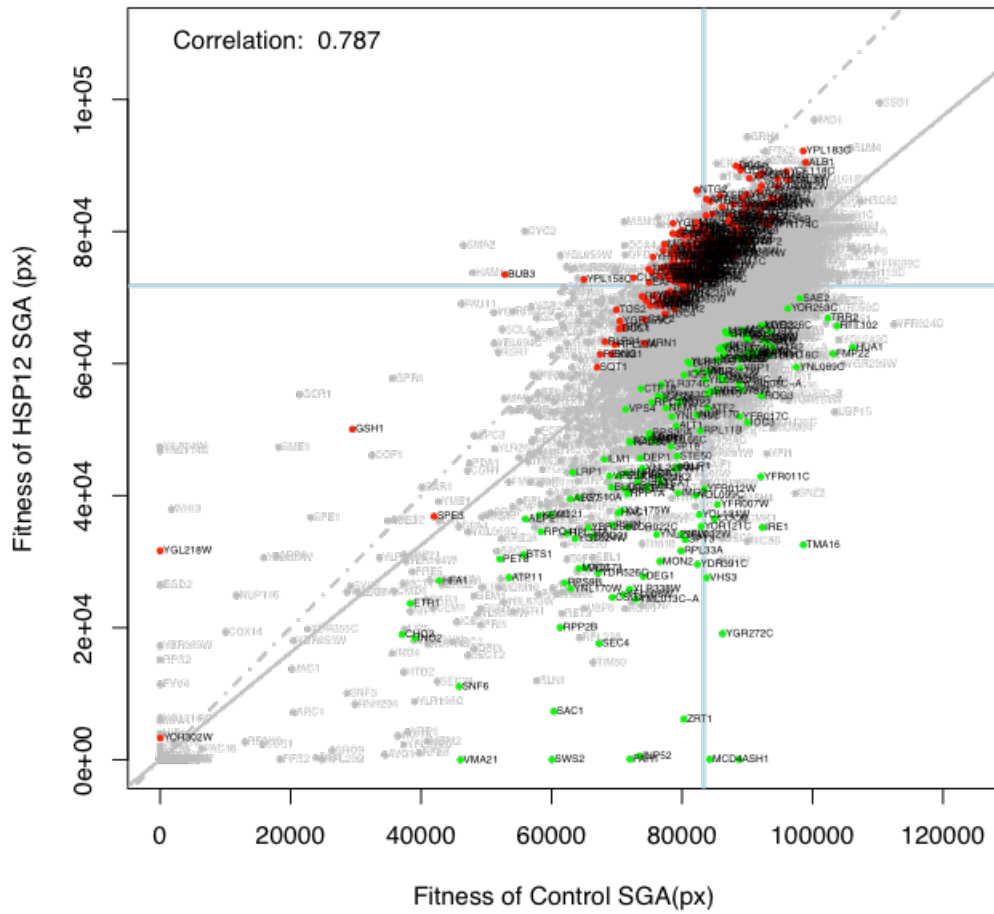
Figure 5.8 Significant genetic interactions of *HSP26* with 0.05% (w/v) glucose. A) Table of genes identified as statistically significant interactors of *HSP26*, falling outside one standard deviation of the mean GIS and with a Q value of < 0.05. B) C) Histograms showing the frequency of GO terms identified by SGA analysis of *HSP26* with 0.05% (w/v) glucose (red) and the corresponding genome frequency (black). SGA analysis with 0.05% (w/v) glucose revealed an over representation of genes involved in mitochondrion organization, golgi vesicle transport, organelle inheritance, cytokinesis, chromosome segregation, invasive growth to glucose limitation and pseudohyphal growth. Data from the *Saccharomyces* genome database using GO Slim Mapper.

5.3.6 Synthetic genetic array analysis of *HSP12* with standard conditions.

SGA analysis was next performed on the *hsp12Δ* query strain to help shed light on the roles *HSP12* plays on longevity. Significant negative genetic interactions were determined as described previously (genes that fall outside one standard deviation of the mean GIS and have a Q value of <0.05). Genes on the same chromosome as *HSP12* (chromosome 6) were excluded. With standard conditions, comparing the frequency of GO terms identified by SGA to the corresponding genome frequency identified an over representation of genes associated with lipid metabolic process, regulation of DNA metabolic process, protein lipidation and ion transport. In addition, there was a high frequency of genes involved in cell wall organization and biogenesis, membrane fusion, vacuole organization, cellular respiration and pseudohyphal growth (Figure 5.11). Comparing the genes identified for *HSP12* to those identified for *HSP26* with standard conditions revealed only one gene, *YMLO13C-A*, which was common to both. When comparing the GO terms of the genes identified for *HSP12* to those described for *HSP26*, this also showed some common GO terms, notably, transcription from RNA polymerase II and pseudohyphal growth (Figure 5.11).

A

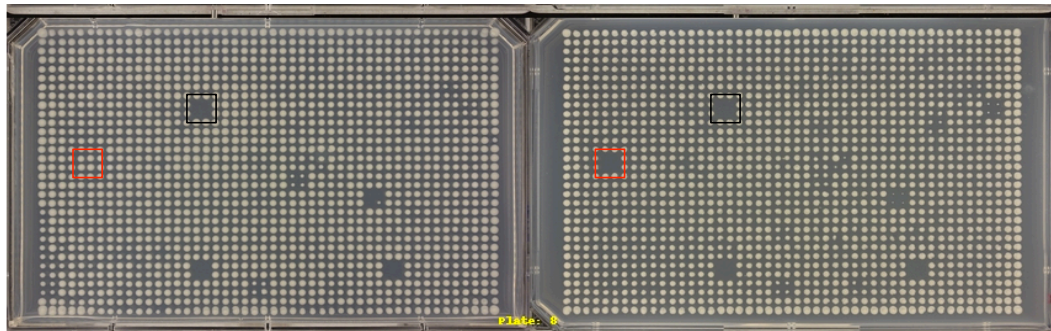
HSP12 SGA at 30C (t-test)



B

Control strain

Hsp12Δ query strain



C

ORF	Gene	P	Q	GIS	QueryFitnessSummary	ControlFitnessSummary	mean:	standard deviation:	lower limit:
YKL185W	ASH1	5.65E-05	0.00616637	-72065.754	37.51727889	88727.92103	-49733.023	12014.78764	-73762.599
YKL165C	MCD4	0.0014663	0.03395812	-68396.818	44.16561312	84221.2291			
YNL106C	INP52	9.84E-05	0.00928655	-59146.84	574.9133224	73491.63104			
YGL255W	ZRT1	6.01E-07	0.00043726	-59095.909	6149.563625	80288.93806			
YMR165C	PAH1	0.00049313	0.01820656	-58453.466	81.82408439	72031.60747			
YGR272C	YGR272C	4.20E-05	0.005625	-50957.29	19111.83724	86224.76978			
YNL081C	SWS2	7.20E-07	0.00045864	-48768.384	26.66752143	60045.58951			
YOR252W	TMA16	3.30E-05	0.00504602	-47578.818	32541.39836	98593.31019			
YKL212W	SAC1	0.00191173	0.03805317	-41709.558	7321.68192	60336.23542			
YOR054C	VHS3	0.00243561	0.04258009	-40494.107	27576.88876	83765.93509			
YHR079C	IRE1	1.25E-05	0.00335019	-39847.855	35194.78949	92345.01739			
YGR105W	VMA21	2.57E-05	0.00465696	-37427.578	0	46057.1495			
YDR391C	YDR391C	1.70E-05	0.00381616	-37343.083	29589.86914	82365.49557			
YML013C-A	YML013C-A	1.13E-05	0.00320657	-34976.868	24316.12685	72964.01432			

Figure 5.9 SGA analysis of *hsp12Δ* with standard conditions. A) Epistasis plot, the mean fitness of each gene deletion mutant is plotted against the mean fitness of each gene deletion in combination with *hsp12Δ*. The solid grey line represents a line of best fit of the expected fitness of the control strain. Any genes that deviate significantly from the solid grey line represents a genetic interaction, those genes that have passed a statistical test for significance are coloured green for enhancers or red for suppressors. B) Agar plates showing growth of control strain vs *hsp12Δ* query strain, a synthetic sick phenotype between *HSP12* and *ASH1* is highlighted by the red square, there is a visual reduction in growth with the *hsp12/ash1Δ* double mutant in comparison to the control strain. The black squares highlight genes, which are not significant as they show a synthetic lethal interaction for with the control strain. C) Table showing those genes identified as having a statistically significant genetic interaction with *HSP12*.

Gene	GO: Cellular	GO: Molecular	GO: Biological
ASH1	Cellular bud, nucleus	core promoter proximal region sequence-specific DNA binding, RNA polymerase II core promoter proximal region sequence-specific DNA binding transcription factor activity involved in negative regulation of transcription	negative regulation of G1/S transition of mitotic cell cycle by negative regulation of transcription from RNA polymerase II promoter, negative regulation of mating type switching by negative regulation of transcription from RNA polymerase II promoter, positive regulation of pseudohyphal growth by positive regulation of transcription from RNA polymerase II promoter
MCD4	Endoplasmic reticulum, fungal cell wall, fungal cell vacuole, integral to membrane	mannose-ethanolamine phosphotransferase activity	ATP transport, GPI anchor biosynthetic process
INP52	actin cortical patch, cytoplasm	phosphatidylinositol-3,5-bisphosphate 5-phosphatase activity, phosphatidylinositol-3-phosphatase activity, phosphatidylinositol-4,5-bisphosphate 5-phosphatase activity, phosphatidylinositol-4-phosphate phosphatase activity	phosphatidylinositol dephosphorylation
ZRT1	Integral to plasma membrane	high affinity zinc uptake transmembrane transporter activity	high-affinity zinc ion transport
PAH1	cytosol, extrinsic to membrane, nuclear membrane, vacuole, cytoplasm	phosphatidate phosphatase activity	aerobic respiration, lipid particle organization, phospholipid biosynthetic process, plasmid maintenance, vacuole fusion, non-autophagic
YGR272C	Unknown	Unknown	Unknown
SWS2	cytoplasm, mitochondrial small ribosomal subunit	structural constituent of ribosome	mitochondrial translation regulation of sporulation resulting in formation of a cellular spore
TMA16	colocalizes with ribosome, nucleus	Unknown	Unknown
SAC1	integral to endoplasmic reticulum membrane, integral to Golgi membrane, SPOTS complex, mitochondrial outer membrane, mitochondrion	phosphatidylinositol-3,5-bisphosphate 5-phosphatase activity, phosphatidylinositol-3-phosphatase activity, phosphatidylinositol-4-phosphate phosphatase activity	phosphatidylinositol dephosphorylation
VHS3	phosphopantothenoylcy steine decarboxylase complex	contributes to phosphopantothenoylcy steine decarboxylase activity, phosphopantothenoylcy steine decarboxylase activity, protein phosphatase inhibitor activity	cellular monovalent inorganic cation homeostasis, coenzyme A biosynthetic process
IRE1	endoplasmic reticulum, nucleus	endoribonuclease activity, protein homodimerization activity, protein serine/threonine kinase activity, unfolded protein binding, protein kinase activity	endoplasmic reticulum unfolded protein response, fungal-type cell wall organization, inositol metabolic process, protein phosphorylation, protein phosphorylation
VMA21	endoplasmic reticulum membrane	Unknown	vacuolar proton-transporting V-type ATPase complex assembly
YDR391C	cytoplasm, nucleus	Unknown	Unknown
YML013C-A	Unknown	Unknown	Unknown

Table 5.10 Table showing gene ontology (GO) terms for genes identified as having a significant genetic interaction with *HSP12*. Data from the *Saccharomyces* genome database.

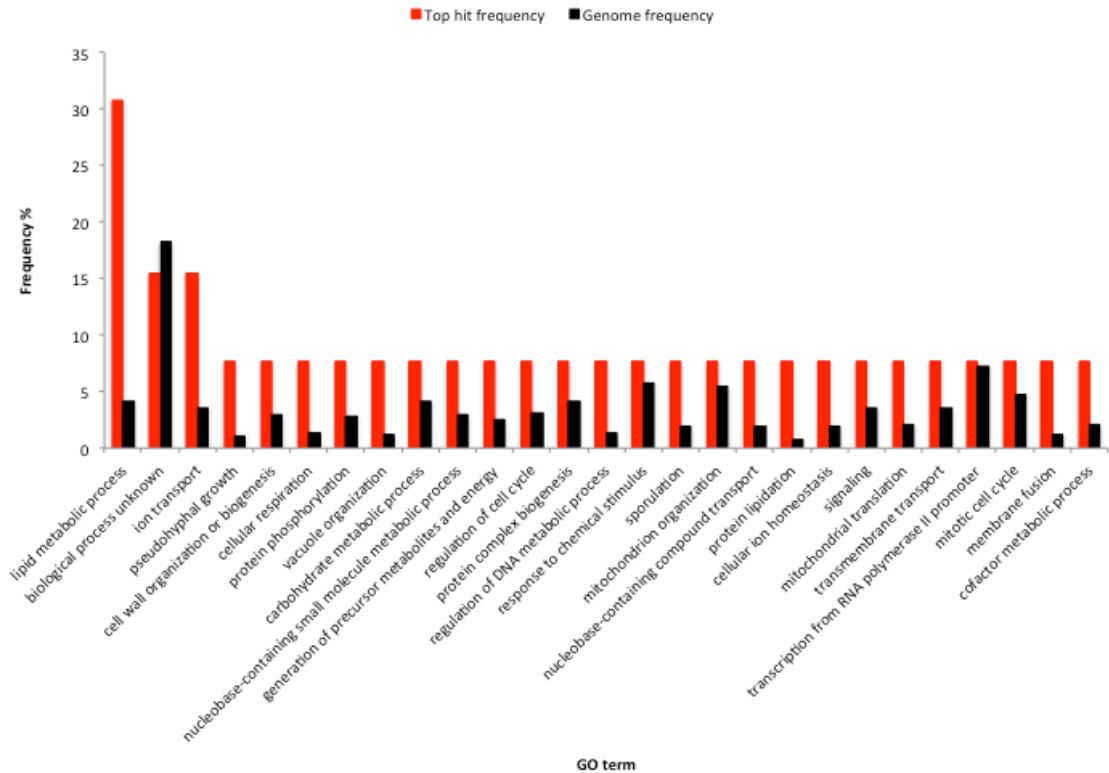


Figure 5.11 Histograms showing the GO terms of the genes identified by SGA analysis of *HSP12*. The GO term frequency of genes identified by *HSP12* SGA analysis (red) is compared to that of the genome frequency (black). SGA analysis of *HSP12* with standard conditions revealed an over representation of genes involved in lipid metabolic process, regulation of DNA dependent metabolic process, protein lipidation, ion transport, vacuole organisation, cellular respiration, cell wall organisation and biogenesis, membrane fusion and pseudohyphal growth. Data from the *Saccharomyces* genome database using GO Slim Mapper.

5.3.6 Synthetic genetic array analysis of *HSP12* with glucose depletion.

SGA analysis of *HSP12* was next performed on media supplemented with 0.5% and 0.05% (w/v) glucose. As described earlier, statistically significant genes were determined as those genes, which fall outside one standard deviation of the mean GIS and have a Q value of <0.05 in both the control and matched data sets.

SGA analysis with 0.5% (w/v) glucose identified an over representation of genes involved in cell wall organisation and biogenesis, protein phosphorylation, protein dephosphorylation, protein acylation, response to DNA damage stimulus, chemical stimulus, signalling, transmembrane transport, ion transport, pseudohyphal growth, invasive growth in response to glucose limitation, organelle inheritance, peptidyl amino acid modification and peroxisome organisation (Figure 5.12). With 0.05% (w/v) glucose, SGA analysis revealed an over representation of genes involved in peptidyl amino acid modification, protein alkylation, DNA dependent transcription termination, translation initiation, carbohydrate transport, organelle inheritance and protein maturation (Figure 5.13).

Like that reported for *HSP26*, genes involved in response to glucose limitation, chemical stimuli, DNA damage stimuli and pseudohyphal growth were also identified in SGA analysis of *HSP12*. In addition SGA analysis of *HSP12* identified genes involved in transcription from RNA polymerase II and regulation of cell cycle, suggesting that both Hsp12 and Hsp26 overlap in some processes (Figures 5.5, 5.8, 5.11 & 5.12).

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ORF	Gene	P	Q	GIS	CONTROL DATA		MATCHED DATA	
					QueryFitnessSummary	ControlFitnessSummary	QueryFitnessSummary	ControlFitnessSummary
YDR392W	SPT3	3.49E-05	0.00364846	-18412.496	15183.05991	67605.7969	15183.05991	38427.53458
YHR079C	IRE1	0.00104376	0.01473063	-16051.533	21207.6078	74978.18681	21207.6078	58304.39299
YGL255W	ZRT1	0.0006403	0.01136171	-14373.168	3188.653969	35340.41683	3188.653969	54839.63832
YKL081W	TEF4	0.00160807	0.01811881	-10728.723	15805.01304	53394.98998	15805.01304	43740.26075
YJL095W	BCK1	1.57E-05	0.00285493	-7987.5718	26713.71423	69830.90472	26713.71423	50874.66412
YIL090W	ICE2	0.00536682	0.03396761	-7986.6485	12268.28691	40759.88612	12268.28691	36065.45199
YLR214W	FRE1	8.40E-05	0.00475362	-6787.0589	29712.99643	73450.64631	29712.99643	54382.03658
YLR103C	CDC45	0.0005163	0.01019589	-6091.6738	32100.70359	76856.1795	32100.70359	58073.45821
YBL046W	PSY4	0.00032355	0.00779306	-5178.4233	32017.39219	74850.75465	32017.39219	58161.59317

B

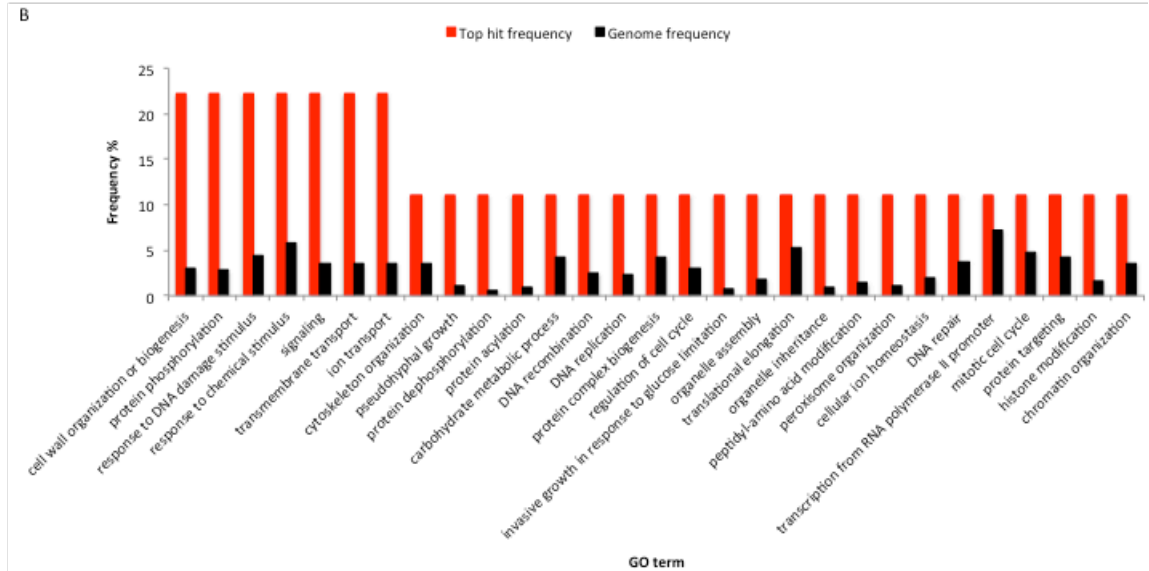


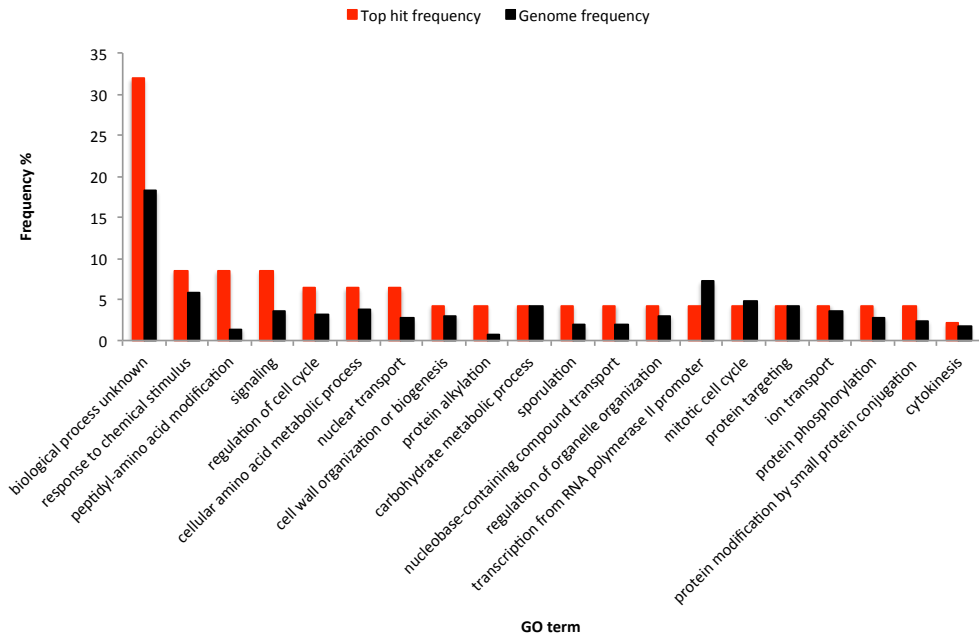
Figure 5.12 Significant genetic interactions of *HSP12* with 0.5% (w/v) glucose. A) Table of genes identified as statistically significant interactors of *HSP12*, falling outside one standard deviation of the mean GIS and with a Q value of < 0.05. B) Histogram showing the GO term frequency of genes identified by *HSP12* SGA analysis with 0.5% (w/v) glucose (red) and the corresponding genome frequency (black). SGA analysis with 0.5% (w/v) glucose revealed an over representation of genes involved in cell wall organisation and biogenesis, protein modifications, response to DNA damage stimulus and chemical stimulus, signalling, transmembrane transport, ion transport, pseudohyphal growth, invasive growth to glucose limitation, organelle inheritance, peptidyl amino acid modification and peroxisome organisation. Data from the *Saccharomyces* genome database using GO Slim Mapper.

Chapter 5. An unbiased approach to identify genetic interactions of *HSP12/HSP26*

A

ORF	Gene	P	Q	GIS	CONTROL DATA		MATCHED DATA	
					QueryFitnessSummary	ControlFitnessSummary	QueryFitnessSummary	ControlFitnessSummary
YGL059W	YGL059W	3.21E-05	0.00043522	-11472.999	10586.93629	72193.38659	10586.93629	25114.57321
YCR073W-A	SOL2	2.89E-06	9.69E-05	-11432.028	10836.80593	72877.02841	10836.80593	20635.89617
YNL244C	SUI1	1.60E-06	6.90E-05	-10733.696	13016.09244	77723.60081	13016.09244	22070.05998
YMR130W	YMR130W	0.00028003	0.00185347	-10250.016	17452.54422	90659.45046	17452.54422	32699.92078
YGL117W	YGL117W	5.21E-05	0.00060662	-9921.5759	15043.38929	81700.39192	15043.38929	25660.33342
YGL034C	YGL034C	0.00010142	0.00093607	-9838.1502	14116.95183	78395.51188	14116.95183	23670.18823
YHR014W	SPO13	6.75E-05	0.0007195	-9784.2776	12825.49169	73992.77358	12825.49169	22266.77328
YHR094C	HXT1	1.54E-06	6.80E-05	-9718.0301	13531.70158	76087.11568	13531.70158	22983.69841
YGR069W	YGR069W	9.52E-06	0.00020155	-9499.4018	12923.6915	73381.8572	12923.6915	24666.22119
YGL166W	CUP2	2.85E-05	0.00039791	-9374.8742	13370.91418	74437.90981	13370.91418	25187.28636
YHR079C	IRE1	0.00065172	0.0033305	-9317.7109	13593.1684	74978.18681	13593.1684	24428.93264
YGL083W	SCY1	5.11E-06	0.00013988	-9283.783	12743.73602	72087.30027	12743.73602	23113.45245
YGR121C	MEP1	5.23E-06	0.00013988	-9211.8145	13115.42184	73068.15565	13115.42184	23371.92712
YGR295C	COS6	0.00067873	0.00340717	-9203.9832	13139.63106	73121.75408	13139.63106	23159.22328
YLR436C	ECM30	1.38E-05	0.00024621	-9187.4295	14443.80462	77335.62139	14443.80462	24012.7699
YML131W	YML131W	2.41E-05	0.00036492	-9179.9802	13867.49572	75425.21311	13867.49572	22909.26695
YML109W	ZDS2	7.06E-05	0.00073775	-9157.4321	14834.25944	78515.25498	14834.25944	27564.41223
YBR034C	HMT1	1.99E-11	2.53E-08	-9129.8102	12853.48925	71942.58703	12853.48925	21476.85416
YDL146W	LDB17	0.00230705	0.00847471	-9080.9632	15058.24592	78998.0212	15058.24592	24208.13286
YGL159W	YGL159W	4.00E-07	3.14E-05	-9076.6076	14325.78905	76586.72747	14325.78905	23430.64642
YHR047C	AAP1	1.06E-06	5.75E-05	-9072.1628	14168.98082	76059.01032	14168.98082	24869.20092
YMR209C	YMR209C	0.00234761	0.00857739	-8948.0987	19876.5087	94331.46451	19876.5087	35511.66406
YGL170C	SPO74	7.07E-05	0.00073775	-8945.2444	13869.01317	74661.98234	13869.01317	22946.62379
YIL105C	SLM1	8.19E-05	0.00080517	-8930.6871	15042.49059	78454.6664	15042.49059	26667.13744
YIL047C	SYG1	0.00015214	0.00122845	-8749.0835	14241.42386	75238.7777	14241.42386	22763.96139
YGR018C	YGR018C	2.09E-08	5.66E-06	-8712.204	14154.27593	74832.88513	14154.27593	23173.81808
YGR001C	YGR001C	2.85E-07	2.59E-05	-8696.1133	13102.0918	71336.84716	13102.0918	23428.4513
YJR010C-A	SPC1	6.48E-05	0.00070081	-8666.4952	16021.58302	80794.25126	16021.58302	25544.28613
YGL032C	AGA2	9.50E-06	0.00020155	-8594.7213	12926.3575	70429.92306	12926.3575	21594.1592
YJR010W	MET3	2.77E-06	9.36E-05	-8531.1727	16705.08574	82588.22672	16705.08574	29324.83635
YJL116C	NCA3	0.00078947	0.00382624	-8480.4503	20070.61899	93436.28298	20070.61899	30219.60445
YHR185C	PFS1	0.00028328	0.00186507	-8468.0769	13733.00542	72655.30379	13733.00542	30219.60445
YHR109W	CTM1	2.56E-06	8.94E-05	-8427.4639	14779.47809	75947.08198	14779.47809	24758.80292
YML013C-A	YML013C-A	3.04E-07	2.72E-05	-8404.1952	6433.735897	48558.64125	6433.735897	14977.56188
YIL156W	UBP7	0.00021267	0.00153042	-8393.7881	14709.66123	75608.39179	14709.66123	24409.5528
YBL013W	FMT1	2.02E-06	7.82E-05	-8384.6168	11761.77188	65931.1096	11761.77188	21440.79819
YKL191W	DPH2	1.60E-05	0.00026933	-8196.4317	12612.27361	68098.60818	12612.27361	22868.64183
YGR209C	TRX2	4.82E-07	3.61E-05	-8150.9315	14310.35451	73506.84677	14310.35451	22868.64183
YDL235C	YPD1	0.00020444	0.00149658	-8113.9289	13064.67574	69309.14136	13064.67574	21601.95367
YGL255W	ZRT1	0.00032418	0.00203662	-7964.2424	2834.63203	35340.41683	2834.63203	29747.91001
YGL086W	MAD1	1.47E-05	0.00025667	-7955.7355	13109.42332	68937.87846	13109.42332	23227.59838
YGL067W	NPY1	2.69E-07	2.50E-05	-7947.3557	14282.24427	72748.63074	14282.24427	25174.11568
YIL074C	SER33	1.12E-06	5.81E-05	-7675.9986	15681.38331	76439.41194	15681.38331	26263.14861
YKL128C	PMU1	0.00086598	0.00408533	-7645.2876	14233.4994	71600.55945	14233.4994	25953.2517
YBR074W	YBR074W	0.00053069	0.00285819	-7641.3126	17743.13109	83073.17813	17743.13109	29738.03263
YDL066W	IDP1	5.34E-06	0.00014012	-7607.771	14104.65036	71056.11104	14104.65036	23204.97654
YGL141W	HUL5	1.35E-06	6.39E-05	-7574.2916	14863.65598	73430.46955	14863.65598	23844.4254

B



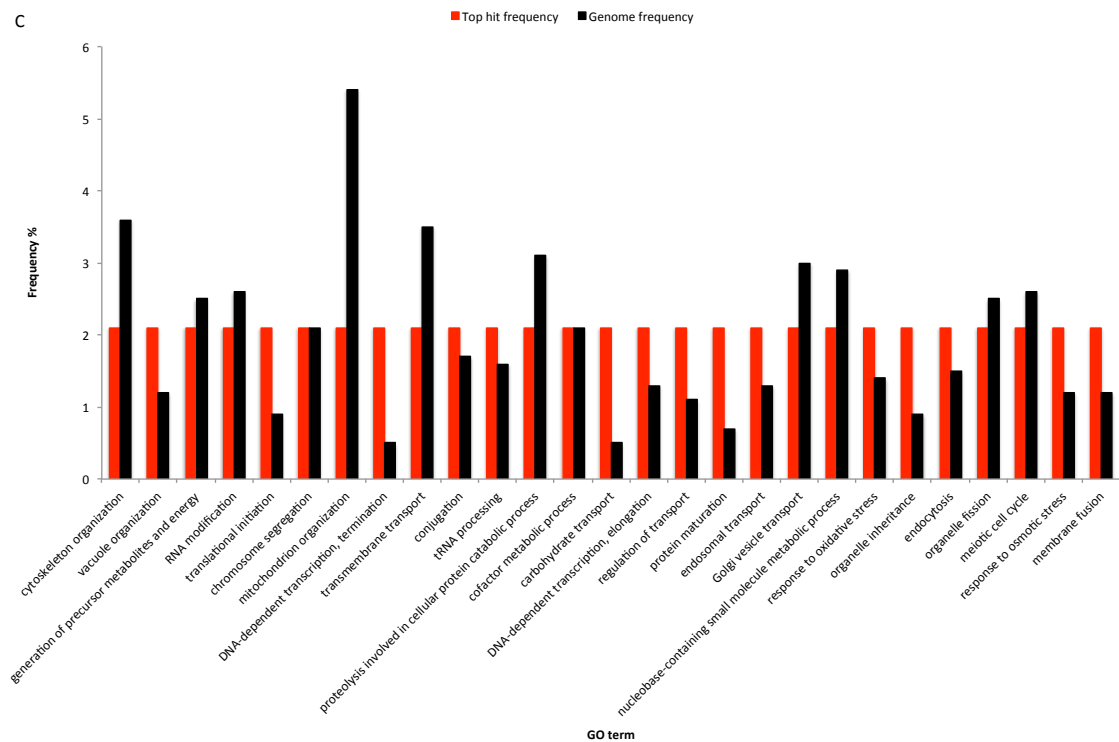


Figure 5.13 Significant genetic interactions of *HSP12* with 0.05% (w/v) glucose. A) Table of genes identified as statistically significant interactors of *HSP12*, falling outside one standard deviation of the mean GIS and with a Q value of < 0.05. B) C) Histogram showing the GO term frequency of genes identified by *HSP12* SGA analysis with 0.05% (w/v) glucose (red) and the corresponding genome frequency (black). SGA analysis of *HSP12* with 0.05% (w/v) glucose revealed an over representation of genes involved in peptidyl amino acid modification, protein alkylation, DNA dependent transcription termination, translation initiation, organelle inheritance and protein maturation. Data from the *Saccharomyces* genome database using GO Slim Mapper.

5.3.7 SGA analysis with stress conditions, a comparison between *HSP12* and *HSP26*.

In addition to 0.5% and 0.05% (w/v) glucose, SGA analysis was also performed with stress conditions known to retard ageing and induce the expression of Hsp12 and Hsp26. SGA analysis was next performed on media supplemented with 10% ethanol, 0.8 M NaCl, 1 mM H₂O₂ and exposure to thermostress by incubation at 37 °C. Statistically significant genetic interactions were determined as described earlier.

We first compared the statistically significant interactions identified for *HSP26* (Appendix table 4) to those identified for *HSP12* (Appendix table 5). From this only 16 genes were common to both (4.7%) (Table 5.14). 8 of these genes did not show any pattern of specificity to a particular stress and GO terms identified their involved in endocytosis, golgi vesicle transport, cell organisation, cell biogenesis, protein modification by small protein conjugation or removal, endosomal transport, transcription from RNA polymerase II promoter and proteolysis involved in cellular protein catabolic process (Figure 5.15).

Interestingly, there was a pattern of genes that were stress specific and common to both *HSP12* and *HSP26*. For example, *TRX2*, *MEP1*, *AAP1* and *NPY1* were all identified as negative genetic interactors of *HSP26* and *HSP12* with 0.05% (w/v) glucose. Analysis of the GO terms indicates a role for Hsp26 and Hsp12 in vacuole organisation and golgi vesicle transport during glucose depletion. Not surprisingly, genes identified by SGA analysis with 0.05% (w/v) glucose also play a role in carbohydrate metabolism, generation of precursor metabolites and energy and response to oxidative stress (Figure 5.16A). SGA analysis with 10% ethanol identified *ECM30* and *TSA1* for both *HSP26* and *HSP12*, exposure to 1 mM hydrogen peroxide identified *YGL015C*, which was found to be common to both *HSP26* and *HSP12* and incubation at 37 °C, identified *OST3* as a significant interactor of *HSP26* and *HSP12* (Table 5.14). The biological processes of *ECM30* and *YGL015C* are unknown. However, analysis of the GO terms known for *TSA1* show a role in regulation of the cell cycle, protein folding and response to stresses such as chemical stimuli, oxidative and DNA damage. Whereas, the GO terms known for *OST3* reveal a role in protein glycosylation and protein complex biogenesis.

Hsp12 & Hsp26	Hsp12 & Hsp26	Hsp12	
YML013C-A	TRX2	ZRT1	General stress response
RCY1	ECM30	IRE1	Specific stress response common to both Hsp12 and Hsp26
YGR272C	MEP1	YML013C-A	Common to Hsp12 and Hsp26
HUL5	AAP1	SPT3	
CSR2	NPY1		
RUD3	OST3		
YGR272C	TSA1		
	YGL015C		

Table 5.14 Comparison of identified genetic interactions between *HSP26* and *HSP12*. Comparing the total genes identified with standard and stress conditions for *HSP26* and *HSP12* reveals some common genes. Genes highlighted in blue are common to both *HSP26* and *HSP12* but show no specificity to a particular stress. In contrast, genes highlighted in yellow are common to both *HSP26* and *HSP12* and show specificity to particular stresses, notably, 0.05% (w/v) glucose and exposure to ethanol, hydrogen peroxide and incubation at 37 °C. Genes highlighted in orange represent a group of genes identified as negative genetic interactors of *HSP12* with multiple stresses indicating their involvement in a general stress response.

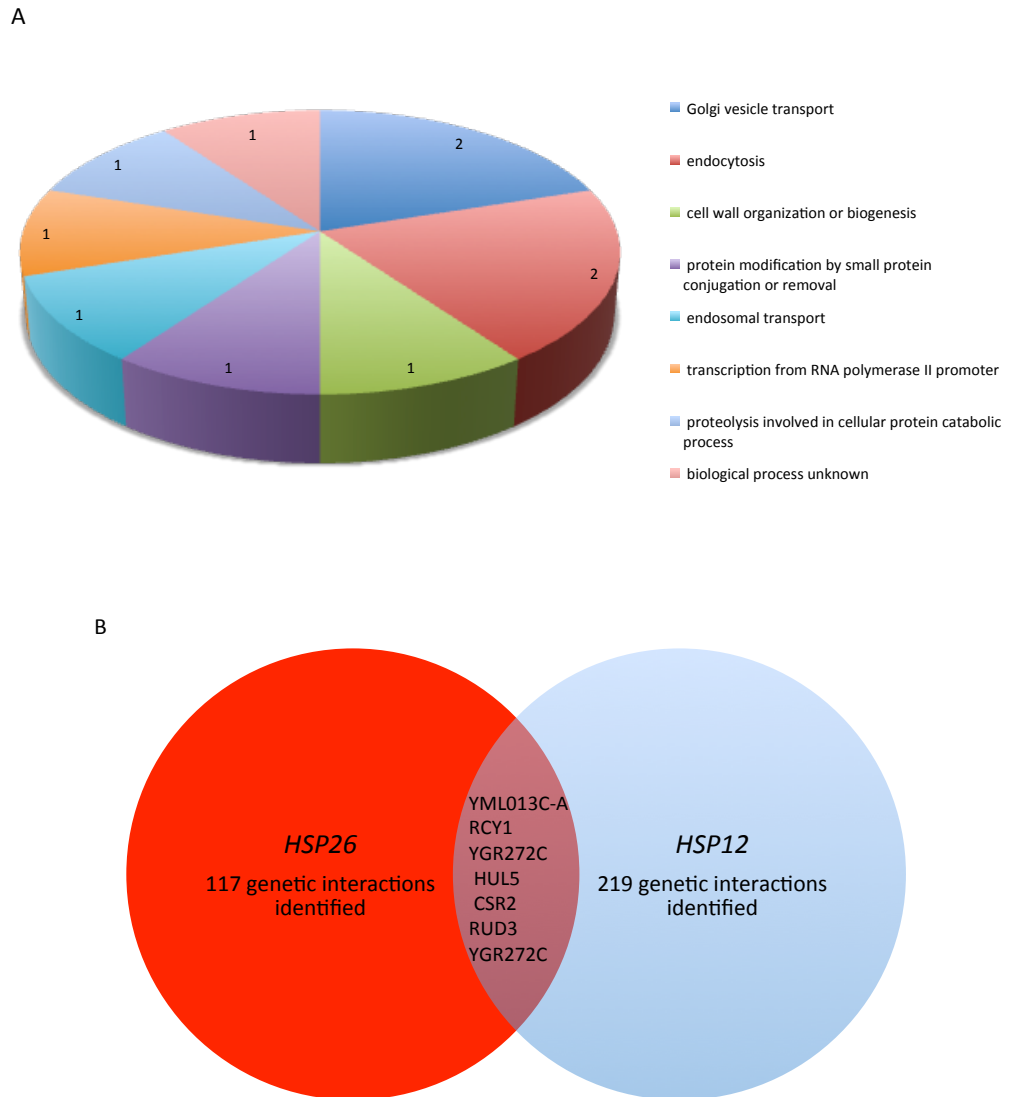
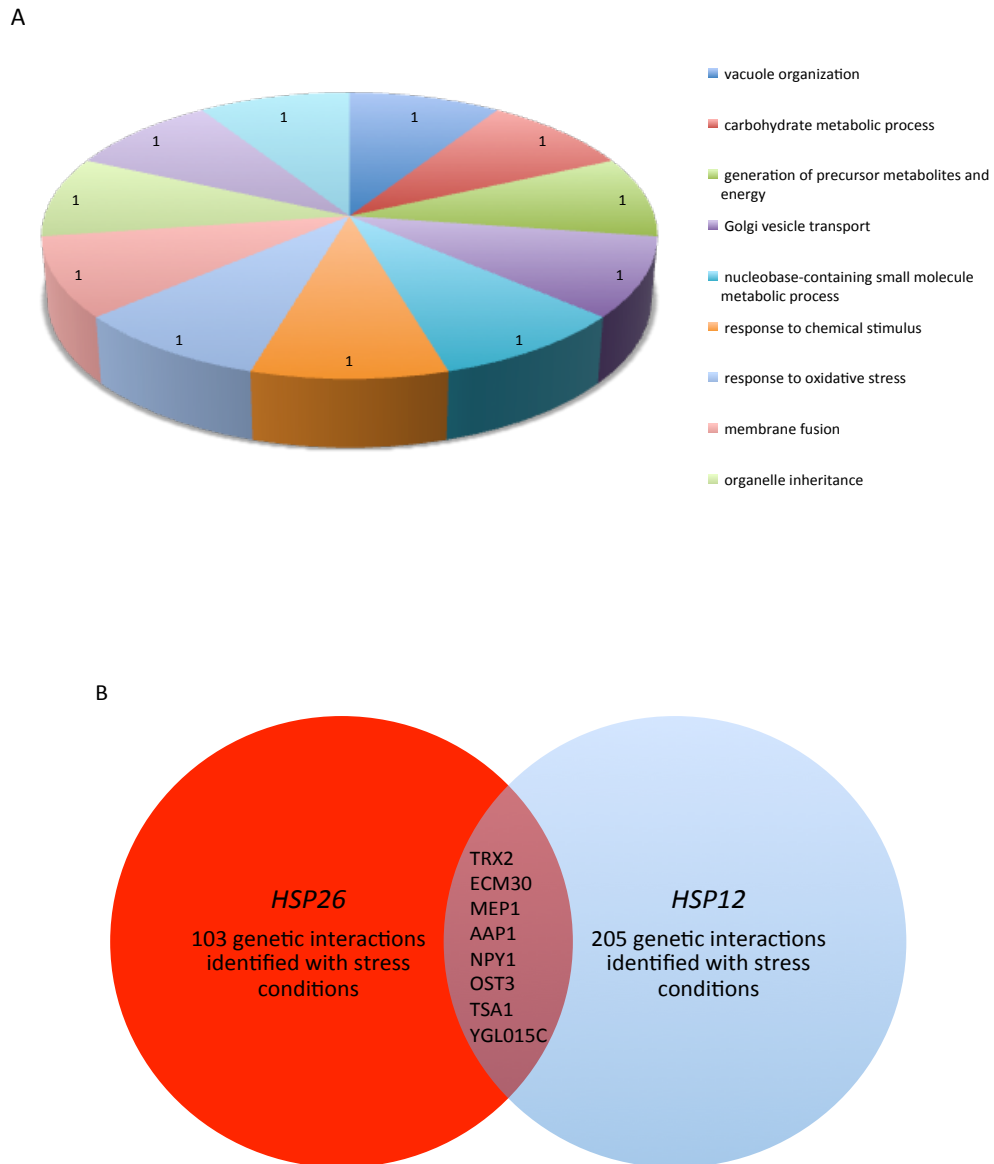


Figure 5.15. Genes identified as being common to both *HSP26* and *HSP12*. A) Pie chart showing the GO terms for genes identified by SGA analysis with standard and stress conditions that are common to both *HSP26* and *HSP12*, the numbers of genes involved in a particular GO terms are shown on the pie chart. The GO terms suggest an association of Hsp12 and Hsp26 with golgi vesicle transport, endocytosis, cell wall organisation and biogenesis, protein modification, endosomal transport, transcription from RNA polymerase II promoter and proteolysis. B) Venn diagram showing genetic interactions common to both *HSP12* and *HSP26*. Data from the *Saccharomyces* genome database using GO Slim Mapper.



5.3.8 Identification of genes associated with the mitochondria and vacuoles.

A recent theory for how DR may mediate lifespan extension proposes that DR maintains an acidic pH within the vacuoles thereby preventing subsequent mitochondrial dysfunction (Hughes and Gottschling, 2012). With this in mind, it was important to determine if any of the genes identified as significant interactors of *HSP12* or *HSP26* are associated with the mitochondria or vacuoles, as this may help explain why the *hsp12/hsp26Δ* double mutant has a reduced RLS with DR.

Analysing the GO terms of genes identified by SGA analysis of *HSP12* and *HSP26* revealed 13 genes associated with the mitochondria. These genes showed many different GO terms but included mitochondrion organisation, mitochondrion translation, involvement in cellular respiration and the generation of precursor metabolites and energy (Table 5.17 & 5.18). Further analysis, revealed 15 genes identified by SGA analysis of *HSP12* and *HSP26* that were associated with the vacuoles. One gene, *TRX2*, was found to be common to both *HSP12* and *HSP26*. These genes showed involvement in protein targeting, endosomal transport, vacuole organisation, golgi vesicle transport, lipid metabolic process, membrane invagination, organelle assembly and endocytosis (Table 5.19).

Transcriptome studies have reported the up-regulation of *HSP12* and *HSP26* during ageing and in this study we have shown induced expression of Hsp12 and Hsp26 at the protein level during DR (Lesur and Campbell, 2004, Lin et al., 2001). Mitochondria are considered to play a role in ageing and also to be a target of the ageing process (Hughes and Gottschling, 2012). During DR, mitochondrion respiration and autophagy are reportedly induced (Hansen et al., 2008). Since both Hsp12 and Hsp26 are up-regulated during DR, the genes identified by SGA analysis of *HSP12* and *HSP26* may suggest that the *hsp12/hsp26Δ* double mutant is defective in mitochondrial or vacuole related processes which ultimately results in a reduced RLS.

Hsp26		Hsp12		
MDJ2	MRL1	SWS2	PAH1	associated with vacuoles
HOT13	TRX2	NCA3	VMA21	associated with mitochondria
PUF3	ATG2	FMT1	VPS5	
HSP78	VPS30	HCM1	VPS74	
FMP36			VPS21	
YDJ1			VPS1	
MRS4			TSC13	
MDM34			MON2	
MNP1			ATG3	
			VPS53	
			TRX2	

Table 15.17 Genes identified by SGA analysis, which show association with the mitochondria and vacuoles. Table of genes identified as statistically significant interactions of *HSP26* and *HSP12*, which are associated with the mitochondria (yellow) and vacuoles (red). *TRX2* is associated with the vacuoles and identified as significantly interacting with both *HSP12* and *HSP26*.

A

Cellular component	Genes
Mitochondrion	FMT1,HSP78,MZM1,MDJ1,MNP1,MDM34,HOT13,MRS4,PUF3,SWS2
Mitochondrion envelope	MDJ1,MDM34,HOT13,MRS4,PUF3
cytoplasm	FMT1,HCM1,HSP78,MZM1,MDJ1,MNP1,MDM34,TRX2,HOT13,MRS4,PUF3,YDJ1,SWS2

B

Biological processes	Genes
mitochondrion organization	HCM1,HSP78,MZM1,MDJ1,MDM34,NCA3,HOT13,PUF3,YDJ1,SWS2
protein folding	HSP78,MDJ1,YDJ1
protein targeting	HSP78,HOT13,YDJ1
cellular respiration	MNP1,PUF3
generation of precursor metabolites and energy	MNP1,PUF3
response to chemical stimulus	HCM1
response to oxidative stress	HCM1
response to heat	HSP78,MDJ1
mitochondrial translation	HSP78,SWS2
transmembrane transport	HSP78,HOT13
proteolysis involved in cellular protein catabolic process	MDJ1,YDJ1
ion transport	MDM34,MRS4
cytoskeleton organization	HCM1
RNA catabolic process	PUF3
RNA modification	FMT1
protein complex biogenesis	MZM1
sporulation	SWS2
regulation of organelle organization	HSP78
lipid transport	MDM34
transcription from RNA polymerase II promoter	HCM1
regulation of translation	HSP78
mitotic cell cycle	HCM1
tRNA processing	FMT1

Table 5.18 Genes identified by SGA analysis of *HSP26* and *HSP12* associated with the mitochondria. A) Table showing the cellular component of mitochondrial genes. B) Table showing the biological processes in which mitochondrial related genes play a role.

A

Cellular component	Genes
cytoplasm	TSC13,VPS74,VMA21,TRX2,VPS53,VPS1,PAH1,ATG2,MON2,ATG3,VPS5,VPS21,VPS30,MRL1
membrane	TSC13,VPS74,VMA21,VPS1,PAH1,ATG2,MON2,VPS5,VPS21,VPS30
endomembrane system	TSC13,VMA21,PAH1,VPS5
vacuole	TRX2,VPS1,PAH1,VPS30
mitochondrion	TSC13,VPS1,VPS21
mitochondrial envelope	VPS1,VPS21
endoplasmic reticulum	TSC13,VMA21
Golgi apparatus	VPS74,VPS53
nucleus	VPS74,PAH1
peroxisome	VPS1
cell cortex	VPS1
cytoskeleton	VPS1

B

Biological processes	Genes
protein targeting	VPS1,ATG2,MON2,ATG3,VPS21,VPS30
endosomal transport	VPS53,MON2,VPS5,VPS21,VPS30
vacuole organization	TRX2,PAH1,ATG2,ATG3,VPS21
Golgi vesicle transport	VPS74,TRX2,VPS53,MON2,VPS21
lipid metabolic process	TSC13,PAH1,VPS30
membrane invagination	ATG2,ATG3,VPS30
organelle assembly	ATG2,ATG3,VPS21
endocytosis	VPS1,MON2,VPS21
peroxisome organization	VPS1,ATG2,VPS30
response to starvation	ATG2,ATG3,VPS30
response to chemical stimulus	VPS74,TRX2
organelle inheritance	TRX2,VPS21
membrane fusion	TRX2,PAH1
cytoskeleton organization	VPS1
cellular respiration	PAH1
generation of precursor metabolites and energy	PAH1
protein complex biogenesis	VMA21
response to oxidative stress	TRX2
protein lipidation	ATG3
organelle fission	VPS1

Table 5.19 Genes identified by SGA analysis of *HSP26* and *HSP12* associated with the vacuoles. A) Table showing the cellular component of vacuole related genes. B) Table showing the biological processes in which vacuole related genes play a role.

5.4 DISCUSSION

5.4.1 Comparison of findings with other genetic screens.

There have been a number of genetic screens performed that have identified genetic interactions of *HSP12* and *HSP26* (Addinall et al., 2011, Duennwald et al., 2012, Moehle et al., 2012, Costanzo et al., 2010, Sharifpoor et al., 2012, Krause et al., 2012). In these genetic screens *HSP12* and *HSP26* have been identified as the prey from other gene baits, so only a fraction of the potential genetic interactions have been tested. In contrast, this is the first study that has identified the genetic interactions of *HSP12* and *HSP26* when these genes are used as the genetic bait. This study has only focused on identifying the genetic interactions of *HSP12* and *HSP26*. This differs to other genetic screens which have analysed up to 5.4 million gene-gene interactions providing genetic interaction profiles for approximately 75% of all genes in *S. cerevisiae* (Costanzo et al., 2010). Since this study was focused on only two genes the genetic interactions identified are more likely to be reliable than those generated from genome-scale screens which may be prone to more mistakes.

In this study only negative genetic interactions were considered. Negative genetic interactions were easier to visualise from the colony images than positive genetic interactions. Also strains growing nearby to strains with a synthetic sick or lethal genetic interaction grew better because of an increase in the availability of nutrients. These strains may therefore look to have a positive genetic interaction and give a positive GIS when this increase in growth is actually not a result of a positive interaction between two genes. In addition, negative genetic interactions were easier to re-confirm when performing spot tests. Previous SGA analysis experiments have also highlighted the significance of negative genetic interactions over positive genetic interactions since virtually all of negative genetic interactions contain at least one essential gene for a particular process (Baryshnikova et al., 2010). It was concluded that negative genetic interactions were more reliable than positive genetic interactions; therefore the latter was excluded from this study.

A genetic interaction between *HSP12* and *HSP26* has not been reported by SGA analysis performed by other groups. However, the reduced RLS of the

hsp12/hsp26Δ double mutant identified and discussed in chapter 3 suggests that *HSP12* and *HSP26* genetically interact. Disappointingly, SGA analysis performed in this study did not identify a genetic interaction between *HSP12* and *HSP26*. SGA analysis is a well-established technique for determination of strong genetic interactions. However, because of the methodology of replica plating from solid cultures, SGA analysis can sometimes not detect when there is a slight reduction in the growth of a double mutant in comparison to the control. In contrast, the methodology used in QFA involves the dilution of solid cultures into liquid media thereby providing much more detailed fitness measurements and allowing identification of slight genetic interactions. It will therefore be interesting to determine if QFA identifies a genetic interaction between *HSP12* and *HSP26*. Alternatively, it may be that *HSP12* and *HSP26* do not genetically interact with one another but interact with common genes.

Comparing the genetic interactions of *HSP26* identified in this study to those identified by other genetic studies revealed only one identical genetic interaction, *YDJ1*, that is a consistent finding in this study and a study performed by Duennwald et al, (Duennwald et al., 2012). *YDJ1* encodes a type 1 Hsp40 co-chaperone involved in the functional regulation of Hsp90 and Hsp70 (Caplan and Douglas, 1991). Hsp26 has been shown to interact physically with Hsp104 and Hsp70 during the reactivation of proteins (Haslbeck et al., 2005). Furthermore, Hsp104, Hsp70 and Hsp40 are known to be essential for yeast cell viability (Xu et al., 2013). This result suggests that *HSP26* and *YDJ1* interact genetically and may overlap functionally to some extent.

Comparing the genetic interactions of *HSP12* identified in this study to those identified in other genetic studies did not reveal any identical genetic interactions. In this study we identified genetic interactions between *HSP12* and *CDC9* and *CDC45*. Similar to this, Costanzo et al, reported a genetic interaction between *HSP12* and *CDC3* (Costanzo et al., 2010). *CDC3* is a component of the septin ring and required for cytokinesis (Takizawa et al., 2000). *CDC9* and *CDC45* are involved in DNA replication and differ in functions to *CDC3* (Willer et al., 1999, Takizawa et al., 2000, Tye, 1999). Although it may appear that the genetic interactions are similar because of the CDC gene class, this is misleading as they actually perform very

different roles in the cell. This underlies the need to do GO term analysis rather than rely on gene class names as an indicator of function.

This study also identified a similar genetic interaction of *HSP26* to that reported by Costanzo et al. In this study a genetic interaction was identified between *HSP26* and *RPL22A*. *RPL22A* encodes a protein component of the large (60S) ribosomal subunit and this result was found to be consistent with that reported by Costanzo et al, (Venema and Tollervey, 1999) (Costanzo et al., 2010). Costanzo et al., reported *HSP26* to have a negative genetic interaction with *RRP1*, which encodes a protein necessary for biogenesis of 60S ribosomal subunits (Horseley et al., 2004).

There were only a limited number of identical genetic interactions of *HSP26* consistent in this study when compared to the existing literature. Despite this, there were similarities in GO terms identified by this study and by other genetic studies. For example, a genetic interaction has been reported for *HSP26* and *NPL3* (Moehle et al., 2012). *NPL3* encodes an RNA-binding protein involved in repressing translation initiation and mRNA processing (Windgassen et al., 2004). This study identified a genetic interaction between *HSP26* and genes involved in translation initiation and mRNA processing.

5.4.2 The genetic link between the *HSP12* and *HSP26* and genes involved in stress response.

SGA analysis was used as an unbiased approach to identify genetic interactions of *HSP12* and *HSP26* on a genome wide scale to help shed light on the longevity roles these proteins play. Not surprisingly, SGA analysis identified a genetic link between *HSP12* and *HSP26* and genes involved in stress responses, notably response to heat, oxidative stress, glucose limitation and DNA damage. Both Hsp12 and Hsp26 are reported to be induced in response to stresses including heat, osmotic stress, low pH, oxidative stress and during glucose depletion (Carmelo and Sa-Correia, 1997, Amoros and Estruch, 2001, Lindquist, 1986, Lanks, 1986). The link identified by SGA analysis between these sHsps and stress responses is therefore consistent with the existing literature. When yeast cells are exposed to stresses, Hsp26 is thought to act to suppress the aggregation of one-third of the cytosolic proteins thereby maintaining proteome stability in collaboration with

other Hsps (Haslbeck et al., 2004). In contrast, Hsp12 has little anti-aggregation activity, and is thought to act to stabilise membranes during exposure to stresses (Welker et al., 2010, Herbert et al., 2012). In response to stresses the Hsps provide broad cytoprotective properties and this has been linked to longevity. In fact there is strong interest in discovering and developing pharmacological agents capable of inducing stress responses (Cornelius et al., 2013). It is thought that stresses such as DR activate hormetic pathways, which then enhances defense and stress resistance homeostasis impacting on longevity (Cornelius et al., 2013).

5.4.3 The genetic link between *HSP26* and genes involved in transcription from RNA polymerase II.

When reviewing the GO terms for those genes identified as significant interactors of *HSP26*, a common finding was the regulation of transcription from RNA polymerase II. Furthermore, SGA analysis of *hsp26Δ* with standard conditions identified a cluster of genes, *NUT1*, *MED1* and *SRB8*, which are all associated with the Mediator complex. However, spot tests did not reproduce a synthetic sick interaction between *HSP26* and *NUT1*, so this gene will be excluded from the discussion. Mediator is a large multi-subunit complex, identified originally in yeast and conserved in humans (Kim et al., 1994, Koleske and Young, 1994, Guglielmi et al., 2004, Myers and Kornberg, 2000). The Mediator complex serves as an adaptor for regulatory information from enhancers, operators and promoters to modulate transcription in a promoter-dependent way through RNA polymerase II (Myers and Kornberg, 2000) (Ries and Meisterernst, 2011). Transcription by RNA polymerase II is divided between two tasks. In the first task, RNA polymerase II synthesizes RNA chains by selecting an appropriate nucleoside triphosphate (NTP) substrate complementary to the DNA template and catalyzes phosphodiester bond formation (Kaplan, 2013). In the second task, transcription is divided between three phases, initiation, elongation and termination, of which all phases have a large number of associated transcription factors functioning to regulate gene expression (Kaplan, 2013). Interestingly, other commonly identified GO terms for interactors of *HSP26* include DNA-dependent transcription initiation, elongation and termination. This suggests that Hsp26 plays a role in pathways which regulate transcription. The

hypothesis that Hsps may play a role in regulation of transcription is not novel. In fact, Hsp90 has been shown to bind to transcription start sites (TTS) and is involved in RNA polymerase II pausing, and regulation of target genes expression (Sawarkar et al., 2012).

In *S. cerevisiae*, the Mediator complex comprises 21 subunits, interestingly; global patterns of gene transcription can be affected by post-translational modifications of specific Mediator subunits (Bjorklund and Gustafsson, 2005). Both *MED1* and *SRB8* are subunits of the Mediator complex, essential for transcriptional regulation and associate with core polymerase subunits to form RNA polymerase II holoenzyme (Hengartner et al., 1995, Balciunas and Ronne, 1995, Balciunas et al., 1999). *SRB8* has also been shown to play a role in glucose repression along with 2 other subunits of the RNA polymerase II complex, cyclin C and cyclin-dependent kinase (CDK) encoded by *GIG2* and *GIG3* respectively (Balciunas and Ronne, 1995).

In addition to *NUT1*, *MED1* and *SRB8*, SGA analysis of *hsp26Δ* with standard conditions also identified 3 genes, *CTK1*, *ACE2* and *SFL1*, which also play a role in the regulation of transcription. In yeast, the C-terminal domain kinase (CTK1) forms a three-subunit complex that regulates the C-terminal repeat domain (CTD) of the largest subunit of RNA polymerase II (Sterner et al., 1995). *CTK1* plays essential roles in the regulation of transcription and translation and humans contain two evolutionary relatives to yeast *CTK1*, hCDK12 and hCDK13 (Bartkowiak et al., 2010). In addition to regulation of transcription, SGA analysis of *HSP26* also identified GO terms including regulation of translation, translation initiation and mRNA processing correlating with the known roles of *CTK1*.

ACE2 is a transcription factor required for septum destruction following cytokinesis (O'Conallain et al., 1999). *SFL1* is a transcriptional repressor and activator involved in the activation of stress responsive genes (Galeote et al., 2007). Deletion of *SFL1* enhances pseudohyphal growth and invasive growth, both of which are common GO terms identified by SGA analysis of *HSP12* and *HSP26* (Robertson and Fink, 1998, Galeote et al., 2007). Interestingly, Sfl1 is involved in the transcriptional activation of *HSP30*, an sHsp member of *S. cerevisiae*, and electrophoretic mobility shift and chromatin immuno-precipitation assays have

demonstrated that Sfl1 is present at the *HSP26* promoter (Conlan and Tzamarias, 2001, Galeote et al., 2007).

SGA analysis therefore suggests that Hsp26 may play a role in the regulation of transcription and have an association with subunits of the Mediator complex. Consistent with this hypothesis, a study in *S. cerevisiae* has shown that in response to temperature change Mediator modules are selectively recruited to the promoter regions of *HSP* genes, in which Hsf1 plays a central role (Kim and Gross, 2013). In fact, Mediator is a coactivator critically required for *HSP* gene activation, and is recruited to the *HSP* gene promoters in both yeast and *Drosophila* (Fan et al., 2006, Park et al., 2001). In *Drosophila*, immunofluorescence analysis of polytene chromosomes has shown a striking colocalisation of HSF and Mediator at native heat shock factor loci (Park et al., 2001). In *S. cerevisiae*, Mediator is strongly recruited to enhancers, *SSA4*, *HSP104* and *HSP26* and activated by Hsf1 (Fan et al., 2006). In contrast, Mediator association at *HSP12* is relatively poor as Msn2 and Msn4 primarily activate the *HSP12* promoter, and collective data suggests that Hsf1, but not Msn2 and Msn4, can efficiently recruit Mediator (Fan et al., 2006). In addition, Mediator has also been shown to regulate RNA polymerase II elongation at both heat shock and non-heat shock protein genes, and has been shown to specifically to regulate the expression of *HSP82* (Kremer et al., 2012).

5.4.4 The genetic link between *HSP12* and genes associated with lipid metabolism, protein lipidation, cytoskeleton organisation and membrane fusion.

SGA analysis of *HSP12* identified a genetic link with genes involved in lipid metabolic process, protein lipidation, membrane fusion and cytoskeleton organization. These findings are consistent with the findings from a recent study which has shown a role for Hsp12 as a membrane-stabilising lipid chaperone (Welker et al., 2010). Hsp12 has been shown to exist both in the cytoplasm and associated with the plasma membrane (Welker et al., 2010). Two recent studies have shown Hsp12 to be monomeric and natively unfolded in solution, however in the presence of lipids, Hsp12 switches to a 4-helical conformation (Herbert et al., 2012, Welker et al., 2010). Through this change in conformation Hsp12 is able to interact with the plasma membrane not to alter the overall lipid composition but to

increase membrane stability during exposure to stresses (Welker et al., 2010). The genetic link identified in this study between *HSP12* and processes associated with lipid metabolism and membrane fusion further support the finding that Hsp12 functions as lipid chaperone.

5.4.5 The genetic link between *HSP12* and *HSP26* and genes associated with the mitochondria.

SGA analysis of *HSP12* and *HSP26* identified a number of genes involved in mitochondrial organization, mitochondrial translation and cellular respiration. The link between the sHsps and genes associated with the mitochondria is of huge interest since mitochondria are well documented to play a role in the ageing process. Mitochondria are highly dynamic organelles that are evolutionarily conserved and play a key role in a number of metabolic and signalling pathways in addition to programmed cell death (Zeng et al., 2013, Bratic and Larsson, 2013). The primary function of mitochondria is to produce ATP by a process known as oxidative phosphorylation, which occurs in the inner mitochondrial membrane (Bratic and Larsson, 2013). During this process electrons are passed through four protein complexes (complex I, II, III and IV) leading to the reduction of molecular oxygen (Merksamer et al., 2013). Occasionally, electrons escape the electron transport chain prematurely leading to the formation of superoxide anion O_2^{*-} (Merksamer et al., 2013). Other reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^*) are also formed by incomplete reduction of molecular oxygen (Merksamer et al., 2013). The presence of ROS in cells can cause an oxidative stress and damage DNA, proteins and lipids and lead to the production of high molecular weight protein aggregates (Mirzaei and Regnier, 2008). The presence of ROS and protein aggregates then induces a stress response to prevent further damage and degrade protein aggregates (Merksamer et al., 2013).

To maintain protein homeostasis, mitochondria contain a protein quality control system made up of chaperones and ATP-dependent proteases that promote proper protein folding (Bender et al., 2011). Autophagy is the primary recycling pathway of cells that play a critical role in mitochondrial quality control and homeostasis (Joo et al., 2011). Selective mitochondrial autophagy (mitophagy)

eliminates damaged or excess mitochondria and is a fundamental process that depends on autophagy-related double-membrane vesicles, which are restricted to the mitochondria (Kanki et al., 2011, Eiyama et al., 2013). Interestingly, Mitophagy is induced in response to DR and requires specific AuTophagy related genes, *ATG11*, *ATG32*, *ATG17*, *ATG29* and *ATG31* (Eiyama et al., 2013). SGA analysis of *HSP12* and *HSP26* identified a genetic interaction with AuTophagy related genes, *ATG2* and *ATG3*, which are required for mitochondrion degradation and autophagic vacuole assembly. Mitophagy is conserved from yeast to humans and defects in mitophagy are linked with mitochondrial genome instability and neurodegeneration (Eiyama et al., 2013).

Chaperones associated with the mitochondria include Hsp90, which is known to play a role in stress response to govern mitochondrial homeostasis (Joo et al., 2011). In addition, Hsp60 is reported to play a key role in protein folding in the mitochondria, in combination with Hsp10, which is also an essential component of the mitochondrial protein folding apparatus (Hohfeld and Hartl, 1994). Hsp60 and Hsp70 are mitochondrial chaperones and known to protect mitochondrial enzymes from stress-induced aggregation (Bender et al., 2011). In *Drosophila*, Hsp22 is a mitochondrial protein whose expression is up-regulated with ageing (Morrow et al., 2000). Interestingly, flies overexpressing Hsp22 live longer but also display up-regulation of genes related to mitochondrial energy production and protein biosynthesis (Kim et al., 2010). In correlation with this, mitochondrial homeostasis is thought to be at the center of Hsp22 beneficial effects on lifespan (Kim et al., 2010).

In humans, there are multiple reports that sHsps exist in mitochondria and confer stress resistance by maintaining mitochondrial homeostasis (Zeng et al., 2013). Hsp27, for example, plays a critical role in intracellular trafficking during mitophagy, and loss of Hsp27 leads to mitochondrial fragmentation, decreased aerobic respiration and ATP production (Zeng et al., 2013). Hsp27 contains a α -crystallin domain and shares sequence similarity to yeast Hsp26 (Datskevich et al., 2012, White et al., 2006). Hsp27 is known to prevent mitochondrial injury and apoptosis in normal and cancer cells and is reported to exert its neuroprotective effects through protective actions on mitochondria (Zeng et al., 2013, Bidmon et al., 2004). With this in mind, it may be that Hsp26 also plays a key role in the

mitochondrial protein folding apparatus and/or in mitochondrial homeostasis through the process of mitophagy. Since Hsp12 is known to display negligible anti-aggregation activity, instead of playing a role in the mitochondrial protein folding apparatus, Hsp12 may function to stabilise mitochondrial membranes, in keeping with its role as a lipid chaperone.

Mitochondria are considered to play a central role in the ageing process as well as being a target of it (Hughes and Gottschling, 2012). Transcriptome analysis has shown the down-regulation of mitochondrial genes with ageing and mitochondrial dysfunction is regarded as a hallmark of ageing (Zeng et al., 2013, Hughes and Gottschling, 2012, Lopez-Otin et al., 2013). Mitochondrial dysfunction is a characteristic of ageing in all eukaryotes and characterised by a decline in ATP generation and an increase in ROS as a consequence of the decline in the respiratory chain efficacy (Lopez-Otin et al., 2013). Increased levels of ROS generated by mitochondrial dysfunction are then thought to cause further mitochondrial deterioration and global cellular damage (Harman, 1956).

A more recent theory has implicated the role of the mitochondria in regulating lifespan by a process known as the mitochondrial unfolded protein response (UPR^{mt}) (Houtkooper et al., 2013). In a study by Houtkooper et al, knockdown of mitochondrial ribosomal proteins (MRPs) in *C. elegans* was shown trigger mitonuclear protein imbalance, reducing mitochondrial respiration and activating the UPR^{mt} (Houtkooper et al., 2013). Furthermore, treatment of *C. elegans* with carbenicillin, doxycycline and ethidium bromide to pharmacologically mimic *mrp* knockdown was shown to extend lifespan. This increase in lifespan was brought about by an imbalance between nuclear DNA and mitochondrial DNA, termed mitonuclear protein imbalance, which then activates the UPR^{mt} (Houtkooper et al., 2013). This mechanism was also conserved in mammalian cells (Houtkooper et al., 2013). The UPR^{mt} is induced by mitochondrial stress and activates transcription of the chaperones; in *C. elegans* these include HSP-6 and HSP-60, which then act to restore mitochondrial proteostasis (Houtkooper et al., 2013, Haynes and Ron, 2010, Zhao et al., 2002). The mitochondria have dedicated molecular chaperones and proteases that play a role in the UPR^{mt} and promote proteostasis and quality control (Haynes and Ron, 2010). The genetic interactions identified for *HSP12* and *HSP26*

may indicate that both proteins play an important role in the UPR^{mt} elicited by mitochondrial stress and the increased generation of ROS. This may further support the hypothesis that Hsp12 and Hsp26 play a role in mitochondrial homeostasis. An alternative hypothesis may be that the *hsp12/hsp26Δ* double mutant has defects in the UPR^{mt} or an enhanced mitochondrial dysfunction, which may be contributing to its reduced RLS.

DR is the most widely researched environmental manipulation known to extend lifespan, and in yeast, DR has been described as having a mitohormetic effect (Sharma et al., 2011). DR appears to decrease ROS-mediated oxidative damage and is positively implicated in cellular stress response by activating hormetic pathways (Cornelius et al., 2013). A recent study has shown that mitochondrial dysfunction in replicatively aged yeast cells can be suppressed by maintaining an acidic pH within the vacuoles, which prevents a decline in pH-dependent amino acid storage in the vacuolar lumen (Hughes and Gottschling, 2012). This study also found that DR could extend lifespan at least in some part by maintaining an acidic pH within the vacuoles (Hughes and Gottschling, 2012). Interestingly, this study identified a link between the vacuoles and mitochondria in yeast. Consistent with this finding, SGA analysis of *HSP12* and *HSP26* also identified a number of genes associated with the mitochondria and vacuoles. Although fluorescent microscopy experiments did not reveal any obvious differences in the structure of mitochondria or vacuolar acidification for the *hsp12/hsp26Δ* double mutants in comparison to the wildtype (chapter 4). It may be that deletion of *HSP12* and *HSP26* leads to a loss of pH-dependent amino acid storage in the vacuolar lumen thereby leading to mitochondrial dysfunction and premature ageing. The genetic link identified by SGA analysis for *HSP12* and *HSP26* strongly suggests that these proteins play a role in processes associated with the mitochondria and vacuoles; the latter will be discussed next.

5.4.6 The genetic link between *HSP12* and *HSP26* and genes associated with the vacuoles.

As mentioned earlier, SGA analysis of *HSP12* and *HSP26* identified a number of genes that are associated with the vacuole. This is of particular interest as there is a vast amount of evidence linking the vacuoles with ageing and DR mediated

lifespan extension. The vacuole is often described as the yeast counterpart of the mammalian lysosome, and serves a role in many processes; it is the destination for cellular elements targeted for degradation by autophagic processes, it acts as a regulator of cellular ion homeostasis and as a storage reservoir for nutrients, metal ions and other metabolites (Tsuchiyama and Kennedy, 2012). There is evidence to suggest that the vacuoles are important in yeast longevity. During DR, the yeast vacuoles undergo vacuolar fusion events leading to the presence of one large vacuole, which replaces the normal 4-5 present during physiological conditions (Tsuchiyama and Kennedy, 2012). Interestingly, two genes, which are required for vacuolar fusion, *ERG6* and *NYV1*, are also required for lifespan extension by DR (Gaber et al., 1989, Nichols et al., 1997, Tang et al., 2008). In addition a set of 7 OSH (oxysterol binding) genes are also required for DR mediated lifespan extension (Tsuchiyama and Kennedy, 2012). Deletion of all 7 OSH genes leads to fragmentation of vacuoles, whereas overexpression of *OSH6* has been shown to extend yeast RLS (Gebre et al., 2012, Tsuchiyama and Kennedy, 2012). Interestingly, the vacuolar membranes of replicatively old yeast cells have been shown to become disordered (Gebre et al., 2012, Tang et al., 2008). It may be that Hsp12 is required to stabilise the vacuolar membranes with increasing age, in keeping with its known role as a lipid chaperone (Welker et al., 2010, Herbert et al., 2012).

Analysis of the genes identified as being associated with the vacuoles revealed one gene, *TRX2*, which was common to both *HSP12* and *HSP26*. *TRX2* (ThioRedoxin) is a cytoplasmic thioredoxin isoenzyme, part of the thioredoxin system in yeast and is required for maintenance of redox homeostasis (Trotter and Grant, 2002). In addition to redox homeostasis, it is thought that *TRX2* plays a role in many cellular processes, two of which, include protein folding and repair of oxidatively damaged proteins (Grant, 2001). *TRX2* plays a huge role in protection of the cell against oxidative stress and deletion of the *TRX2* gene results in extreme sensitivity to H_2O_2 (Kuge and Jones, 1994). Both *HSP12* and *HSP26* play a crucial role in protein homeostasis and protection of the cell against stresses. The finding that *TRX2* also overlaps in cellular functions is of high significance. During exposure to oxidative stress, *TRX2* may interact with *HSP12* and *HSP26* to prevent protein misfolding and aid in the correct folding of oxidatively damaged proteins. Further

experiments will now be performed to re-confirm the genetic interaction between *TRX2* and *HSP12* and *TRX2* and *HSP26* in multiple yeast backgrounds. It will also be important to perform *in vivo* protein aggregation assays on *trx2/hsp12Δ* and *trx2/hsp26Δ* double mutants to determine if they show increased protein aggregation in comparison to single mutants and wildtype.

Autophagy occurs in the lysosome/vacuole and functions to degrade intracellular components and recycle building blocks during DR and exposure to stresses (Aris et al., 2013, Yen and Klionsky, 2008). Exposure to stresses causes proteins to unfold and impairs proper folding of nascent proteins. Unfolded proteins are either refolded by Hsps or are targeted for destruction by one of three processes: chaperone-mediated autophagy (CMA) endosomal microautophagy or by chaperone-assisted selected autophagy (Kaushik and Cuervo, 2012).

Interestingly, autophagy is linked to DR and ageing. Autophagy is required for chronological lifespan extension in yeast by DR and promotes mitochondrial respiration proficiency during ageing with DR conditions (Yen and Klionsky, 2008). SGA analysis of *HSP12* and *HSP26* identified two genes, *ATG2* and *ATG3*, which play a role in autophagy and are also linked with response to starvation. It is thought that induction of autophagy in response to DR is required for removal of damaged proteins to maintain proper metabolism, which is crucial for cell survival (Yen and Klionsky, 2008). The link between *HSP12* and *HSP26* and genes associated with the vacuole suggests that these proteins play a role in autophagy processes including mitophagy. Since the *hsp12/hsp26Δ* double mutant has a reduced RLS it may be that deletion of both these proteins leads to defects in autophagy, mitophagy or an abnormality in vacuolar membranes, which has deleterious effects on the cell. An alternative explanation may be that deletion of *HSP12* and *HSP26* leads to a loss of pH-dependent amino acid storage in the vacuolar lumen. The latter, has been implicated in a recent theory linking the vacuoles and the mitochondria in yeast ageing (Hughes and Gottschling, 2012). In this theory, altered vacuolar pH is linked with a loss of pH-dependent amino acid storage in the vacuolar lumen leading to mitochondrial dysfunction and ageing (Hughes and Gottschling, 2012). The vacuoles and mitochondria play a huge role in ageing and the link identified by SGA analysis

between these two organelles and *HSP12* and *HSP26*, may pinpoint the longevity roles of these proteins to processes specific to the vacuoles and mitochondria.

5.7 CONCLUSION

SGA analysis of *HSP12* and *HSP26* has helped to shed light on the processes in which the proteins encoded for by these genes play a part. SGA analysis also provides a novel way of identifying genetic interactions during stress conditions. The results show a significant correlation between *HSP26* and the Mediator complex and *HSP12* and *HSP26* and regulation of transcription from RNA polymerase II. In addition, SGA analysis has shown a strong association between *HSP12* and *HSP26* with genes associated with the mitochondria and vacuoles, both of which have been shown to be important players in DR mediated lifespan extension and ageing. With this in mind, future work will now focus on analysis of QFA data to re-confirm the genetic interactions identified by SGA analysis. Ultimately, we aim to use the data collected from SGA analysis and QFA to then pinpoint the areas in which Hsp12 and Hsp26 play a role. If genetic interactions identified by SGA analysis are re-confirmed by QFA analysis, future work will then focus on functional assays to explore the possible roles Hsp12 and Hsp26 play in the regulation of transcription and in processes specific to the vacuoles and mitochondria.

6.1 SHSPS EXPRESSION DURING DIETARY RESTRICTION.

6.1.1 Hsp12 and Hsp26 are strongly induced by DR.

Both Hsp12 and Hsp26 are important components of the heat shock response whose expression is up-regulated by exposure to a variety of stresses. In addition to heat shock, osmostress, ethanol exposure and entry into stationary phase, microarray data also report the up-regulation of *HSP12* and *HSP26* in response to glucose depletion (Kasambalides and Lanks, 1983, Susek and Lindquist, 1990). This finding is of significance since DR can be mimicked in yeast by reducing the glucose concentration of the growth medium from 2% to 0.5% (w/v) or below (Kaeberlein et al., 2007). Furthermore, 2-D gel electrophoresis and mass spectrometry performed by the Morgan laboratory identified the induced expression of Hsp12 in response to 0.5% (w/v) glucose (Herbert et al., 2012). In this study, Western blotting has identified a strong induction of Hsp12 and Hsp26 at the protein level in response to 0.5% (w/v) and 0.05% (w/v) glucose with the strongest induction with severe DR (0.05% (w/v) glucose). The enhanced expression of these proteins during glucose depletion indicates that Hsp12 and Hsp26 play a role in DR-mediated lifespan extension.

6.2 THE ROLE OF HSP12 AND HSP26 IN DR-MEDIATED LIFESPAN EXTENSION.

6.2.1. Hsp12 and Hsp26 are essential for DR-mediated lifespan extension.

The sHsps have been implicated in ageing in invertebrate models, notably, *Hsp22* in *Drosophila* longevity and HSP-16 in *C. elegans* longevity (Morrow et al., 2004b, Morrow et al., 2004a, Walker and Lithgow, 2003). In addition, published work from the Morgan laboratory has shown that *HSP12* is essential for DR-mediated lifespan extension in yeast (Herbert et al., 2012). Notably, lifespan extension by moderate DR is abolished in a *hsp12Δ* knockout strain (Herbert et al., 2012). In yeast, transcriptome analysis has shown the up-regulation of *HSP12* and *HSP26* with old age (Lesur and Campbell, 2004, Lin et al., 2001). In addition to their up-regulation during yeast ageing, this study also revealed Hsp12 and Hsp26 up-regulation at the protein level in response to moderate and severe DR. Considering the existing literature and the necessity for *HSP12* in DR-mediated lifespan

extension in yeast, it was important to investigate longevity phenotypes of *hsp26Δ* and *hsp12/hsp26Δ* knockout mutants.

RLS performed with severe DR (0.05% (w/v) glucose) extended the mean lifespan of the wildtype strain, as expected. Consistent with the finding that *HSP12* is necessary for DR-mediated lifespan, this study also found that *HSP12* is sufficient for DR-mediated lifespan. Notably, overexpression of *HSP12* extended lifespan, which was unable to be further extended with the addition of DR. These findings suggest that *HSP12* plays an important role in yeast ageing. Unlike other studied sHsps, Hsp12 has negligible anti-aggregation activity (Herbert et al., 2012, Welker et al., 2010). Instead of preventing protein aggregation, Hsp12 is thought to bind to lipids within the plasma membrane, endosomes and possibly vacuoles to stabilise membranes during exposure to stresses (Welker et al., 2010, Herbert et al., 2012). The longevity phenotype of *HSP12* may therefore be linked to its functional role as a membrane-stabilising lipid chaperone. This hypothesis is consistent with a study that showed Ssd1 extends RLS by increasing the stability of the plasma membrane (Kaeberlein et al., 2004a).

Like that reported for *HSP12*, DR was unable to extend the lifespan of the *hsp26Δ* single mutant indicating that *HSP26* is also necessary for DR-mediated lifespan extension in yeast. Furthermore, *hsp12/hsp26Δ* double mutants showed a striking reduction in RLS compared to the wildtype and *hsp26Δ* single mutant when grown with severe DR. Collectively these findings indicate that both *HSP12* and *HSP26* play a role in yeast ageing. Unlike Hsp12, Hsp26 is a well-established chaperone with 100-fold more anti-aggregation activity (Franzmann et al., 2008). Hsp26 is known to overlap in its substrate proteins with its cytosolic family member, Hsp42 (Haslbeck et al., 2004). Consistent with this finding, the chaperones are thought to work together and have overlapping functions and Hsp26 is also reported to collaborate with Hsp70 and Hsp104 during the reactivation of proteins (Haslbeck et al., 2005). Since the functional properties of Hsp12 and Hsp26 are very different it is likely that these proteins affect yeast longevity in different ways. The loss of either gene leads to a reduction in RLS, however, the collective loss of both genes results in a more dramatic phenotype.

6.2.3 *Hsp12/hsp26Δ* double mutants do not appear to be defective in processes associated with yeast ageing.

Western blotting and RLS analysis revealed a role for *HSP12* and *HSP26* in DR-mediated lifespan extension in yeast. The *hsp12/hsp26Δ* double mutant was characterised to determine the presence of any defects in processes known to affect yeast ageing.

Both Hsp12 and Hsp26 are known to play protective roles in the cell, Hsp12 by stabilising membranes and Hsp26 by preventing protein aggregation (Haslbeck et al., 2004, Welker et al., 2010). However, the *hsp12/hsp26Δ* double mutant did not show an increased susceptibility compared to the wildtype when exposed to a variety of stresses, consistent with that reported by other groups (Petko and Lindquist, 1986, Praekelt and Meacock, 1990, Fu et al., 2012).

One of the common theories of DR-mediated lifespan extension in yeast implicates the activation of Sir2 (Guarente, 2011). Transcriptional silencing assays at the telomeres and rDNA revealed no differences in Sir2 activity in the *hsp12/hsp26Δ* mutant in comparison to the wildtype. The longevity phenotypes identified for *HSP12* and *HSP26* are therefore not attributable to any effect on Sir2 activity.

Ageing is characterised by the accumulation of oxidized, misfolded and aggregated proteins which are then thought to effect cellular homeostasis (Gelino and Hansen, 2012). However, aggregation assays performed in this study did not suggest that the *hsp12/hsp26Δ* double mutant has increased levels of insoluble protein aggregates in comparison to the wildtype. Future experiments will use *in vivo* chaperone assays on magnetically sorted old yeast cells. These experiments should provide more detailed information about the levels of protein aggregation than the *in vitro* aggregation assays performed in this study. Furthermore, analysing yeast cells at different age points will shed important information on the asymmetry of damaged proteins between mother and daughter cells. This will help determine if the *hsp12/hsp26Δ* double mutant has a reduced RLS due to an increased inheritance of damaged proteins by the daughter cell.

A recent study in yeast ageing has linked the mitochondria and vacuoles with DR-mediated lifespan extension (Hughes and Gottschling, 2012). However, in contrast to that reported by Hughes and Gottschling 2012, this study did not find an

increase in vacuolar acidity or suppression of mitochondrial dysfunction by DR. Future work will now be focused on analysing mitochondrial dysfunction and vacuolar acidity in mother and daughter cells which have been magnetically sorted at various ages. Hughes and Gottschling used the MEP to sort cells at different ages. In this study, yeast cells were not analysed at various ages and this may explain the differences in results obtained.

6.2.4 Genome wide analysis of genetic interactions of *HSP12* and *HSP26*.

The *hsp12/hsp26Δ* double mutant did not appear to be defective in processes associated with yeast ageing including stress resistance, Sir2 activity, protein aggregation and mitochondrial and vacuolar dysfunction. SGA analysis was therefore performed as an unbiased approach to identify genetic interactions of *HSP12* and *HSP26* and to map the functional relationships of these genes to help understand their roles in yeast ageing.

Not surprisingly, SGA analysis identified a genetic link between *HSP12* and *HSP26* and genes involved in stress responses, notably, response to heat, oxidative stress, glucose limitation and DNA damage. This finding is consistent with existing literature which report *HSP12* and *HSP26* induction in response to heat, osmotic stress, low pH, oxidative stress and during glucose depletion (Carmelo and Sa-Correia, 1997, Amoros and Estruch, 2001, Lindquist, 1986, Lanks, 1986).

SGA analysis of *HSP26* revealed a genetic link with genes involved in the regulation of transcription from RNA polymerase II and genes associated with the Mediator complex. Consistent with this finding, a study in *S. cerevisiae* has shown that in response to temperature change Mediator modules are selectively recruited to the promoter regions of *HSP* genes (Kim and Gross, 2013). In addition, Mediator has been shown to be a coactivator critically required for *HSP* gene activation, and is recruited to the *HSP* gene promoters in both yeast and *Drosophila* (Fan et al., 2006, Park et al., 2001).

A genetic link between *HSP12* and genes involved in lipid metabolism, membrane fusion and cytoskeleton organization was identified by SGA analysis. This finding is consistent with the recent finding that Hsp12 interacts with lipids and

functions to stabilise membranes during exposure to stresses (Welker et al., 2010, Herbert et al., 2012).

A genetic link was identified between *HSP12* and *HSP26* and genes involved in mitochondrial organization, mitochondrial translation and cellular respiration. Since the mitochondria are well documented to play a role in the ageing process this genetic link may help explain the roles of Hsp12 and Hsp26 in yeast longevity. It is possible that Hsp12 and Hsp26 play crucial roles in response to mitochondrial stress, in mitochondrial homeostasis, in the mitochondrial unfolded protein response (UPR^{mt}) and autophagic processes. Therefore the absence of *HSP12* and *HSP26* leads to detrimental effects in these processes and resulting in a reduction in RLS.

SGA analysis identified a genetic link between *HSP12* and *HSP26* and genes related to the vacuoles. The vacuoles play an important role in autophagic processes and have been linked with yeast longevity and DR-mediated lifespan extension (Tsuchiyama and Kennedy, 2012, Hughes and Gottschling, 2012). During DR, the yeast vacuoles undergo vacuolar fusion events and two genes which are required for vacuolar fusion, *ERG6* and *NYV1*, are also required for lifespan extension by DR (Gaber et al., 1989, Nichols et al., 1997, Tang et al., 2008). Autophagy is required for CLS extension in yeast by DR and promotes mitochondrial respiration proficiency during ageing with DR conditions (Yen and Klionsky, 2008). There is a link between the processes associated with the mitochondria and processes associated with the vacuoles (Hughes and Gottschling, 2012). The reduced RLS of *hsp12/hsp26Δ* double mutant may be a result of defects in the mitochondria, autophagy, mitophagy or abnormalities in vacuolar and/or mitochondrial membranes, which has deleterious effects on the cell.

Future work will now focus on analysis of QFA data to re-confirm the genetic interactions identified by SGA analysis. QFA provides much more detailed information than SGA analysis on the fitness measurements of double mutants. This is achieved because yeast strains are grown in liquid culture and not on solid agar like in SGA analysis. The yeast strains are then diluted and plated onto selective media and photographed every 4-6 hours generating growth curves. If genetic interactions identified by SGA analysis are re-confirmed by QFA analysis, future

work will then focus on functional assays to explore the possible roles Hsp12 and Hsp26 play in the regulation of transcription from RNA polymerase II. Future experiments may include transcriptional assays in *hsp12Δ*, *hsp26Δ* and *hsp12/hsp26Δ* double mutants. Experiments will also focus on investigating the roles Hsp12 and Hsp26 play in processes specific to the mitochondria and vacuoles. These experiments will include fluorescent microscopy on magnetically sorted cells to analyse mitochondrial structure, vacuolar structure and acidity at various ages.

6.3 CONCLUSION.

In conclusion, both Hsp12 and Hsp26 are induced in response to DR and are necessary for DR-mediated lifespan extension in yeast. Yet *hsp12/hsp26Δ* double mutants are not defective in processes known to affect yeast ageing. An unbiased genome-wide approach provided a novel method of identifying genetic interactions of *HSP12* and *HSP26* under various stress conditions including DR. SGA analysis suggests an important role for *HSP12* and *HSP26* in processes associated with the mitochondria and vacuoles. Future work will now focus on QFA and functional assays to understand how Hsp12 and Hsp26 affect yeast ageing, in particular DR-mediated lifespan extension.

Name	Genotype	Background reference
BY4741 PARENT	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Abgene, Fisher Scientific
BY4741 <i>hsp26Δrcy1Δ</i>	BY4741 <i>hsp26Δ::NATMX rcy1Δ::KANMX</i>	This study
BY4741 <i>hsp26Δbud27Δ</i>	BY4741 <i>hsp26Δ::NATMX bud27Δ::KANMX</i>	This study
BY4741 <i>hsp26Δctk1Δ</i>	BY4741 <i>hsp26Δ::NATMX ctk1Δ::KANMX</i>	This study
BY4741 <i>hsp26Δmed1Δ</i>	BY4741 <i>hsp26Δ::NATMX med1Δ::KANMX</i>	This study
BY4741 <i>hsp26Δnut1Δ</i>	BY4741 <i>hsp26Δ::NATMX nut1Δ::KANMX</i>	This study
DLY7325	<i>MATα lyp1::HPH::LEU2 can1Δ::STE2pr-Sp_his5 his3Δ leu2Δ ura3Δ met15Δ LYS+</i>	Professor David Lydall
DLY7325 <i>hsp26Δ</i>	DLY7325 <i>hsp26Δ::NATMX</i>	This study
DLY7325 <i>hsp12Δ</i>	DLY7325 <i>hsp12Δ::NATMX</i>	This study
JS128 PARENT	<i>MATα his3Δ200 leu2Δ1 ura3-167 RDN1(NTS1)::Ty1-mURA3</i>	Professor Jeffrey Smith
JS128 <i>hsp26Δ</i>	JS128 <i>hsp26Δ::KANMX</i>	This study
JS128 <i>hsp12Δhsp26Δ</i>	JS128 <i>hsp12Δ::HIS3MX6 hsp26Δ::KANMX</i>	This study
AEY1017 PARENT	<i>MATα ade2-1 ura3-1 his3-11 leu2 -3 112 trp1-1 can 1-100 TELVIL::URA3</i>	Abgene, Fisher Scientific
AEY1017 <i>hsp12Δhsp26Δ</i>	AEY1017 <i>hsp12Δ::HIS3MX6 hsp26Δ::KANMX</i>	This study
DLY7388 <i>his3Δ</i> (Control strain for SGA)	<i>MATα lyp1::HPH::LEU2::NAT can1Δ::STE2pr-Sp_his5 his3Δ leu2Δ ura3Δ met15Δ LYS+</i>	Professor David Lydall

Appendix Table 1. Genotypes of yeast strains used in this study. Yeast strains were either made in-house (in this study), purchased from Fisher Scientific or obtained as a kind gift from Professor Jeffrey Smith or Professor David Lydall.

Primer name:	Sequence:
RCY1 forward	GTGTTTCAAGGCTAACTTTTGCCG
RCY1 reverse	AGTCTTTTATTTCCGCACTTCATACCTATATAGAG
BUD27 forward	GGCGATGAGCTGCTCTTAAAGG
BUD27 reverse	GAGGAACTGCTGAGCTTATTCGTACAAG
CTK1 forward	GCACTATTCTTTGCACTAGAATAACACAGGG
CTK1 reverse	GATGTTTCGTATCATATTTACGGGTATCGCG
MED1 forward	CATGCAGGTGCAGAATATTGGTGTAG
MED1 reverse	CCATCGAGTGTACGGTCCAC
NUT1 forward	CCACCTCAACAAAGCTATTCGTGGC
NUT1 reverse	GCGGCAAGTGCATTGGCATG

Appendix Table 2. Gene specific primers designed for use in chapter 5. Primers designed for amplification of ORFs to confirm double mutants made by replica plating, as described in chapter 5.

ORF:	Systematic name:	Primer combination:	Product (bp):
RCY1	YJL204C	RCY1 forward/RCY1 reverse (parent)	2711
		RCY1 forward/RCY1 reverse (Δ)	1688
BUD27	YFL023W	BUD27 forward/bud27 reverse (parent)	2621
		BUD27 forward/bud27 reverse (Δ)	1730
CTK1	YKL139W	CTK1 forward/bud27 reverse (parent)	1587
		CTK1 forward/bud27 reverse (Δ)	1500
MED1	YPR070W	MED1 forward/bud27 reverse (parent)	2012
		MED1 forward/bud27 reverse (Δ)	1819
NUT1	YGL151W	NUT1 forward/bud27 reverse (parent)	3648
		NUT1 forward/bud27 reverse (Δ)	1749

Appendix Table 3. Expected band sizes of wildtype and knockout mutants using designed primers used in chapter 5. Table showing expected band sizes for wildtype and knockout mutants using designed primers to confirm double mutants made in chapter 5.

HSP26 30 °C	HSP26 0.5% GLUCOSE	HSP26 0.05% GLUCOSE	HSP26 37 °C	HSP26 H ₂ O ₂	HSP26 EtOH	HSP26 NAACL
STE50	ULP2	YNR018W	CSR2	MRS4	BEM2	PBS2
BUD27	TAF12	HOT13	CLN2	YGR226C	CIK1	HOG1
SPF1	DUG1	HUL5	SFI1	YNL165W	REC102	RVS167
NUT1	YFR032C	PUF3	HUT1	YML020W	DBP7	MNP1
MED1	CAF130	DSE1	SRC1	YGL015C	SUR4	YPR097W
SRB8	YGR272C	MEK1	RUD3	CTL1	MDM34	CH55
YML013C-A	YNL211C	CAR2	ARO2	YOR289W	YLR338W	SYT1
APQ12	GCN1	TRX2	EFT2	PFA3	PER1	SDA1
RCY1	OPY2	RSN1	CCH1	CTI6	YJL007C	YLR338W
PSF3	YPR022C	CTF19	HSP78	FET3	TSA1	ORC6
RPL22A	YDR444W	MCM22	OST3	YGR287C	YFR039C	TPO3
CTK1	ROG3	RAX1	FMP36			SRN2
ACE2	YGL108C	YER184C	YPL272C			
SFL1	MDJ2	ECM30	YIR003W			
	YMR291W	IMD4	YDJ1			
	MRL1	MEP1	ATG2			
	MBP1	DUG1	YJL007C			
	ADE2	YOR318C	GSY2			
		AAP1	YOR050C			
		YIP3	VPS30			
		YOR356W	YNL324W			
		NPY1	YPL261C			
		JLP2				
		YPL229W				
		DIG2				
		YPL108W				
		YMR158C-B				
		MEI5				
		YPL199C				

Appendix Table 4. List of genes identified by SGA analysis as significant negative genetic interactors of *HSP26*. With standard conditions statistically significant genes were determined as those that fall outside one standard deviation of the mean GIS, have a Q value <0.05 and show a visual difference in growth to the control strain. For all other conditions, statistically significant genes were identified as those that fall outside one standard deviation of the mean GIS and have a Q value <0.05 in the control and matched data sets.

HSP12 30 °C	HSP12 0.5% GLUCOSE	HSP12 0.05% GLUCOSE	HSP12 37 °C	HSP12 H ₂ O ₂	HSP12 EtOH	HSP12 NA CL
ASH1	SPT3	YGL059W	SEC66	KIP1	SSD1	IRE1
MCD4	IRE1	SOL2	IRE1	SPT3	IRE1	SPT3
INP52	ZRT1	SUI1	VPS5	IRE1	COG8	MON2
ZRT1	TEF4	YMR130W	SPT3	ZRT1	TCO89	ZRT1
PAH1	BCK1	YGL117W	YNL234W	YGR237C	RAV1	YNL234W
YGR272C	ICE2	YGL034C	ZRT1	GLG1	RUD3	ATG3
SWS2	FRE1	SPO13	VPS74	RPN10	LYS20	SSD1
TMA16	CDC45	HXT1	SLA1	MON1	GUK1	SUI1
SAC1	PSY4	YGR069W	OCA2	TEF4	MUP1	YML013C-A
VHS3		CUP2	YOL098C	YML013C-A	MCM2	ICE2
IRE1		IRE1	FEN1	YKL037W	ELC1	YLL020C
VMA21		SCY1	VPS21	FEN1	CSR2	RPL14A
YDR391C		MEP1	VPS1	YDR391C	YNL234W	ADH4
YML013C-A		COS6	ALG5	IPT1	YGL024W	YNL226W
		ECM30	TEF4	YGL015C	SPT3	VPS53
		YML131W	INO2	SLG1	ERV14	YNL200C
		ZDS2	OST3	BCK1	GPI15	YNL296W
		HMT1	ALG6	YDR271C	ZRT1	YDR271C
		LDB17	GET3	ERV2	RCY1	FCF1
		YGL159W	NPR1		YKE2	RAD27
		AAP1	RVS161		YDL114W	PEX1
		YMR209C	RPF2		ELM1	SPT8
		SPO74	MUP1		SIR1	HMT1
		SLM1	YGL024W		GSG1	YKR033C
		SYG1	RAD27		ICE2	RPS0A
		YGR018C	ELC1		TSC13	OSH3
		YGR001C	ALG8		ARP1	YKL063C
		SPC1	YML013C-A		SLT2	DIA2
		AGA2	HHT1		MAC1	PEX30
		MET3	CDC45		YCR062W	BNS1
		NCA3	BCK1		SPT8	ACF2
		PFS1	IXR1		MEH1	
		CTM1			SKY1	
		YML013C-A			CWH41	
		UBP7			YAE1	
		FMT1			TSA1	
		DPH2			SGN1	
		TRX2			NPR1	
		YPD1			MON2	
		ZRT1			CDC9	
		MAD1			GUP1	
		NPY1			ARP4	
		SER33			YLR364W	
		PMU1			AGE2	
		YBR074W			YNL140C	
		IDP1			ECM30	
		HUL5			FAT1	
					LDB18	
					PMU1	
					PMT2	
					CNB1	
					ADH4	
					APS1	
					YML013C-A	
					CTF8	
					DPH2	
					OST6	
					NTC20	
					APL4	
					ICY2	
					HCM1	
					BUD21	
					COS6	
					NAM8	
					YJR100C	
					FLC2	
					OPI1	

Appendix Table 5. List of genes identified by SGA analysis as significant negative genetic interactors of *HSP12*. Statistically significant genes were identified as those that fall outside one standard deviation of the mean GIS and have a Q value <0.05 in the control and matched data sets.

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