DOCTOR OF PHILOSOPHY

Saccharomyces Cerevisiae as a biotechnological tool for ageing research

studies on translation and metabolism

Stephanie Cartwright

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Aston University

SACCHAROMYCES CEREVISIAE AS A BIOTECHNOLOGICAL TOOL FOR AGEING RESEARCH: STUDIES ON TRANSLATION AND METABOLISM

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Thesis summary

The yeast *Saccharomyces cerevisiae* is an important model organism for the study of cell biology. The similarity between yeast and human genes and the conservation of fundamental pathways means it can be used to investigate characteristics of healthy and diseased cells throughout the lifespan. Yeast is an equally important biotechnological tool that has long been the organism of choice for the production of alcoholic beverages, bread and a large variety of industrial products. For example, yeast is used to manufacture biofuels, lubricants, detergents, industrial enzymes, food additives and pharmaceuticals such as anti-parasitics, anti-cancer compounds, hormones (including insulin), vaccines and nutraceuticals. Its function as a cell factory is possible because of the speed with which it can be grown to high cell yields, the knowledge that it is generally recognized as safe (GRAS) and the ease with which metabolism and cellular pathways, such as translation can be manipulated. In this thesis, these two pathways are explored in the context of their biotechnological application to ageing research: (i) understanding *translational* processes during the high-yielding production of membrane protein drug targets and (ii) the manipulation of yeast *metabolism* to study the molecule, L-carnosine, which has been proposed to have anti-ageing properties.

In the first of these themes, the yeast strains, *spt3Δ, srb5Δ, gcn5Δ* and yTHC*BMS1*, were examined since they have been previously demonstrated to dramatically increase the yields of a target membrane protein (the aquaporin, Fps1) compared to wild-type cells. The mechanisms underlying this discovery were therefore investigated. All high yielding strains were shown to have an altered translational state (mostly characterised by an initiation block) and constitutive phosphorylation of the translational initiation factor, eIF2α. The relevance of the initiation block was further supported by the finding that other strains, with known initiation blocks, are also high yielding for Fps1. A correlation in all strains between increased Fps1 yields and increased production of the transcriptional activator protein, Gcn4, suggested that yields are subject to translational control. Analysis of the 5´ untranslated region (UTR) of *FPS1* revealed two upstream open reading frames (uORFs). Mutagenesis data suggest that high yielding strains may circumvent these control elements through either a leaky scanning or a re-initiation mechanism.

In the second theme, the dipeptide L-carnosine (β-alanyl-L-histidine) was investigated: it has previously been shown to inhibit the growth of cancer cells but delay senescence in cultured human fibroblasts and extend the lifespan of male fruit flies. To understand these apparently contradictory properties, the effects of L-carnosine on yeast were studied. *S. cerevisiae* can respire aerobically when grown on a non-fermentable carbon source as a substrate but has a respiro-fermentative metabolism when grown on a fermentable carbon source; these metabolisms mimic normal cell and cancerous cell metabolisms, respectively. When yeast were grown on fermentable carbon sources, in the presence of L-carnosine, a reduction in cell growth and viability was observed, which was not apparent for cells grown on a non-fermentable carbon source. The metabolism-dependent mechanism was confirmed in the respiratory yeast species *Pichia pastoris*. Further analysis of *S. cerevisiae* yeast strains with deletions in their nutrient-sensing pathway, which result in an increase in respiratory metabolism, confirmed the metabolism-dependent effects of L-carnosine.

Key words: yeast; Fps1; L-carnosine; translation initiation; metabolism

For my Mother, Father and

Brother

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Contents

Abbreviations

3´UTR	3´ Untranslated region
5´UTR	5´ Untranslated region
$A_{2a}R$	Adenosine $_{2a}$ receptor
Aβ	Amyloid Beta
AD	Alzheimer's disease
ADA	Ada-transcriptional adaptor complex
A	Adenine
AQP	Aquaporin
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AUG	Translational start codon
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
C	Cytosine
CFUs	Colony forming units
CLS	Chronological lifespan
CML	Carboxymethyl lysine
CN1	Serum carnosinase
CN2	Cytosolic carnosinase
CNS	Central nervous system
CoA	CoenzymeA
CRC	Carnosine related compound
CSM	Complete synthetic medium
Cu^{2+}	Copper ion
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DO	Drop out
DTT	Dithiothreitol
EAP1	eIF4E-binding protein
E. coli	*Escherichia coli*
eEF	Eukaryotic elongation factor
eIF	Eukaryotic initiation factor
ER	Endoplasmic reticulum
Fe	Iron
GAAC	General amino acid control
GABA	Gamma-aminobutyric acid

GAP	GTPase-activating protein
Gcd	General control derepressed
Gcn	General control non-inducable
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GPCR	Guanine nucleotide binding protein coupled receptor
GPD1	Glyerol-3-phosphate dehydrogenase
GPD2	Glycerol-3-phosphatase
GRAS	Generally regarded as safe
GTP	Guanosine triphosphate
G	Guanine
H^+	Proton
HC	Homocysteine
HIS3	Imidazoleglycerol-phosphate dehydrogenase (in histidine biosynthesis)
HisRE	Histidyl-tRNA synthetase linked domain
4-HNE	4-Hydroxy-trans-3-nonenal
HOG	High osmolarity glycerol
HSP	Heat shock protein
IECs	Intestinal epithelial cells
IRE	Iron responsive element
IRES	Internal ribosome entry sites
ITAFs	IRES trans-acting factors
LB	Luria-Bertani
LPO	Lipid peroxidation
MDa	Megadalton; unit of mass
MDA	Malondialdehyde
MG	Methyl glyoxal
Met-$tRNA_i$	Methionine tRNA; starting tRNA
µg	Micrograms
mg	Milligrams
MIP	Major intrinsic protein
mRNA	Messenger RNA
NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced NAD^+
NMD	Nonsense mediated decay
NMDA	N-methyl-D-aspartate
OD	Optical density
ONPG	O-nitrophenyl-β-D-galactoside

ORF	Open reading frame
PABP	Poly(A) binding protein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing Tween 20
PCR	Polymerase chain reaction
P_i	Inorganic phosphate
pH	Negative logarithm of hydronium ion concentration
PIC	43S pre-initiation complex
P. pastoris	*Pichia pastoris*
PPP	Pentose phosphate pathway
RFUs	Relative fluorescence units
RLS	Replicative lifespan
RLUs	Relative light units
RNA	Ribonucleic acid
RNA	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Revolutions per minute
rRNA	Ribosomal RNA
S	Sulphur
S	Svedberg unit; measure of sedimentation (When applied to ribosomal subunits, e.g. 80S)
SAGA	Spt-Ada-Gcn5-acetyltransferase complex
SAM	Senescence accelerated mice
S. cerevisiae	*Saccharomyces cerevisiae*
SEM	Standard error of the mean
SD	Shine-Dalgarno
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
SRP	Signal recognition particle
SR	SRP receptor
SR	Sarcoplasmic reticulum
TAM	Thermal activity monitor
TBP	TATA-binding protein
TC	Ternary complex
TCA	Tricarboxylic acid cycle
T	Thymine
TOR	Target of rapamycin

tRNA	Transfer RNA
U	Uridine
uAUG	Upstream AUG
UDP	Uridine diphosphate
uORF	Upstream ORF
UPR	Unfolded protein response
URA3	Orotidine-5´-phosphate decarboxylase, an enzyme in uracil biosynthesis
WT	Wild-type
YPD	Yeast peptone dextrose

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Chapter 1: Introduction

The yeast *Saccharomyces cerevisiae* is an important model organism for the study of cell biology. The similarity between yeast and human genes and the conservation of fundamental pathways (Rose and Harrison, 1971) means it can be used to investigate cellular mechanisms (Feldmann, 2010) and cellular pathways that are involved in ageing (Dilova et al., 2007). Yeast is an equally important biotechnological tool that has long been the organism of choice for the production of alcoholic beverages, bread and a large variety of industrial products. For example, yeast is used to manufacture biofuels, detergents, industrial enzymes, food additives and pharmaceuticals such as anti-parasitics, anti-cancer compounds, hormones (including insulin), vaccines and nutraceuticals (Celik and Calık, 2012; Ferrer-Miralles et al., 2009). Its function as a cell factory is possible because of the speed with which it can be grown to high cell yields, the knowledge that it is generally recognized as safe (GRAS) (Ferrer-Miralles et al., 2009) and the ease with which its cellular and metabolic pathways can be manipulated (Celik and Calık, 2012). In this thesis, two pathways are explored in the context of their biotechnological application to ageing research: (i) understanding *translational* processes during the high-yielding production of membrane protein drug targets and (ii) the manipulation of yeast *metabolism* to study the molecule, L-carnosine, which has been proposed to have anti-ageing properties (McFarland and Holliday, 1994). The need for novel drugs is set to increase dramatically with the demands of an ageing world population (Byass, 2008; Christensen et al., 2009).

1.1. *Saccharomyces cerevisiae*

The yeast species, *Saccharomyces cerevisiae*, belongs to the Fungi kingdom and is classically associated with its use in bread and beer production. It is a budding yeast 2-10μM in size that divides by a daughter cell or "bud" growing from a mother cell (Figure 1.1). The optimal doubling time of *S. cerevisiae* is around 1.6 h when grown on complex media in the presence of glucose, which is its preferred carbon source (Werner-Washburne et al., 1993). In addition to its culinary use, *S. cerevisiae* is one of the best studied organisms in the biological sciences: it is a eukaryote and has similar cellular mechanisms, which have been conserved, to those that occur in human cells. The complete genome sequence of *S. cerevisiae* was published in 1993 revealing around 6,200 genes on 16 chromosomes (Werner-Washburne et al., 1993). The open availability of the *Saccharomyces* Genome Database (http://www.yeastgenome.org/) and a culture of sharing molecular and microbiological tools and

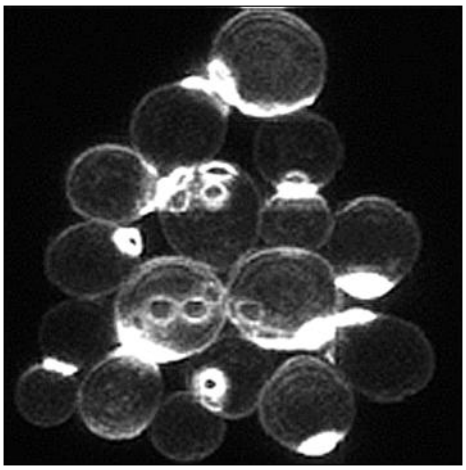


***Figure 1.1: Confocal microscope image of wild-type* S. cerevisiae *at a magnification of 100**. The yeast cell walls are stained with calcofluor. The circles on the yeast are bud scars. The image shown was recorded on a Leica TCS SP5 X instrument as described in Chapter 2.*

knowledge mean that this yeast has been used to address a wide range of biological and biotechnological questions. Moreover the ease at which experiments can be performed and at low costs further encourages its use (Rose and Harrison, 1971). Consequently, *S. cerevisiae* has been used for the study of many different cellular mechanisms, such as metabolism, translation, transcription and the cell cycle (the latter leading to a Nobel Prize for Paul Nurse in 2001). Yeast has also been valuable in the study of diseases that affect humans, for instance the cellular mechanisms for ageing and mitochondrial dysfunction (Feldmann, 2010).

1.1.1. S. cerevisiae *growth characteristics*

In a closed system, yeast growth comprises a lag phase, a log phase and a stationary phase (Werner-Washburne et al., 1993). The lag phase is the result of yeast cells changing their metabolism to enable growth on a new carbon source. The log phase is due to exponential growth. During the log phase on a fermentable carbon source, such as glucose, yeast cells have a respiro-fermentative metabolism (see section 1.2.1) and ethanol is released into the medium. When the fermentable carbon source has been used, the diauxic shift occurs (Figure 1.2) and the cells switch from a respiro-fermentative metabolism to respiration (Werner-Washburne et al., 1993) and begin to consume the ethanol. This period is known as the post-diauxic shift during which the yeast cells grow slowly and increase their carbohydrate stores. Once all the ethanol has been consumed, yeast cells enter stationary phase. During stationary phase, there is no increase in cell number due to limiting nutrient/s or a build-up of toxic products. Stationary phase cells have an increase in stress resistance, thick, less porous cell walls and a decrease in the levels of transcription and translation (Werner-Washburne et al., 1993). The production of glycogen stores begins just before the depletion of glucose and peaks at the diauxic shift (Werner-Washburne et al., 1993), whilst stores of trehalose begin to accumulate during the post-diauxic shift and peak as the cells enter stationary phase. Yeast cells can often survive for weeks or months in stationary phase, depending on the medium or strain (Werner-Washburne et al., 1993).

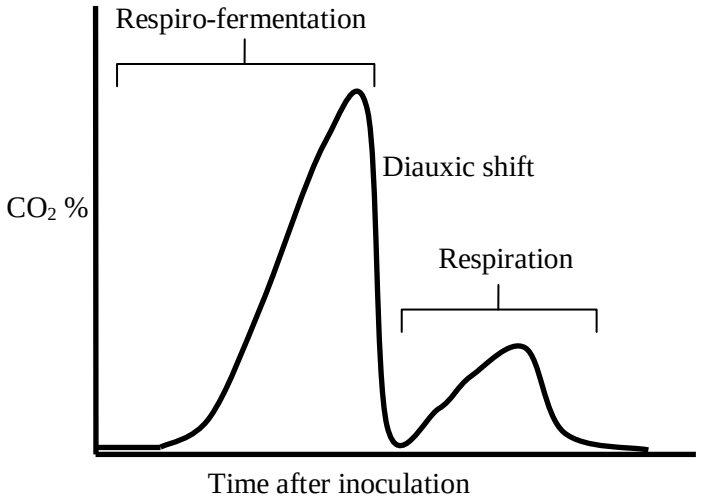


Figure 1.2: Representation of carbon dioxide gas profile. *The diauxic shift occurs once all the glucose has been used. The cells then begin to respire the ethanol that was produced during exponential growth.*

1.1.2. Cultivation of S. cerevisiae

Yeast can be grown either in solid or liquid media, where either complex or defined media can be used. When grown in liquid media, the cells can either be grown in shake flasks; where the environmental conditions are roughly controlled, such as pH and temperature. Alternatively, yeast can be cultivated in bioreactors, where the environmental conditions are tightly controlled. There are three different types of culturing processes:

- *Batch culture:* Is a closed system, where no medium is added or removed. As a result, the culture conditions will change over time.
- *Fed batch:* Is when medium or a particular substrate is added to the culture throughout the process. The culture conditions will change, but the exponential phase may be elongated depending on the substrate added. In the case of *S. cerevisiae*, fed batch is used to prevent the formation of ethanol and to increase the biomass (Ferndahl, 2006). Fed batch is also used for *P. pastoris*; methanol is added after exponential growth to trigger the activation of the *AOX1* promoter to induce expression of a protein.
- *Continuous culture*: Is an open system, where an equal volume of medium is added to that which is removed. The culturing conditions as a result do not change and the yeast remain in a defined phase (e.g. the log phase) for a prolonged period of time (Ferndahl, 2006).

In both studies for this thesis, batch culture conditions were used, where yeast cells were grown in shake flasks.

1.2. *Saccharomyces cerevisiae* as a model organism

Yeast has been valuable in the study of diseases that affect humans and is particularly important as a tool to study cellular mechanisms of ageing (Feldmann, 2010). For instance calorie restriction has been shown in *S. cerevisiae, Caenorhabditis elegans, Drosophila melanogaster* and mice to increase lifespan (Dilova et al., 2007). The pathways that calorie restriction target in both yeast and mice have been found to be similar. In yeast, calorie restriction has been shown to lower the activities of the nutrient sensing pathways, which involves Ras, Sch9 and TOR1 (Fabrizio et al., 2005). In mice calorie restriction, results in a decrease in insulin and thus a decrease in the insulin signalling pathway, which also involves Ras, mTOR and AKT (homologue of Sch9) (Fabrizio et al., 2005; Kaeberlein and Kennedy, 2007). These signalling pathway also play a role in yeast metabolism, and can influence the levels of respiration (Bonawitz et al., 2007; Lin et al., 2002). In yeast calorie restriction increases both the chronological and replicative lifespan (CLS and RLS) (Koubova and Guarente, 2003), where CLS is how long a yeast can survive in stationary phase, whilst RLS is the number of times a mother cell divides (Fabrizio and Longo, 2003; Kaeberlein et al., 2004). *S. cerevisiae* has also been suggested as a cancer model on account of its metabolism, which mimics that of cancer cells (Diaz-Ruiz et al., 2009). A focus of this thesis was the unique metabolic characteristics of yeast.

1.2.1. The metabolism of S. cerevisiae

Yeast can metabolise a range of hexose sugars such as fructose, mannose and galactose, but the fermentation of mannose and galactose is slower than the preferred carbon source glucose. Other sugars, such as pentose and sugar alcohols are not fermented (Rose and Harrison, 1971).

As a facultative anaerobic organism, *S. cerevisiae* can produce ATP in the presence or absence of oxygen. In the absence of oxygen, anaerobic metabolism takes place where glucose is metabolised to ethanol and carbon dioxide, during glycolysis (Figure 1.3). This form of metabolism results in the production of the carbon skeletons required for growth and the production of two ATP molecules. Ethanol is produced so that the NADH, which has been produced as a by-product of glycolysis, can be recycled to NAD^+, enabling further rounds of glycolysis (Rose and Harrison, 1971). During growth in the absence of oxygen, some NADH is also recycled to NAD^+ via the production of glycerol from dihydroxyacetone phosphate (Figure. 1.3).

Growth in the presence of oxygen results in aerobic metabolism, where glucose is fully metabolised to carbon dioxide and water, as respiration can take place and oxygen is used as the terminal electron acceptor. However, if large quantities of glucose are available to *S. cerevisiae*, this results in the partial inhibition of respiration, known as the Crabtree effect, where some of the glucose in the presence of oxygen is metabolised to ethanol and carbon dioxide from pyruvate, and the rest is fully metabolised to water and carbon dioxide: The Crabtree effect was first described by Herbert Crabtree in 1929 (Crabtree, 1929). Yeast species can either be divided in to Crabtree positive or Crabtree negative organisms. Many Crabtree negative yeast can ferment, but only in the absence of oxygen (Postma et al., 1989). *S. cerevisiae* is a Crabtree positive yeast (De Deken, 1966; Díaz-Ruiz et al., 2008) and, as there is inhibition of respiration during growth on glucose in the presence of oxygen, this results in mostly a respiro-fermentative metabolism. However, at higher glucose concentrations, complete inhibition of respiration may occur. This increase in fermentation and the production of ethanol results in rapidly growing cells, however there is a reduction in biomass compared to slow growing respiratory cells. There is also an increase in the production of glycerol from dihydroxyacetone phosphate (Frick and Wittmann, 2005). Glycolysis and respiration are not only important for the production of ATP, but many of the glycolytic products are anabolic precursors for the formation of amino acids, nucleotides and lipid synthesis, such as alanine from pyruvate, (Van Urk et al., 1990). During fermentation, since glycolytic rates are higher than during respiration, anabolic precursors can be made at a quicker rate, enabling quicker proliferation rates (Dang, 2012).

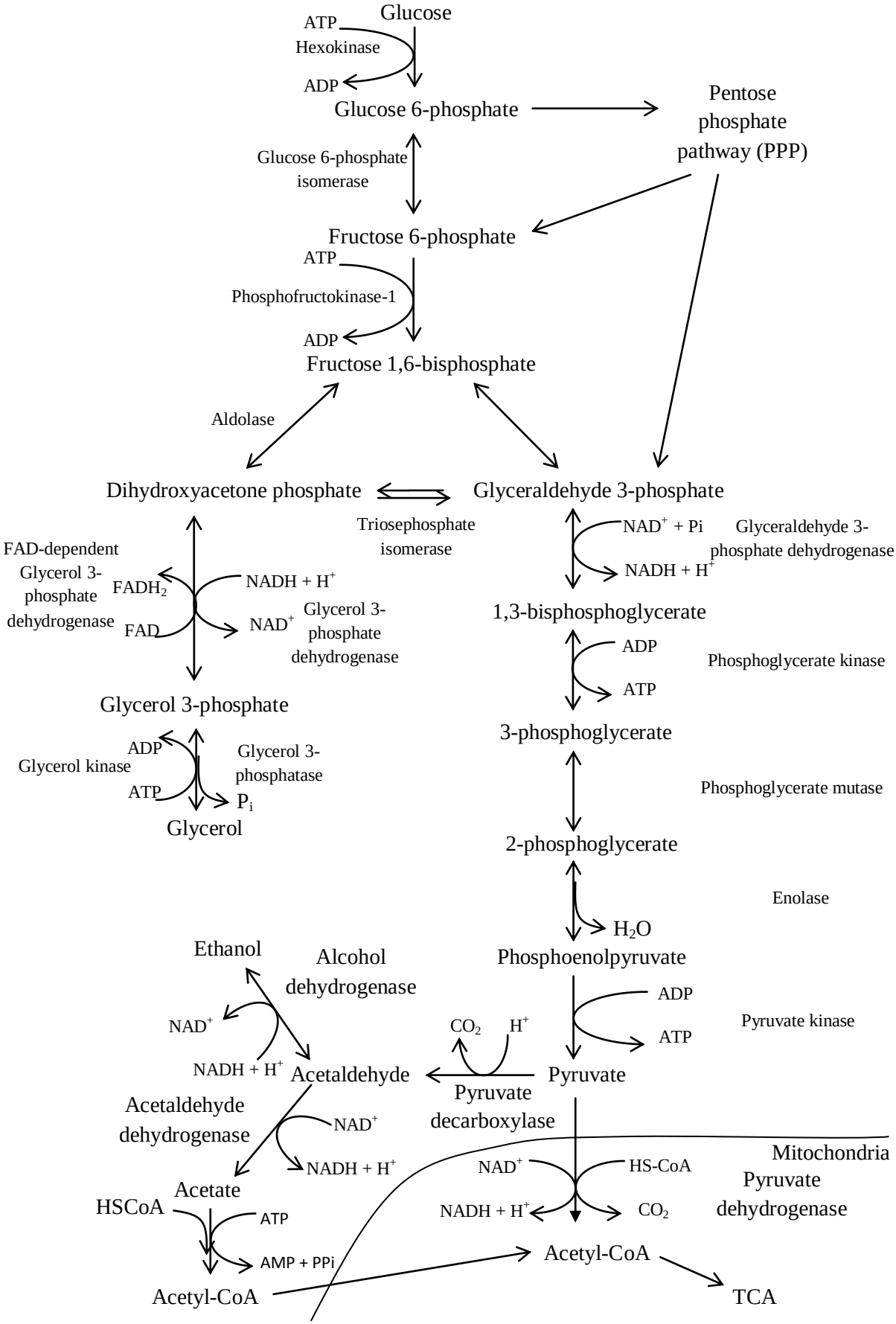


Figure 1.3: Representation of glycolysis and entry into the tricarboxylic acid cycle (TCA). *The diagram shows the individual steps of glycolysis, and where ATP is produced. Pyruvate is either taken up by the mitochondria or converted to acetaldehyde where it could either be converted to ethanol, during fermentation, or acetate. Moreover, the metabolism and production of glycerol is shown, adapted from (Diaz-Ruiz et al., 2009; Frick and Wittmann, 2005; Horton et al., 2006).*

The mechanisms behind the Crabtree effect are poorly understood. Current theories include (i) metabolic overflow from pyruvate to acetaldehyde and ethanol, as it is has been shown that the activities of pyruvate decarboxylase and alcohol dehydrogenase increase (Diaz-Ruiz et al., 2011), (ii) the competition of ADP and P_i between glycolysis and respiration (Diaz-Ruiz et al., 2011) or (iii) the inhibition of respiration by glycolytic intermediates (Diaz-Ruiz et al., 2011); for instance it has been shown that fructose 1,6-bisphosphate can inhibit respiration by inhibition of Complexes 3 and 4 of isolated *S. cerevisiae* mitochondria, but not respiration from Crabtree negative yeast (Diaz-Ruiz et al., 2011). Moreover the deletion strain *hxk2Δ*, which has lower levels of fructose 1,6-bisphosphate has higher respiratory rates than wild-type cells in the presence of glucose (Díaz-Ruiz et al., 2008).

Metabolic shifts from respiration to fermentation are associated with an increase in the rate at which glucose is taken up by yeast cells, an increase in the rate of glycolysis and glycolytic enzyme activities and a decrease in the metabolic flux of glucose to the pentose phosphate pathway. This results in a decrease in the availability of pentose phosphate precursors for the synthesis of nucleotides and a decrease in NADPH. It is likely, therefore, that the pentose phosphate pathway operates at high levels during respiration for the synthesis of NADPH, rather than to produce precursors for nucleotide synthesis; many pentose phosphate pathway products are filtered back into fructose-6-phosphate and glyceraldehyde-3-phosphate. There is also a decrease in the flux of pyruvate to mitochondria, resulting in a decrease in the TCA cycle and a decrease in TCA enzyme levels (Frick and Wittmann, 2005).

During fermentation, there is a decrease in gluconeogenic enzymes and enzymes involved in the metabolism of other sugars (Sierkstra et al., 1992). Pyruvate, rather than entering mitochondria is instead converted to acetaldehyde; it is important to note that during respiratory growth, a large amount of pyruvate is converted to acetaldehyde rather than entering the mitochondria. During fermentative growth this acetaldehyde is converted to ethanol, which results in the oxidation of NADH to NAD^+, enabling NAD^+ to be used for glycolysis. Under respiratory growth acetaldehyde is converted to acetate by acetaldehyde dehydrogenase, this acetate is then converted to acetyl-CoA, by acetyl-CoA synthetase, which then enters the mitochondria, and used in the TCA cycle (Frick and Wittmann, 2005). It is thought that there is a change in metabolic flux during a change from respiration to fermentation; this is because the K_M of pyruvate dehydrogenase is lower than that of pyruvate decarboxylase, thus under respiratory conditions, where concentrations of pyruvate are low, pyruvate enters the TCA cycle. This is also true for alcohol dehydrogenase, where its K_M is 100 times higher than acetaldehyde dehydrogenase (Van Dijken et al., 1993). Furthermore, pyruvate dehydrogenase and acetaldehyde dehydrogenase are thought to be working at full capacity during respiration, thus when glycolysis is increased, pyruvate and acetaldehyde levels increase and result in the formation of ethanol (Frick and Wittmann, 2005). Overall these metabolic changes in *S. cerevisiae* results in a respiro-fermentative metabolism when cells are grown in glucose-containing media even in the presence of oxygen.

When yeast is grown in concentrations of glucose above 5%, respiration is fully inhibited, resulting in a metabolism similar to that seen during anaerobic growth (Rose and Harrison, 1971). Respiratory growth results in the complete metabolism of glucose and generates about 15 times more energy in the form of ATP compared with glycolysis (Ferreira, 2010), but with slower growth rates (Pathania et al., 2009). The ability to ferment sugars in the presence of oxygen is thought to give *S. cerevisiae* a competitive advantage over other microorganisms as it can grow rapidly, while the production of ethanol prevents the growth of other microbes (Darby et al., 2012; Werner-Washburne et al., 1993). Respiration occurs in the mitochondria, where pyruvate is further metabolised during the tricarboxylic acid (TCA) cycle. During the TCA cycle the intermediates reduce NAD^+ to NADH and carbon skeletons are broken down to carbon dioxide. The organelles of the mitochondria have two membranes, resulting in the intermembrane space and the matrix. The innermembrane is folded to form cristea, and the electron transport chain and Complex 5 are located in the inner membrane, whilst the TCA enzymes are located in the matrix (Pathania et al., 2009). Succinate dehydrogenase, which is part of the TCA cycle, is also known as Complex 2 in the electron transport chain, however, unlike the other complexes, it does not transport hydrogen ions.

The electrons from NADH and $FADH_2$ can enter the electron transport chain through Complex 1 and Complex 2 respectively. The electrons are then transferred sequentially to uniquinol, Complex 3, cytochrome C, and finally to Complex 4, at which point they are used to reduce oxygen to water. When electrons are moving from one complex to the next, this provides energy to each complex, which allows it to pump hydrogen ions from the matrix and into the intermembrane space, to create a proton gradient. This proton gradient is then used to drive Complex 5 to synthesize ATP from ADP and P_i (Pathania et al., 2009).

1.2.2. S. cerevisiae: *a model for cancer cells*

S. cerevisiae has been suggested to be a good model system for studying cancer cells on account of its metabolic properties (Diaz-Ruiz et al., 2011). Cancer cells have uncontrolled cellular division, metastatic properties and a change in metabolism due to genetic mutations. Cancer cells have also been shown to have an inhibition in respiration, in the presence of oxygen, which was first documented by Otto Warburg in 1923, and as a result is referred to as the Warburg effect (Diaz-Ruiz et al., 2009). Due to the inhibition of respiration this results in the majority of ATP being produced from glycolysis, similar to yeast cells (Diaz-Ruiz et al., 2009). Moreover this inhibition leads to the production of lactic acid from pyruvate, by lactate dehydrogenase, to enable the production of NAD^+ from NADH, similar to the formation of ethanol in yeast (Figure 1.4) (Diaz-Ruiz et al., 2011). It is still unknown why many tumour cells down-regulate respiration, but it is thought that by adopting this phenotype it may give the cancer cells certain advantages (Diaz-Ruiz et al., 2009). These might include: the ability to grow in hypoxic conditions, due to poor vascularisation; the ability to avoid apoptosis as mitochondria play a role in cellular apoptosis; to become more invasive; to rapidly generate anabolic precursors; for rapid growth similar to yeast cells or being the result of mitochondrial dysfunction, due to mutations in

mitochondrial genes (Dang, 2012; Diaz-Ruiz et al., 2011; Pelicano et al., 2006). It is interesting to note that the mitochondria of cancer cells are still functional as they can produce anabolic precursors, such as fatty acids (Pathania et al., 2009).

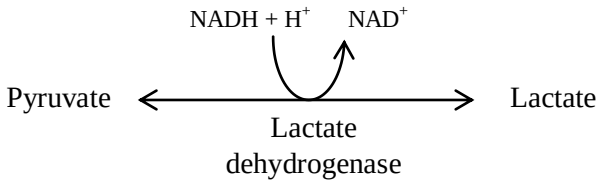


Figure 1.4: Formation of lactate. *In mammalian and cancer cells, if there is a reduction in respiration pyruvate is converted to lactate to enable the recycling of NAD^+, adapted from (Diaz-Ruiz et al., 2009).*

Similar to yeast cells, cancer cells have an increase in the rate of glycolysis and a decrease in TCA cycle activity (Diaz-Ruiz et al., 2011). The rate of glycolysis is increased in both yeast and cancer cells, due to an increase in the uptake of glucose, moreover some cancer cells have been shown to over-express glucose transporters (Diaz-Ruiz et al., 2011); the fact that cancer cells uptake glucose more rapidly than somatic cells is used in their identification by assaying with fluorodeoxyglucose (Ferreira, 2010). Furthermore, hexokinase which converts glucose to glucose-6-phosphate is inhibited in somatic cells by the levels of glucose-6-phosphate; in cancer cells this does not happen, which is similar to yeast cells as Hxk2 is not inhibited by glucose-6-phosphate. Moreover, the activities of phosphofructokinase and lactate dehydrogenase are increased in cancer cells; phosphofructokinase and alcohol dehydrogenase are increased in yeast cells (Diaz-Ruiz et al., 2011). The rate of glycolysis is also increased by mutations in signalling pathways which control growth and metabolism, such as phosphatidylinositide 3-kinase (PI3K) and the protein kinase B, AKT, which can activate glycolysis by increasing enzyme expression and glucose uptake. The PI3K pathway can have an increase in activity by genetic mutations in "phosphatase and tensin homolog" (PTEN) (Pathania et al., 2009), whilst AKT stimulates the mTOR pathway, which links cellular growth to nutrient availability, and can stimulate protein and lipid synthesis and ribosomal biogenesis (Cairns et al., 2011)

The TCA cycle is reduced in cancer due to the decreased expression of TCA enzymes, this also occurs in *S. cerevisiae* under respiro-fermentative conditions. In cancer cells this may be the result of mutations; for instance mutations in succinate dehydrogenase and fumate hydrate are associated with the formation of cancer (Dang, 2012; DeBerardinis, 2008). Also there may be a decrease in TCA cycle activity due to a change in metabolic flux from pyruvate entering the mitochondria to the formation of lactate, this change in metabolic flux can be the result of hypoxic conditions, which are often seen in cancer cells, resulting in hypoxic-inducible factor (HIF-1) activating pyruvate dehydrogenase kinase (PDK1) which inhibits the activity of pyruvate dehydrogenase (Dang, 2012).

Cancer treatments rely on differentiating between cancer cells and somatic cells; as a result, drugs that target their different metabolisms can be used. The theory is that as cancer cells rely on glycolysis for the generation of ATP, inhibition of glycolysis will result in cancer cell death. The drugs 3-bromopyruvate, which inhibits hexokinase, genilstein, which decreases glucose uptake, and oxalate, which inhibits pyruvate kinase, are all examples of molecules targeted at glycolysis (Cairns et al., 2011; Pelicano et al., 2006). However as cancers are a heterogenous population that can readily evolve, these types of drugs are most likely to be used in dual-hit therapies; in addition cancers can have many different mutations, making drug therapies difficult (Diaz-Ruiz et al., 2011).

1.2.3. L-Carnosine: a dipeptide with opposing effects on cancer and somatic cells

L-carnosine was first identified by Valdimir Gulevitsch in 1900 and is a dipeptide of L-histidine and β-alanine (Figure 1.5). Much of the early work on L-carnosine was done in Russia by Severin and Boldyrev (Boldyrev, 2012), but its precise physiological role is still unknown. However, it has been ascribed many properties such as buffering muscle pH, scavenging reactive oxygen species (ROS), preventing glycation, preventing lipid peroxidation and minimizing DNA damage (Boldyrev et al., 1999) (See appendix A.2. for a detailed account of L-carnosine properties). Due to its antioxidant properties, L-carnosine may have potential to be used in the treatment of age-related diseases (Hipkiss et al., 2013); furthermore L-carnosine can also inhibit the growth of cancer cells (Holliday and McFarland, 1996).

1.2.3.1. Biosynthesis and cellular localisation of L-carnosine

L-carnosine is synthesised in muscle and nerve cells by the ATP-dependent enzyme, carnosine synthetase (Figure 1.5), which was discovered in 1954. This enzyme has broad range specificity and can also produce carnosine-related compounds (CRC; such as homocarnosine and anserine), but at slower rates (Bauer, 2005; Bellia et al., 2011; Boldyrev, 2012). Carnosine synthetase can also produce β-alanyl-lysine and β-alanyl-arginine, which have been detected in muscle at low concentrations since they are degraded by β-alanyl-lysine dipeptidases (Boldyrev, 2012).

L-carnosine and CRC cannot be broken down by regular dipeptidases, due to the presence of the non-proteogenic amino acid, β-alanine (Boldyrev et al., 1999). Instead, L-carnosine is rapidly broken down by the dipeptidase, carnosinase, which was discovered in 1949 (Bellia et al., 2011; Lenney et al., 1985); there are two types of carnosinase, serum carnosinase, CN1, and cytosolic carnosinase, CN2. These two enzymes are structurally related, but have different properties. CN1 is secreted by the liver into the blood serum and has specific activity towards L-carnosine, although it can also break down homocarnosine and anserine at a slower rate. CN1 is also expressed in the central nervous system (CNS) and liver of humans (Bellia et al., 2011). As carnosinase is expressed in human blood serum, most of the L-carnosine taken up from the intestinal lumen is rapidly broken down to β-alanine and L-histidine. CN2 can break down most CRC except homocarnosine (Bellia et al., 2011).

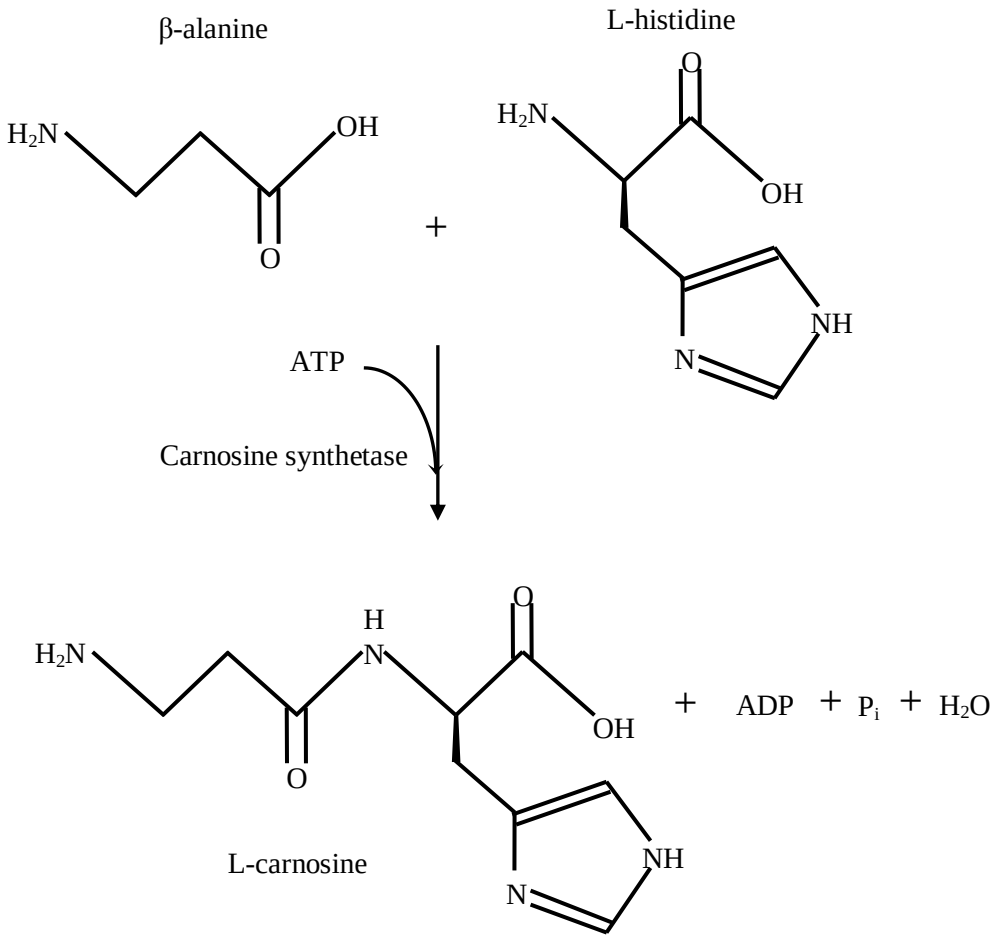


Figure 1.5: L-carnosine synthesis from the amino acids β-alanine and L-histidine. *L-carnosine is synthesised by carnosine synthetase from the amino acids β-alanine and L-histidine, adapted from (Caruso et al., 2012).*

L-carnosine and CRC are found in the skeletal muscle, cardiac muscle and the CNS of vertebrates (Boldyrev, 2012) (Table 1.1 and Figure 1.6). The dipeptides are found at high concentrations in these metabolically active tissues where they act as physiological buffers and protect them from oxidative stress (Kohen et al., 1988). Different CRC have slightly different physiological functions against oxidants, lipid peroxidation products and glycation, while different animals contain different CRC in their muscles at different concentrations. For instance, birds typically contain anserine in their skeletal muscles (Abe, 2000), whereas humans do not (Mannion et al., 1992), marine mammals contain high levels of ophidine, whilst animals that have a hopping locomotion such as kangaroos, rabbits and goats contain higher concentrations of anserine in their skeletal muscles (Abe, 2000). Homocarnosine on the other hand is located in human brain due to the availability of gamma-aminobutyric acid (GABA; GABA is not available in muscle) (Bellia et al., 2011), whilst N-acetylcarnosine is located in the CNS, skeletal muscle and cardiac muscle of humans (Babizhayev et al., 2009). Thus, L-carnosine and CRC levels are controlled by carnosine synthetase and carnosinase, and L-carnosine and CRC serve slightly different physiological functions.

D-carnosine is made from the amino acids β-alanine and D-histidine, is an unnatural dipeptide and is the optical isomer (enantiomer) of L-carnosine (Vigneaud and Hunt, 1936). As a result it has a different three-dimensional orientation, however it does share some properties with L-carnosine: it is a buffer (Boldyrev et al., 1999) and has been shown to protect against lipid peroxidation (Aldini et al., 2011).

Table 1.1: L-carnosine related compounds (CRC) which occur in vertebrates. *A range of different CRC occur in different species and at different concentrations, adapted from (Boldyrev, 2012).*

Carnosine related compounds (CRC)
L-carnosine (β-alanine-L-histidine)
Anserine (β-alanine -N1-methyl-L-histidine)
Homocarnosine (γ-aminobutyryl-L-histidine)
Ophidine (β-alanine –N3-methyl-L-histidine)
Homoanserine (γ-aminobutyryl-N1-methyl-L-histidine)
N-acetylcarnosine (N-acetyl- β-alanine-L-histidine)
N-acetylhomocarnosine(N-acetyl-γ-aminobutyryl-L-histidine)
N-acetylanserine (N-acetyl-B-alanyl-N1-methyl-L-histidine)
Carcinine (β-alanine-L-histidimine)

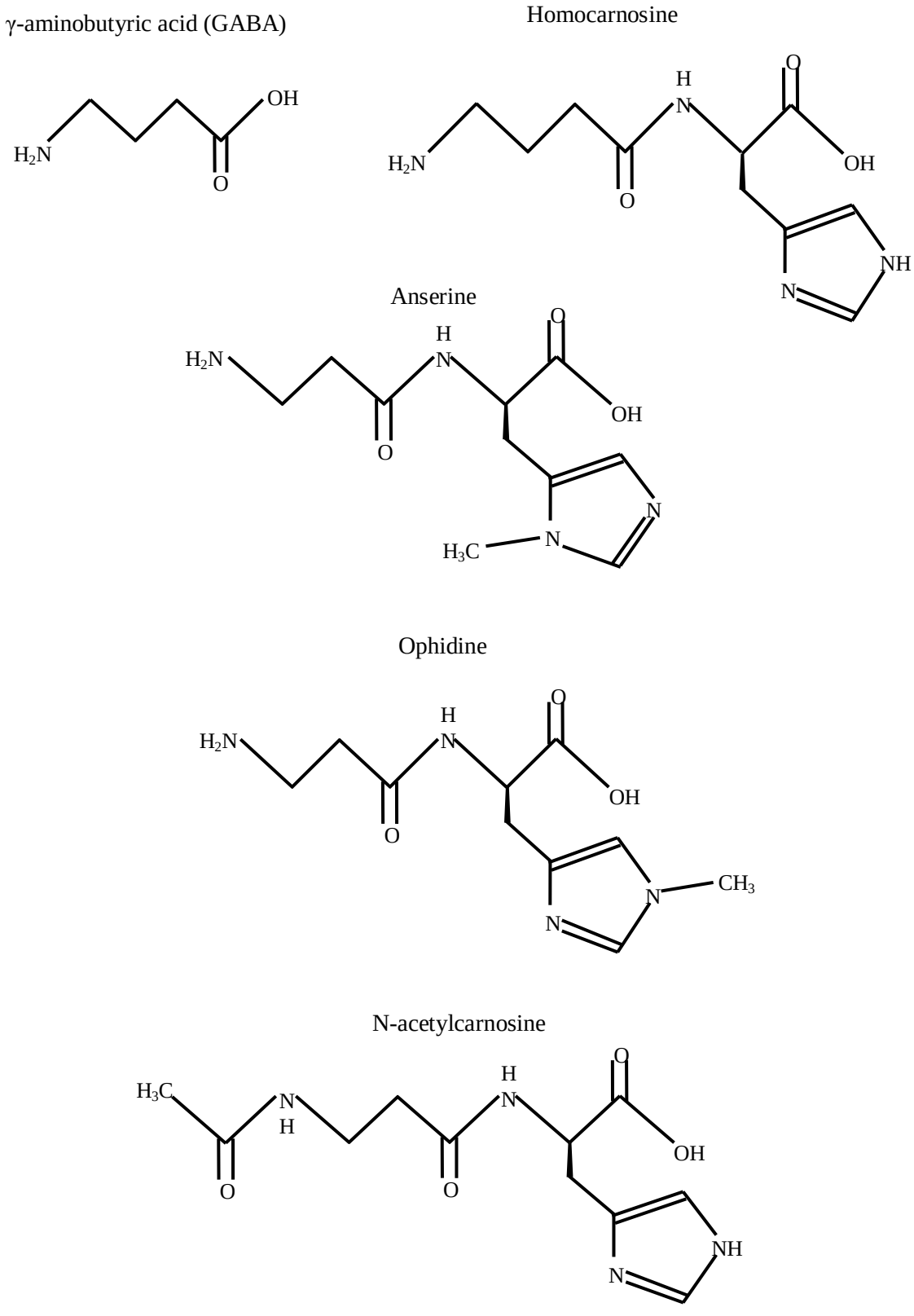


Figure 1.6: Structure of gamma-aminobutyric acid (GABA) and common L-carnosine related compounds (CRC). *Homocarnosine is made from L-histidine and GABA. Anserine and ophindine have methyl groups whereas, N-acetylcarnosine is acetylated, adapted from (Aruoma et al., 1989; Caruso et al., 2012).*

1.2.3.2. Cellular L-carnosine concentrations throughout the lifespan

It is known that L-carnosine concentrations vary greatly in humans, which may be due to differences between males and females (Baguet et al., 2011). It is also known that concentrations vary greatly between young and old (Baguet et al., 2011). Males contain 20-82% higher concentrations of muscle L-carnosine than females. This is probably due to males having a higher proportion of type 2 muscle fibres, which are typically associated with higher L-carnosine concentrations (Baguet et al., 2011; Mannion et al., 1992). Muscle L-carnosine concentrations have been shown to increase in men between pre-pubescence and adolescence and then to rapidly decrease until middle age at which point concentrations begin to plateau. The trend is similar for women; however, there is no significant increase in L-carnosine levels during puberty. It is also known that anaerobic capacity in all adults decreases around the ages of 30-40; this correlates with the decrease in L-carnosine concentrations in skeletal muscle. From studies on dizygotic and monozygotic twins, L-carnosine concentrations are known to be mostly influenced by environmental factors. However, in some muscle groups concentrations seem to be genetically determined (Baguet et al., 2011).

Older adults typically suffer from sarcopenia, which is a loss of muscle tissue and its replacement by fat. As there is also a decrease in muscle L-carnosine concentrations this could contribute to an increase in falls and problems with everyday activities (Del Favero et al., 2012). Del Favero et al., 2012 showed that β-alanine supplementation in old age increases muscle L-carnosine concentrations by 78% and this was marked by an increase in high intensity physical activity. However, there was no increase in the performance of mild exercise suggesting that increased L-carnosine concentrations may not improve the everyday physical activity of older adults.

1.2.3.3. The anti-senescent properties of L-carnosine

There is a correlation between the concentrations of L-carnosine and the lifespan of organisms, and hence it has been celebrated as an "anti-ageing" molecule (Hipkiss, 2000). For instance mice (with a typical lifespan of 3 years) have 1 mM L-carnosine in their skeletal muscle whereas humans (with a typical lifespan of around 70 years) have 20 mM (Hipkiss, 2000). Holliday and McFarland showed in 1994 that the addition of L-carnosine at physiological concentrations to cultured human fibroblasts, HFF-1 and MRC-5, resulted in an increase in population doublings by about 10, compared to control, suggesting that L-carnosine can increase the Hayflick limit (the finite number of cellular divisions that result in a senescent phenotype). L-carnosine also increases the CLS of cells, which is the total number of days cells are cultured before they become non-confluent (where confluence means the ability of cells to cover a culture dish). Moreover, L-carnosine addition to senescent cells resulted in rejuvenation, the senescent phenotype was reversed and the cells survived for longer. Furthermore, when L-carnosine-treated cells stopped dividing they had a less severe senescent phenotype than control cells. Interestingly the addition of L-carnosine slowed the growth rate of the cultures at 30 mM and more so at 50 mM. The addition of D-carnosine, homocarnosine, anserine or β-alanine did not have the same effect as L-carnosine, whilst L-histidine was toxic to fibroblasts.

L-carnosine and CRC have antioxidant and metal chelation properties, which may provide a mechanism for the increase in lifespan and rejuvenation. However, since other CRC do not increase the lifespan of cultured cells (McFarland and Holliday, 1994), and also other antioxidants such as vitamin C and E do not (Hipkiss et al., 2001), this suggests that it may be the anti-glycation abilities contributing to an increase in mammalian cell lifespan; L-carnosine can be glycated at β-alanine, thereby acting as a sacrificial peptide, (See appendix A.2.10 for more information on L-carnosine and glycation). It is known that advanced glycation end-products (AGEs; formed by condensation between an amine group of a protein side chain and a carboxyl group of a sugar) contribute to ageing (Hipkiss et al., 1995; McFarland and Holliday, 1994). L-carnosine may therefore decrease AGEs and as a result increase lifespan and rejuvenate senescent cells (McFarland and Holliday, 1994).

Bacterial lifespan can be decreased by increasing concentrations of glucose as this results in AGE formation, such as the commonly formed AGE carboxymethyl-lysine (CML) (Figure 1.7) which is formed from the glycation of glyoxal. CML can also be formed from the glycation of glucose with lysine to form fructose-lysine, which can be oxidatively cleaved to CML. L-carnosine can increase the CLS of *E. coli* cells in the presence of glucose, as it decreases the formation of CML at physiological concentrations (Pepper et al., 2010).

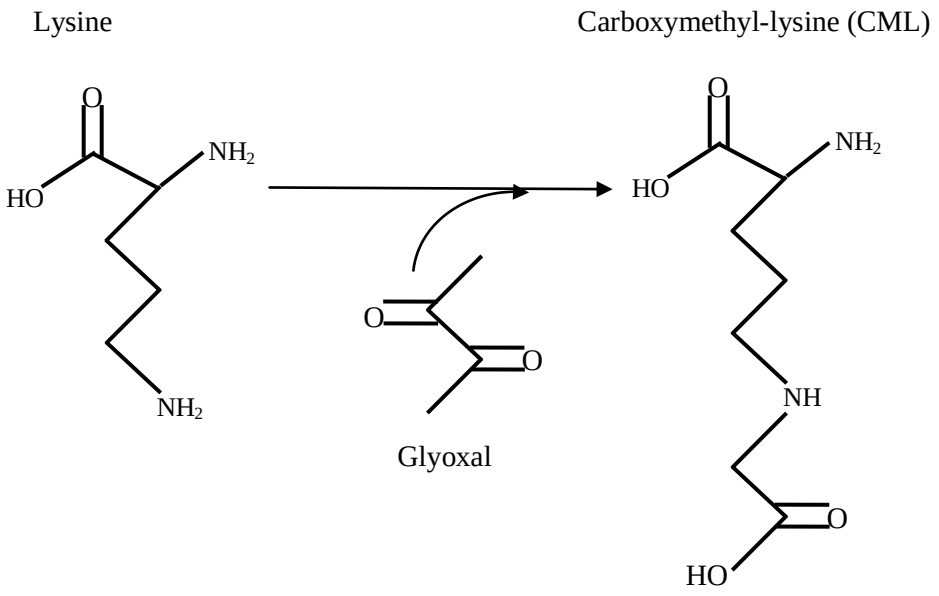


Figure 1.7: Structure of lysine and the common advanced glycation end product (AGE), carboxymethyl-lysine (CML). *Glyoxal reacts with the amine group of lysine's side chain, to form an AGE, adapted from (Price et al., 2001).*

L-carnosine supplementation increased the CLS of male flies since they have decreased oxidative stress resistance compared to female flies. L-Histidine or β-alanine supplementation did not increase the CLS of flies (Yuneva et al., 2002). In addition, Alexander Boldyrev showed in 1999 that L-carnosine supplementation at 100mg/kg of body weight increased the mean lifespan of senescence

accelerated mice (SAM), as SAM have an increase in ROS production. L-carnosine also decreased the age-associated features, so that the SAM looked healthier. B-alanine and L-histidine did not increase the mean lifespan of SAM. L-carnosine treatment also increased the mean lifespan of SAM-resistant mice, but not in a significant manner. Interestingly, even though L-carnosine increased the mean lifespan it did not significantly increase the SAM total lifespan. Also it is important to note that unlike humans, mice do not have as much carnosinase in their blood serum (Boldyrev et al., 1999). In conclusion it appears that L-carnosine supplementation increases mammalian cell lifespan, both replicative and chronological and this is attributed to its anti-glycation properties as it acts as a sacrificial peptide (See appendix A.2.11 for more information on L-carnosine and its anti-ageing properties).

1.2.3.4. The anti-cancer properties of L-carnosine

In 1996, Holliday and McFarland showed that transformed cells, which have cancer-like properties, did not grow in the presence of L-carnosine when there is no pyruvate in the medium. This was also shown to be the case for HeLa cells. Cancer cells in media containing pyruvate, along with L-carnosine grew similar to control cells; if the concentrations of pyruvate were decreased, this affected growth rate. They also showed that the addition of non-essential amino acids such as serine, glycine, alanine and glutamate supported slow growth of cancer cells in the presence of L-carnosine. Moreover, the addition of non-essential amino acids to the medium enabled lower concentrations of pyruvate to support cancer cell growth in the presence of L-carnosine. It is suggested that this may be because non-essential amino acids such as alanine can be metabolised to pyruvate. Cancer cell growth was also supported by the addition of TCA metabolites, oxaloacetate and α-ketogluterate. The addition of homocarnosine, D-carnosine and β-alanine did not have any inhibitory effects on cancer, whereas anserine prevented the growth of HeLa cells in the absence of pyruvate (Holliday and McFarland, 1996). The addition of L-carnosine to immortal embryonic stem cells, did not reduce their growth rate, indicating that L-carnosine does not specifically affect immortal cell lines, of which cancers are an example (McFarland and Holliday, 1999). Tenisetam, which is an anti-glycation product, also inhibited the growth of cancer cells in the absence of pyruvate.

Since pyruvate is central to metabolism, L-carnosine may be affecting metabolism. It is known that cancer cells rely heavily on glycolysis for ATP production, which is known as the Warburg effect. The authors attribute L-carnosine effects to the fact that it reacts with the triose sugars dihydroxyacetone phosphate and glyceraldehyde-3-phosphate *in vitro*, as a result, they suggest that L-carnosine is removing these products and thus decreasing the production of energy, as the triose sugars feed into the energy yielding half of glycolysis. Moreover, this will reduce the levels of pyruvate after glycolysis and also decrease ATP production from the TCA cycle. While normal cells can also make their energy from the TCA cycle, the addition of pyruvate to cancer cells may enable the TCA cycle to function (Holliday and McFarland, 1996).

The addition of L-carnosine to glioblastoma multiforme tumour cells, neuronal cancer cells NIH3T3-HER2 and HCT116 human colorectal carcinoma cells causes a reduction in ATP production in a dose-dependent manner (Iovine et al., 2012; Renner et al., 2010, 2008), specifically from glycolysis and not oxidative phosphorylation (Renner et al., 2010). There may be a decrease in ATP production as L-carnosine may react with essential metabolites such as the reactive sugars, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (Holliday and McFarland, 1996). This decrease in ATP production results in a decrease in proliferation rates (Renner et al., 2008). In 2010, Renner showed L-carnosine injections decreased the growth of NIH3T3-HER2 cancer cells, which had been injected under the skin of mice. However in two of the twelve mice treated with L-carnosine secondary cancers formed, suggesting that L-carnosine may aid in metastasis (Renner et al., 2010). In contrast, L-carnosine does not decrease ATP production in control cells (Iovine et al., 2012).

These data suggest that L-carnosine's ability to act as a sacrificial peptide for glycation and reduce ATP production could be causing the inhibition of cancer cell growth and as such L-carnosine could be used in the treatment of tumour formation. In conclusion L-carnosine has been shown to have many different properties over the last century: it has been shown to scavenge ROS, chelate metal ions, decrease lipid peroxidation and decrease glycation (Boldyrev et al., 1999). Due to these properties L-carnosine increases the lifespan of mammalian cells. This is attributed to its anti-glycation properties since other CRC and antioxidants do not have a similar effect. Due to its life extension properties, it has been proposed for the use in the treatment of age-related diseases. Furthermore, L-carnosine has also been shown to prevent the growth of cancer cells, but the mechanisms behind this are not very well understood; as other CRC and antioxidants do not have the same effect, L-carnosine's action has been attributed to its anti-glycation ability and its reaction with triose sugars which results in decreased ATP production, specifically from glycolysis which cancer cells rely on for energy production. An aim of this thesis was therefore to address the metabolism-dependence of L-carnosine's mechanism of action using yeast as a model organism.

1.3. *S. cerevisiae* as a biotechnological tool for recombinant protein production

A second aim of this thesis was to understand translational processes during the high-yielding production of recombinant proteins. Currently there are over 150 recombinant proteins approved by the US Food and Drug Administration (FDA) as drugs, with about 29% produced by *E.coli*, 18% by *S. cerevisiae*, 12% by hybridoma cells, 40% by mammalian cells, 0.5% by insect cells and 0.5% by transgenic animals (Ferrer-Miralles et al., 2009). This number is set to rise with the demands of an ageing world population (Byass, 2008; Christensen et al., 2009), coupled with an increasing demand for recombinant membrane protein drug targets to enable structure-aided drug design (Bonander and Bill, 2009).

Recombinant protein technologies were developed in the 1970s, with insulin being the first recombinant protein pharmaceutical available, produced from *E. coli* in the 1980s (Martínez et al., 2012). The approach is attractive as it allows the protein of interest to be produced and purified in

much larger quantities than could be naturally obtained. This is achieved by inserting the corresponding gene of interest into a suitable plasmid, with appropriate promoters, tags, signal sequences and genes for selection (Darby et al., 2012). The chosen cell factory is then transformed with the plasmid, which can either be inserted into the host's genome by homologous recombination or remain as an episomal plasmid (Darby et al., 2012); common cell factories are yeasts, bacteria and mammalian cells.

At industrial scale, therapeutic drugs and other products are most often produced in microbes and mammalian cell-lines; yield optimisation relies on a combination of modification of growth conditions, strain engineering and modification of the expression construct. Bacterial cells are used to produce amino acids, vitamins, organic acids, enzymes (such as lactase and α-amylase), and antibiotics such as penicillin from *Penicillium chrysogenum* (Demain and Vaishnav, 2009). *E. coli* is also used to make therapeutic drugs such as insulin, human growth hormone and insulin growth factor 1 using recombinant techniques (Ferrer-Miralles et al., 2009). The eukaryotic microbe *S. cerevisiae* is used in the brewing industry and to produce ethanol as a biofuel. This yeast is also used to produce therapeutic targets such as insulin, glucagon, human growth hormone and vaccines against Hepatitis B and human Papilloma viruses (Celik and Calık, 2012; Ferrer-Miralles et al., 2009). Mammalian cell lines are used to produce therapeutic targets, as well, such as erythropoietin, granulocyte colony stimulating factor and monoclonal antibodies (Demain and Vaishnav, 2009). Overall, different microbes and cell-lines are used for the production of a wide range of industrial relevant products, all of which have advantages and disadvantages that may dictate which one is chosen for a particular application (Table 1.2).

Table 1.2: Comparisons between common cell factories showing advantages and disadvantages. *The table provides information on the advantages of each cell factory, in regards to cost, growth rate, protein production yields, cell densities, post-translational modifications and the presence of cholesterol in the cell membrane.*

	E. coli	***Pichia pastoris***	***Saccharomyces cerevisiae***	**Insect cells**	**Mammalian cells**
Cost	Low	Low	Low	High	High
Growth rate	Very quick	quick	Quick	Slow	Slow
Production yields	High	High	moderate	Low	Low
Cell densities	High	Very high	High	Low	Low
Glycosylation	None	Not sialylated	Hypermannosylated	Not sialylated	Yes
Cholesterol	None	None	None	Yes	Yes

1.3.1. S. cerevisiae *as a cell factory: comparison with other commonly used hosts*

The advantages of using *S. cerevisiae* are that it has a rapid growth, thus products can be obtained quickly, the media are well defined and cheap, making the production of the protein cheaper than mammalian cell lines, it can grow to high cell densities, and high production yields can be achieved compared to mammalian cells. Moreover, it is easy to scale up experiments and the yeast is a GRAS organism. For the expression of human proteins, *S. cerevisiae* is a eukaryote, and as a result shares

similar cellular functions to mammalian cells, such as, translation and the secretory pathway (Ferrer-Miralles et al., 2009; O'Malley et al., 2009). In addition, there is a wealth of knowledge on its cellular processes, as it is used as a model organism for the study of metabolism, ageing, translation and other fundamental processes. Importantly for this study, the genome of the yeast has been sequenced and is freely available with deletion and tetracycline repressible essential gene sets available. Moreover, most post-translational modifications are similar such as disulphide bond formation; however, glycosylation of proteins is different, *S. cerevisiae* hyper-mannosylates N-linked glycans that may be immunogenic in humans (Celik and Calık, 2012). This is important as glycosylation may affect the folding, stability and function of proteins, however there are developments in humanising the glycolytic pathway (Celik and Calık, 2012; Demain and Vaishnav, 2009). Also importantly for membrane proteins, their membranes do not contain the sterol cholesterol, but instead contain the sterol ergosterol (Freigassner et al., 2009).

Pichia pastoris is another popular yeast to use in recombinant protein production, as it shares many of the advantages of *S. cerevisiae*, such as low costs and rapid growth. The key advantages of using *P. pastoris* over *S. cerevisiae* are that it grows to very high cell densities, enabling very high production yields (Ferrer-Miralles et al., 2009); importantly for membrane protein production *P. pastoris* does not hyper-mannosylate glycans nor are they immunogenic (Celik and Calık, 2012). However, the glycosylation pattern is still different from that of humans, most notably as they are not terminally sialylated. To address this *Pichia pastoris* strains have been generated with humanised glycosylation pathways, but these are not commercially available (Darby et al., 2012). In addition, to aid in very high yields of protein production, the promoter *AOX1* is used, which is the promoter for *AOX* genes, which control methanol utilisation; this promoter is very strong and tightly regulated. The main disadvantage of using *P. pastoris* is that it has relatively limited genetic resources available, as the genome was only made publically available in 2009. Moreover, the use of methanol to control a promoter can be dangerous and can lead to culture cell death, if levels are too high. However, other expression vectors with different promoters are available (Darby et al., 2012). Also similar to *S. cerevisiae* it lacks cholesterol in its membrane.

The use of *E. coli* in recombinant protein production has similar advantages to yeast, such as quick growth, thus the product is obtained quickly and at high yields, the experiments are cheap, it is easy to scale up production and there are many different molecular tools available to aid in expression (Demain and Vaishnav, 2009; Ferrer-Miralles et al., 2009). However, there are some key differences with *E. coli*, as it is a prokaryote. For instance its codon usage is different, thus a specific tRNA in *E. coli* may be rarer and affect translation and production (Ferrer-Miralles et al., 2009). Moreover elongation during translation is 4-10 times quicker, which could affect folding of eukaryotic proteins and result in the exposure of hydrophobic segments and aggregation. Importantly for membrane proteins, it has differences in post-translational modifications, as it does not glycosylate, phosphorylate, acetylate or acylate proyeins; this could affect the production of human membrane

proteins. Bacteria membranes also do not contain sterols, polyunsaturated fatty acids or sphingolipids (Freigassner et al., 2009).

Insect and mammalian cells provide an authentic environment for the production of human proteins as they have the right secretory pathway with the correct post-translational modifications for human proteins, thus the protein will be correctly folded. However, there are some disadvantages to these cell factories, which favours the production of human proteins in microbes: they are slow growing, expensive due to their requirement for complex media, have low cell densities, are more prone to contamination and result in low yields of protein (Martínez et al., 2012). Insect cells also have a slight difference in glycosylation compared to mammalian cells (Ferrer-Miralles et al., 2009).

1.3.2. Membrane proteins as recombinant targets

Membrane proteins can be peripheral or transmembrane and can be broadly classed as transporters or receptors, while some membrane proteins are both (Elbing, 2004). Not all molecules need to be transported across the membrane via a transport protein; for instance, gases, ethanol, water and fatty acids can all diffuse freely across the cellular membrane according to the concentration gradient. For those that do need to be transported there are two types of transporters, those that require energy and those that do not. Channels and carriers are involved in facilitated diffusion and do not require energy, they aid transport down a concentration gradient. Channels are pores that allow molecules into the cell based on size, whilst carriers are specific and bind the molecule to enable transport. The transporters that require energy can be split into two groups, primary and secondary active transporters (Elbing, 2004). Primary transporters require energy directly whereas, secondary transporters require energy indirectly (Elbing, 2004).

Receptors on the other hand are involved in cell-cell interactions, relay information from the external environment to the inside of the cell and can affect gene regulation. As a result, membrane proteins are involved in diseases such as neurodegeneration, cardiovascular disease, cancer, neurological diseases and others (Bill, 2001). Obtaining structural and functional information on membrane proteins is essential for the development of new pharmaceutical drugs in a rational manner (Bawa et al., 2011). Currently 50% of pharmaceutical drugs target membrane proteins (Freigassner et al., 2009); membrane proteins are involved in many diseases, as they are often the first protein in cellular pathways (Bawa et al., 2011). Members of the seven transmembrane domain G protein-coupled receptor (GPCR) family, in particular, are key drug targets due to their roles in disease. GPCRs are activated by many different small molecules, such as ions, odours, amino acids and can also be activated by light in the case of rhodopsin (Lundstrom et al., 2006).

1.3.3. The challenge of producing recombinant membrane proteins

Membrane proteins need to be overexpressed for further biochemical and biophysical study as they typically do not occur in high quantities in nature; however there are exceptions such as the GPCR, rhodopsin, and several members of the aquaporin family (Bawa et al., 2011). Often insufficient

quantities of correctly folded, stable protein are produced (Freigassner et al., 2009). The reasons for this are not completely clear, but several factors are likely to be responsible. For example, membrane proteins rely on the membrane to maintain their function and stability, with the GPCR adenosine A_{2a} receptor and rhodopsin relying on the sterol, cholesterol (Opekarová and Tanner, 2003). This interaction with membranes means membrane proteins need to be isolated with detergents, the presence of which make crystallography experiments challenging (Opekarová and Tanner, 2003). The interaction of the membrane protein with the membrane can also affect whether the membrane protein will fold and function correctly in a cell factory, which is not the native cell. Membrane compositions differ between species, as there is a range of different phospholipids, such as, phosphatidylcholine and phosphatidylserine, and also glycolipids and sterols that vary in their amounts and presence. For example the sterol, ergosterol, is present in *S. cerevisiae* membranes rather than cholesterol, which is found in mammalian membranes (Opekarová and Tanner, 2003).

Another difficulty in obtaining the structures of membrane proteins, such as GPCRs, is that they have flexible structures resulting in a heterogeneous population, which in turn affect crystal formation. To improve the stability of GPCRs and aid in crystal formation, some groups have inserted T4 lysosyme in an intracellular loop (Lundstrom et al., 2006). This approach has been successful in the case of the β-adrenergic receptor and adenosine A_{2a} receptor (Cherezov et al., 2007; Jaakola et al., 2008).

Unfortunately the high yields of recombinant protein produced in some systems may not necessarily translate into high yields of functional protein; the lack of correlation between protein production, quantified from western blot, and functional assays have been shown for a range of GPCRs in the yeast *P. pastoris* and mammalian cells (Lundstrom et al., 2006). Strong promoters and high copy number plasmids are used to obtain large quantities of mRNA and thus protein, but this can overload the secretory pathway resulting in misfolding and aggregation. A heterogenous mixture of correctly folded and misfolded proteins then affects subsequent experimentation such as biochemical analysis or crystal formation. It has been shown that by decreasing promoter strength and gene copy number, even though this will decrease protein production, this does not over load the cell and results in an increase in functional protein yield (Griffith et al., 2003). This was shown to be the case for the high yielding Walker strains of *E. coli*, which increased protein production yields. These strains have mutations in the promoter of T7 RNA polymerase (T7RNAP). T7RNAP is often used to drive transcription of recombinant proteins and is a strong promoter, resulting in high amounts of mRNA that would be likely to overload the translocon during translation. In the Walker strains there is a decrease in the levels of T7RNAP; a decrease in mRNA production and thus the cell factory is not overloaded resulting in an increase in functional protein (Wagner et al., 2008).

When a new membrane protein target is chosen, multiple rounds of trial and error experiments are typically performed. This is because when a particular host cell and experimental conditions have been developed for one membrane protein, this set of conditions does not necessarily work for other membrane proteins; even for membrane proteins which come from similar families (Tyo et al., 2012).

Trial and error experiments can include changing the cell factory and redesigning the vector system, such as promoters, tags, signal sequence and vector copy number (Bonander and Bill, 2009). It can also mean changing the growth conditions, such as temperature, aeration, pH and induction (methanol feeds, in the case of *P. pastoris*). Changing the medium composition can also have an effect, for example it has been shown that 2×CBS increases membrane protein yields in comparison to CSM, and this is thought to be due to the extra myo-inositol added (Bonander and Bill, 2012). Furthermore, additives have also been shown to aid in functional membrane protein production, such as, L-histidine (acting as an antioxidant) and DMSO (by increasing lipid biosynthesis) (Bonander and Bill, 2012). Trial and error experiments though can be very time consuming and often do not give a mechanistic understanding of why these sets of conditions increase protein yields (Bonander and Bill, 2012). To aid in decreasing the number of experiments performed, a design of experiments approach can be used (Bawa et al., 2011). In this type of experiment, predictive equations are generated to identify a set of conditions that will maximise protein production. This process is often used in industry, but not to a great extent in academic laboratories (Bawa et al., 2011). As trial and error experiments are very laborious, one aim of this thesis is to understand the mechanisms in high yielding strains so that this can be applied to the production of other proteins.

1.3.4. The production of the recombinant aquaporin, Fps1

In this thesis, the channel protein Fps1 was used for the development and the study of the mechanisms of high yielding *S. cerevisiae* strains. Fps1 has proven to be challenging to obtain in high yields (Bill et al., 2001), and therefore a body of research has built up on the recombinant production of this protein.

1.3.4.1. Function and regulation of Fps1

Fps1 is a *S. cerevisiae* channel protein and a member of the Major Intrinsic Protein (MIP) family. As such it has six transmembrane domains and is 669 amino acids long with very long hydrophilic N- and C- terminal domains which are located inside the cell (Figure 1.8) (Luyten et al., 1995).

Fps1 functions as a homotetramer in yeast (Beese-Sims et al., 2011) and is involved in glycerol transport. Fps1 can also transport polyols, glyceraldehydes, glycine, arsenite and acetic acid (Hohmann et al., 2000; Mollapour and Piper, 2007; Thorsen et al., 2006). When *FPS1* is deleted, yeast can still grow on glycerol as a metabolite with no change in growth rates. This is probably because glycerol can be taken up slowly through the plasma membrane (Luyten et al., 1995). Biologically, it appears that Fps1 is mostly associated with glycerol export. During anaerobic growth, there is an increase in glycerol production from the glycolytic intermediate dihydroxyacetone phosphate. The reason for this is to convert NADH to NAD^+ to maintain redox balance in the absence of oxygen (Luyten et al., 1995). Fps1 is used for glycerol export in these circumstances.

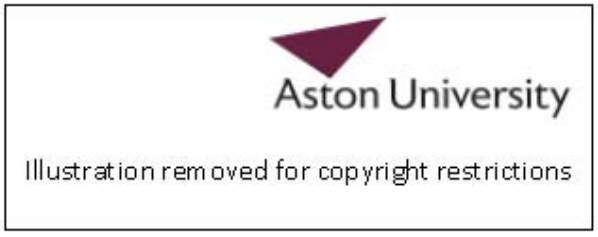


Figure 1.8: Diagrammatic representation of Fps1 in the membrane. *Fps1 has six transmembrane domains and long intracellular N and C-terminal tails, represented by a dotted line. The NPS and NLA sequences stand for asparagine-proline-serine and asparagine-leucine-alanine respectively which forms the pore of the Fps1 channel. Letters A-E represent the loop regions of Fps1, taken from (Bill et al., 2001).*

Fps1 is also involved in osmoregulation. Glycerol is used as a solute to achieve osmotic balance. During hyperosmotic shock there is a decrease in water availability and this can be achieved in the laboratory by the addition of 5% NaCl to the growth medium, which causes the loss of water from the cell. As a result, the high osmolarity glycerol (HOG) pathway is activated which in turn increases the expression of the glycerol producing enzymes, glycerol-3-phosphate dehydrogenase (Gpd1) and glycerol-3-phosphatase (Gpd2) which convert dihydroxyacetone phosphate to glycerol-3-phosphate and then to glycerol respectively (Tamás et al., 1999; Luyten et al., 1995). During this time the Fps1 pore is assumed to be closed in a HOG-independent manner (Luyten et al., 1995); it seems instead that turgor pressure may regulate Fps1 gating (Mollapour and Piper, 2007). *FPS1* mRNA levels have been shown not to be increased during hyperosmotic shock (Tamás et al., 1999) suggesting that regulation is controlled at the protein level. During hypo-osmotic shock, there is a decrease in salt concentrations in the medium, which can be achieved by moving yeast from YPD into a medium containing NaCl at 5% for 2h, and then moving the cells back to YPD (Luyten et al., 1995). This hypo-osmotic shock results in opening of the Fps1 pore as glycerol is released quickly from the cell, to avoid bursting. The levels of glycerol inside the cell can fall by 50-75% in 3 min. Yeast which have had *FPS1* deleted are sensitive to hypo-osmotic shock (Tamás et al., 1999).

It has been found that deletion of 12 amino acids in the N-terminus, near the first transmembrane domain, prevents the regulation of the Fps1 channel and results in an open channel, which is sensitive to hyper-osmotic shock. The same is true for the C-terminal domain where the deletion of 12 amino

acids near the sixth transmembrane domain affects regulation of the channel (Hedfalk et al., 2004; Mollapour and Piper, 2007). Interestingly when arsenite and acetic acid are taken up by the cell through the Fps1 channel this results in the activation of the HOG pathway and at the same time Hog1 has been shown to phophorylate Fps1 at threonine 231 during growth in the presence of arsenite (Thorsen et al., 2006). Growth in the presence of acetic acid on the other hand has been shown to cause phosphorylation at sites threonine 231 and serine 537 by Hog1 and in turn Fps1 is ubiquitinated, then endocytosed and degraded (Mollapour and Piper, 2007).

1.3.4.2. The production of Fps1 in high-yielding strains

In 2005, Bonander and colleagues began to explore the development of high yielding strains in the yeast *S. cerevisiae* using a transcriptomics approach (Bonander and Bill, 2009; Bonander et al., 2005). This resulted in the development of the high yielding strains yTHC*BMS1*, *srb5Δ, gcn5Δ* and *spt3Δ* which could increase the yield of their chosen membrane protein target, Fps1, by 70 fold, bypassing the need for trial and error experiments (Bonander and Bill, 2009; Bonander et al., 2005). In 2009 the same group began to unpick the mechanisms to why these strains are high yielding.

Initially Bonander showed that maximal membrane-bound Fps1 yields are achieved just before the diauxic shift, and moreover crude cell lysates could not be used to give accurate levels of membrane bound Fps1; instead membrane preparations were found to give reliable estimates of membrane inserted Fps1.

It was also shown that the optimal growth conditions of 30°C pH 5 were not the optimal conditions to achieve maximum membrane inserted Fps1, instead lowering the temperature to 20°C at pH5 resulted in an increase, but also resulted in a very slow growth rate of $0.07\mu\ h^{-1}$ (Bonander et al., 2005). A decrease in growth rate is often associated with a decrease in translation, which might enable an improvement in protein folding and membrane insertion (Mattanovich et al., 2004).

The increase in Fps1 yield observed at 20°C, pH5 was shown not to be the result of an increase in *FPS1* transcripts. Thus a transcriptomics approach was used to determine which genes were differentially regulated between normal growth conditions of 30°C pH5 and both low yielding conditions of 35°C pH5 and 35°C pH7. Low yielding conditions were chosen for the comparison as more than one could be identified; this enabled effects due to a change in culture conditions to be excluded. In the final analysis, only genes that changed in the same direction when comparing *both* low yielding conditions with normal growth conditions were selected for further analysis. This gave a total of 39 genes which were differentially regulated in both microarrays (Bonander et al., 2005). This gene set was further reduced when normal growth conditions of 30°C pH5 was then compared to high yielding conditions of 20°C pH5, giving 30 genes which when up-regulated during high yielding conditions were down regulated during low yielding conditions and *vice versa*. Of these genes only two were then shown to increase Fps1 production when altered on their own, this included *SRB6* and *BMS1* (Bonander and Bill, 2009). Related genes to those which were associated with an increase in

Fps1 yield were also analysed and this identified the deletion strains *spt3Δ, gcn5Δ* and *srb5Δ* as high-yielding strains.

Bms1 is involved in 40S ribosomal subunit biogenesis: it is a nuclear protein mostly located in the nucleolus and has been shown to be involved in 35S pre-rRNA processing where it recruits other ribosomal processing factors (Gelperin et al., 2001). It has been shown that defects in Bms1 result in a decrease in 20S rRNA, which results in a decrease in 18S rRNA and thus a decrease in levels of 40S, whereas the levels of the rRNA associated with 60S are unchanged (Gelperin et al., 2001; Perez-Fernandez et al., 2011). *BMS1* is an essential gene thus in the strain yTHC*BMS1*, its promoter has been replaced with a tetracycline titratable promoter that enables regulation of gene expression with doxycycline. When there is no doxycycline in the medium, the promoter is fully activated; doxycycline addition reduces gene expression (Mnaimneh et al., 2004). It was found that the addition of doxycycline which resulted in maximal Fps1 yield in this strain was 0.5µg/mL which gave a 78 fold increase in Fps1 yield over wild-type strains in bioreactors (Bonander et al., 2009). In yTHC*BMS1* (with doxycycline at 0.5µg/mL) there was a decrease in the 40S ribosomal subunit; 40S could not be detected at 10µg/mL doxycycline. Also the levels of 25S to 18S were compared; 25S is an rRNA component of the 60S subunit and 18S is an rRNA component of the 40S subunit. On increasing concentrations of doxycycline, there was a decrease in the levels of 18S, whereas the 25S levels did not change (Bonander et al., 2009).

The strains *spt3Δ, gcn5Δ* and *srb5Δ* increased Fps1 yield (compared to wild-type) in 2×CBS bioreactor cultures by 54 fold, 25 fold and 10 fold, respectively; 2×CBS medium gave higher protein production yields compared to CSM medium. Gcn5 and Spt3 are both part of the SAGA (Spt-Ada-Gcn5 acetyltransferase) complex (Liu et al., 2010). The SAGA complex is composed of around 20 subunits and is a co-activator involved in the initiation of transcription (Jacobson and Pillus, 2009). It is involved in the transcription of ~10% of the yeast genome; many of these genes are associated with stress such as heat shock, starvation, osmotic stress and DNA damage repair (Jacobson and Pillus, 2009; Kim et al., 2004). Gcn5 is a histone acetyl transferase, which acetylates histones and, as a result, DNA is more loosely bound to nucleosomes thus enabling gene transcription. Gcn5 is critical for SAGA activity and was first recognised to play a role in general amino acid control and aid transcription of amino acid biosynthetic enzymes (Liu et al., 2010). Gcn5 also is part of the ADA complex which drives transcription of a different subset of genes (Belotserkovskaya et al., 2000). Spt3 on the other hand has been shown to regulate the recruitment of the TATA-binding protein (TBP) to promoters (Laprade et al., 2007); with some genes it has been shown to aid in the recruitment of the TBP to the TATA box. However with other genes, such as *HIS3*, Spt3 has been shown to only release the TBP to the TATA box during amino acid starvation (Belotserkovskaya et al., 2000).

Srb5 is also involved in gene transcription and is part of the mediator complex (Mukundan and Ansari, 2011). The mediator complex is involved in integrating signals from activator and repressor proteins to the transcriptional machinery and is a co-activator involved in most gene transcription from RNA

polymerase II-transcribed genes. Interestingly mediator subunits can vary. Srb6 which has also been shown to increase Fps1 production is part of the mediator complex (Kremer and Gross, 2009).

All high yielding strains (yTHC*BMS1*, *srb5Δ, gcn5*Δ and *spt3Δ*) did not have changed *FPS1* transcript levels compared to wild-type (Bonander et al., 2009). In addition, the deletion strains *srb5Δ, gcn5Δ* and *spt3Δ,* were shown to have a ~6 fold increase in the levels of *BMS1* transcript (0.1 copies/cell in wild-type cells). The transcript levels in yTHC*BMS1* (with 0.5µg/mL doxycycline) was shown to be 0.5 copies/cell of *BMS1* transcript (Bonander and Bill, 2009; Bonander et al., 2009).

In addition to their effects on Fps1 yield, Bonander showed that the yTHC*BMS1* strain can double functional protein yields of the human adenosine A_{2a} receptor and the soluble protein, Green Fluorescent Protein (GFP) in the presence of 10µg/mL doxycycline (Bonander et al., 2009). Overall, the high yielding strains resulted in a 10-70 fold increase in Fps1 yields, where yTHC*BMS1* (with 0.5µg/mL doxycycline) resulted in the highest levels. A common characteristic amongst all high yielding strains was their increase in *BMS1* transcript levels. Moreover, yTHC*BMS1* (with 0.5µg/mL doxycycline) was shown to have a decrease in the 40S subunit suggesting a role for protein translation in high-yielding cell factories.

1.3.5. Mechanisms of translation

Protein synthesis or "translation" in all cells is performed by ribosomes, which are large complexes made from protein and ribosomal RNA (rRNA). Understanding translation is therefore central to understanding the mechanisms responsible for high recombinant protein yields. Key differences have emerged between translation in prokaryotes and eukaryotes (See appendix A.1.1. for a detailed account of translation in prokaryotes).

1.3.5.1. Ribosomes and their biogenesis

Eukaryotic ribosomes have a large 60S ribosomal subunit and small 40S ribosomal subunit in their cytoplasm, which makes the 80S ribosome (or monosome). The small ribosomal subunit has a mass of 1.4MDa and is comprised of 18S rRNA with about 33 associated ribosomal proteins. The large ribosomal subunit has a mass of 2.9MDa and is comprised of three rRNAs; 5S, 25S and 5.8S and has about 46 associated proteins (Marintchev and Wagner, 2004).

Ribosomal biogenesis is a highly energy consuming process and is tightly coupled to cellular growth and nutrient availability (Tschochner and Hurt, 2003). Ribosomal biogenesis initially occurs in the nucleolus, where the transcription of rRNA takes place (Tschochner and Hurt, 2003). The ribosomal RNA genes for 18S, 25S and 5.8S are all encoded on one transcript, known as the 35S pre-rRNA and are separated by transcribed spacers. The 35S rRNA is transcribed by RNA polymerase I. The 5S rRNA is on a separate transcript and is transcribed by RNA polymerase III. RNA polymerase II transcribes ribosomal proteins (Zemp and Kutay, 2007). Moreover, around 150 non-ribosomal factors are required for ribosomal maturation. Due to the use of three different RNA polymerases and

translational processes for the production of ribosomal proteins and non-ribosomal factors, all aspects of ribosomal biogenesis must be highly coordinated, so that the correct proportions of each component are made (Tschochner and Hurt, 2003).

The pre-35S rRNA along with the 5S rRNA, ribosomal proteins and the non-ribosomal proteins make the 90S pre-ribosome, which is cleaved at the 35S rRNA between the 20S and the 27S, resulting in the pre-40S and pre-60S (Tschochner and Hurt, 2003). The 27S is matured in the nucleoplasm to 25S and 5.8S. Further maturation of the pre-60S occurs in the nucleoplasm before being exported through the nuclear pore complex to the cytoplasm, where it under goes further maturation (Tschochner and Hurt, 2003). The pre-40S, soon after cleavage from the pre-60S, is exported from the nucleus to the cytoplasm, where the 20S rRNA undergoes further cleavage to 18S. The ribosomal proteins and the rRNA aid in ribosomal biogenesis, as they act in the recruitment of non-ribosomal proteins, thus mutations in ribosomal proteins can affect ribosomal biogenesis (Tschochner and Hurt, 2003; Zemp and Kutay, 2007).

1.3.5.2. Cap-dependent mechanism of translation

There are two mechanisms of translation in eukaryotes, cap-dependent and cap-independent mechanisms of translation (See appendix A.1.2. for a detailed account of cap-independent mechanism of translation). Cap-dependent translation can be split into three stages, initiation, elongation and termination (Figure 1.9). Unlike prokaryotes, where translation is initiated by the joining of the small ribosomal subunit to the SD sequence, the majority of eukaryotic translation takes place by ribosome scanning (Kozak, 2005). The scanning model relies on the recruitment of the 40S subunit to the 5′cap (7-methylguanosine cap; 7mG) of mRNA; once recruited, scanning in the 5′-3′ direction takes place until a start codon is located and translation can occur. This scanning model, known as "cap-dependent" translation, was elucidated in the early 1980s in eukaryotes by Kozak (Kozak, 2005).

Ribosomes are in equilibrium between 80S and the component 40S and 60S subunits; for initiation to take place the two ribosomal subunits must be disassociated from each other. Disassociation of 40S is promoted by the eukaryotic initiation factors (eIFs): eIF1, eIF1A, eIF5 and eIF3 (Merrick, 1992). The 40S subunit then associates with the ternary complex (TC), which is composed of eIF2·GTP and Met-$tRNA_i$. eIF2 itself is made from three subunits, α, β and γ. The affinity of eIF2·GTP is greater than that of eIF2·GDP for Met-$tRNA_i$ (Hinnebusch, 2011). Together the 40S subunit and the TC form the 43S-preinitiation complex (PIC). eIF4F is made from the subunits eIF4A, which is a RNA helicase, eIF4E and eIF4G. eIF4B and eIF4F associate with the 5′cap, which is located at the 5′end of the mRNA. mRNAs are thought to be in a closed loop structure as poly(A)-binding protein (PABP), is associated with the 5′ end of the mRNA through the interaction with eIF4G, which binds eIF4E. This closed looped structure is thought to aid in the re-initiation of protein translation. eIF4A which has helicase activity removes secondary structures in the mRNA, providing a region for the PIC to bind. The eIFs at the 5′ cap aid in the recruitment of the 43S PIC to the mRNA, through an interaction with eIF4G and

eIF3 to form the 48S complex. Once associated with the mRNA the PIC then scans for the start codon, AUG, which is an ATP-dependent process.

When the start codon enters the (P-site) of the 40S subunit, this is recognised by the anticodon of the Met-tRNA$_i$ (Merrick, 1992). If the anticodon is changed, this then results in translation from non-AUG (Kozak, 1992). Once it encounters the start codon, eIF5 (which is a GTPase-activating protein; GAP) is recruited and promotes the hydrolysis of eIF2·GTP to eIF2·GDP; this causes eIF2·GDP to disassociate from the 40S subunit, along with the eIFs. For a new TC to form, the eIF2·GDP must be recycled to eIF2·GTP by eIF2B, which is a guanine nucleotide exchange factor (GEF). The large ribosomal subunit 60S is then recruited, aided by eIF5B, to make the 80S ribosome (Hinnebusch, 2011). The association of the 60S ribosomal subunit leads to the formation of three pockets where tRNAs can be situated; the A-site (aminoacyl) is where new aminoacyl-tRNAs joins, the P-site (peptidyl) is where the growing peptide chain is located and the E-site (exit) is where tRNAs, which have been stripped of their amino acid, are located before leaving the ribosome. The starting tRNA, Met-tRNA$_i$ is located in the P-site. Aminoacyl-tRNAs which are recruited are bound by eEF1A·GTP, which binds to the empty A-site of the ribosome at which point if the anti-codon of the tRNA matches the codon of the mRNA eEF1A·GTP is hydrolysed to eEF1A·GDP and is released. eEF1A·GDP is recycled to eEF1A·GTP by eEF1B. A peptide bond is formed between the α-amino group of the aminoacyl-tRNA in the A site with the carbonyl group of the aminoacyl-tRNA in the P site.

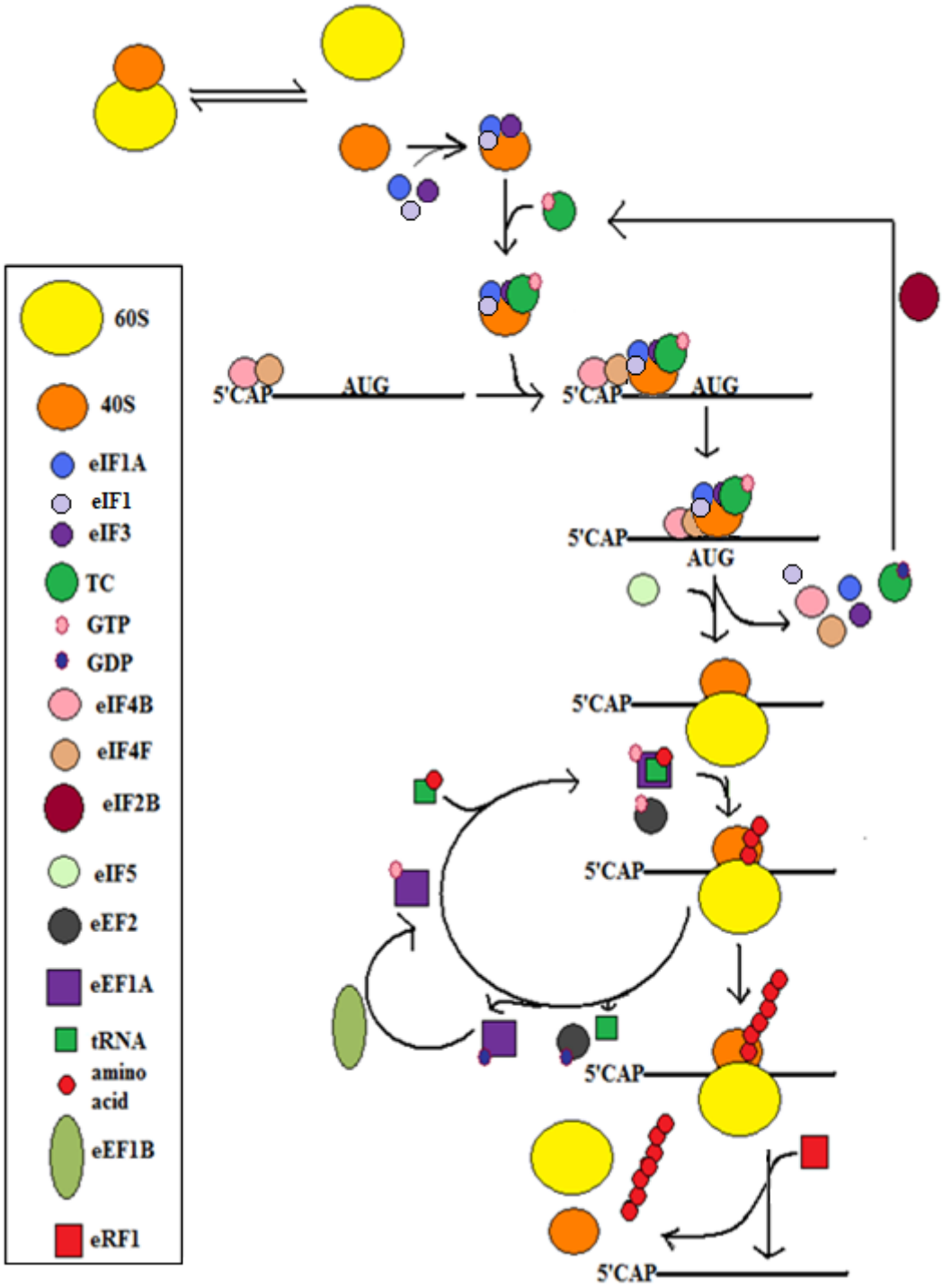


Figure 1.9: Representation of translation initiation. *Translation initiation begins with the joining of the eIFs to the 40S subunit, to create the PIC, which binds to the mRNA and begins to scan for the start codon. Once the start codon has been recognised, the 60S is recruited and the eIFs dissociate, enabling protein synthesis to occur, adapted from (Hinnebusch, 2005).*

When this happens, the stripped tRNA located in the P-site moves to the E-site whilst the one in the A-site moves to the P-site, so that a new aminoacyl-tRNA can enter the A site; this movement is promoted by eEF2·GTP. This process is repeated until a stop codon (UAA, UGA, UAG) is reached. Once a stop codon enters the A-site, a releasing factor, eRF1 enters the site and breaks the bond between the polypeptide, in the P-site and tRNA, causing its release. This is known as termination (Merrick, 1992).

Once termination has happened, the ribosome will either disassociate from the mRNA or will be involved in another round of translation. It is important to note that more than one ribosome can translate a copy of mRNA at a time; this is called a polysome (Figure 1.10). Initiation of translation is often rate limiting, and at times of stress the rate of initiation greatly decreases, resulting in fewer ribosomes attached to a single stand of mRNA. This means that there is an increase in the number of monosomes (single ribosomes per mRNA) or free 80S and a decrease in the number of polysomes in a cell (Merrick, 1992; Shenton et al., 2006).

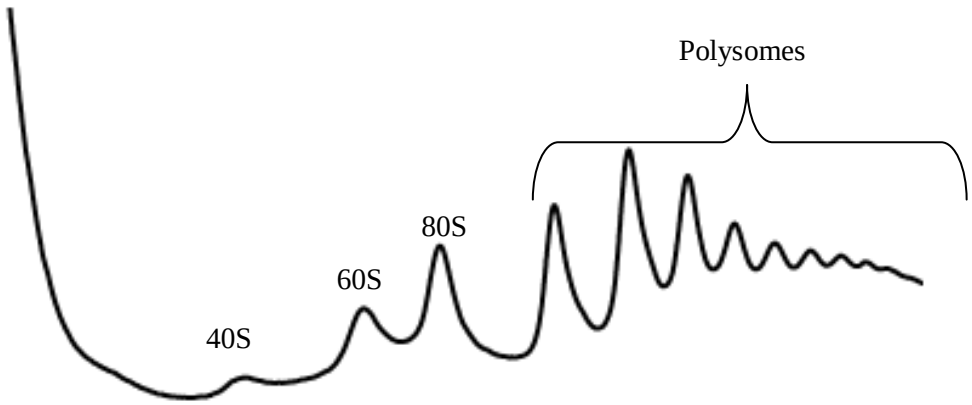


Figure 1.10: Representation of a polysome profile; a snap-shot of translational activity in a cell. *To generate a polysome profile, a cell lysate is isolated and layered on a sucrose gradient, which is used to separate fractions of RNA bound to one or more ribosomes according to size. Nucleic acid concentrations (recorded at 254nm) in each of the fractions are plotted, resulting in peaks which represent translating ribosomes. The 40S and 60S peaks represent free 40S and 60S not associated with mRNA, whilst the 80S (or monosome) peak represents either a single translating ribosome on mRNA or free 80S. The polysome peaks represent two, three, four or more ribosomes translating mRNA. The profile shown was recorded with the Gradient profiler instrument as described in Chapter 2.*

1.3.5.3. Consensus sequence and leaky scanning

Research by Marilyn Kozak in higher eukaryotes showed that a consensus sequence exists around the start codon that is (A/G)NNAUGG, where the A of the start codon is given the coordinate +1. A purine, A/G, at position -3 or a G at position +4 aids in efficient translation from the start codon (Kozak, 1981). Moreover Cs in positions -1, -2, -4 and -5 can also aid in start site recognition, especially when the consensus sequence deviates at positions -3 and +4 (Kozak, 2002). The consensus sequence is thought to aid start site recognition by stalling the scanning PIC. The hydrolysis of GTP on

eIF2 is controlled by pausing, a long pause causes eIF5 to activate hydrolysis. Mutations in eIF2 or eIF5 which causes faster rates of GTP hydrolysis initiate translation at non-AUG, such as UUG (Kozak, 1999). Translation can also start from non-AUG start codons in eukaryotes, but this is rare and when it does occur the same consensus sequence is used (Kozak, 1997). Consensus sequences also exist in *S. cerevisiae*, however they do not effect translation initiation to the same extent as in higher eukaryotes. The change of the A at position -3 can decrease translation from the start codon by 2-3 fold (Vilela and McCarthy, 2003).

During initiation of translation the scanning 40S can bypass start codons if they are surrounded by an unfavourable sequence which deviates from the consensus sequence, this is known as leaky scanning (Kozak, 1989). Leaky scanning also occurs in yeast, but not to the same extent as higher eukaryotes (Hinnebusch, 2011) (See appendix A.1.3. for further detail on leaky scanning).

1.3.5.4. uORF affect translation initiation

Upstream open reading frames are located in the 5´UTR of mRNA and can affect translation of the ORF, usually by down regulation (Lawless et al., 2009). The minimum structure of an uORF is a start codon, a sense codon and a stop codon (Hood et al., 2009). uORF in higher eukaryotes and *S. cerevisiae* are often short, for instance the average length of uORF in *S. cerevisiae* are 16 codons (Lawless et al., 2009)

uORFs can decrease translation at ORFs because the uORF is translated and the ribosomes dissociate from the mRNA after the translation of the uORF; for those ribosomes which remain attached, reinitiation of translation may not be able to occur before the ORF. Moreover, leaky scanning of the uORF can occur if the uORF's start codon deviates from the Kozak sequence, thus small amounts of translation of the ORF could take place. For instance, some proteins inside cells are required in small amounts as they can be detrimental to the cell, for example proteins which are cell regulators, such as cytokines, growth factor, kinases and transcription factors (Kozak, 2002). An example of such a protein is the yeast gene *CLN3*, which is a cyclin and is involved in the progression of cell cycle from G1 to S phase. *CLN3* contains an uORF to decrease levels of protein synthesis; if the uORF is deleted this causes an acceleration through the cell cycle (Vilela and McCarthy, 2003).

Reinitiation of translation can occur after the translation of an uORF; it is thought during termination of translation at an uORF, the 60S dissociates and depending on the sequence surrounding the termination codon, the 40S can remain attached (Kozak, 1987). A sequence rich in A/U promotes retention of the ribosome, whilst sequences rich in GC promote dissociation and prevent translation of the ORF (Vilela and McCarthy, 2003). If a 40S remains attached it will continue scanning for the next start codon. Reinitiation of translation is affected by the distance between the termination codon of the uORF and the start codon of the ORF; Kozak found that as the distance increased this aided in reinitiation. A distance of 79 nucleotides aided efficient reinitiation, the reason for this is the TC needs to be recruited for the identification of the start codon (Kozak, 1987); the levels of the TC can effect

reinitiation and regulate gene expression, such as with the yeast transcription factor *GCN4* (Vilela and McCarthy, 2003).

The translation of the ORF can also be affected by the length of the uORF, it has been shown that a codon length above 23 codons can decrease reinitiation and a length greater than 35 codons in *S. cerevisiae* can prevent reinitiation. It has also been shown that if the rate of elongation is decreased by the insertion of a pseudoknot in the uORF, this too decreases reinitiation. It is thought that reinitiation is decreased due to the loss of initiation factors (Kozak, 2001; Vilela and McCarthy, 2003) (See appendix A.1.4 for detail on yeast uORFs).

1.3.5.5. Translation initiation and stress

1.3.5.5.1. eIF2α phosphorylation decreases translation initiation

During times of stress, such as decreased amino acid and glucose availability, heat shock and viral infection (Merrick, 1992), translation is down regulated, often caused by a block in translation initiation. This may be to prevent errors occurring in new polypeptide chains, during error prone conditions. It could also occur to enable reprogramming of the cell, to enable genes which are required for survival during the stress to be made and to enable damage to be repaired (Shenton et al., 2006). The production of Gcn4, during amino acid starvation is an example of this.

An initiation block can be achieved by the phosphorylation of eIF2α at serine-51. In the case of amino acid starvation, phosphorylation of eIF2α is achieved by the protein kinase Gcn2. This phosphorylation of eIF2α causes it to competitively inhibit eIF2B, as it has a higher affinity for eIF2B than non-phosphorylated eIF2, and thus the recycling of eIF2·GDP to eIF2·GTP is reduced. As a result the number of TC decreases and an initiation block occurs. Only a small amount of phosphorylated eIF2α is required for an initiation block (Hinnebusch, 2011, 2005). Gcn2, which functions as a dimer, is activated during amino acid starvation by uncharged tRNAs binding to its histidyl-tRNA synthetase like domain (HisRE), at the C-terminal domain, which causes Gcn2 to autophosphorylate at threonine 882 and threonine 887 in the protein kinase domain (Cherkasova and Hinnebusch 2003). Gcn2 also has a ribosome binding domain, at the C-terminal domain, and forms a complex with Gcn1 and Gcn20 at the translating ribosomes as its N-terminal domain binds Gcn1 (Hinnebusch 2005). This is thought to help with the binding of uncharged tRNAs that enter the translating ribosome at the A site (Wang et al., 2000).

1.3.5.5.2. GCN4 *translation*

Gcn4 is a transcriptional activator and was initially shown to participate in the general amino acid control (GAAC) under amino acid starvation, resulting in the activation of over 30 different amino acid biosynthetic genes, even though only one amino acid had been removed (Hinnebusch and Fink, 1983; Hinnebusch, 2005). Moreover it has been shown that Gcn4 regulates the transcription of over 500 genes (Cherkasova and Hinnebusch, 2003). Gcn4 protein levels are controlled at the level of translation

as the *GCN4* transcript levels remain constant (Thireos et al., 1984). Under normal, non-starved conditions, the levels of Gcn4 protein are low, however during histidine starvation, the levels of Gcn4 protein increase six fold, which has been shown many times using the fusion transcript *GCN4-LacZ* (Hinnebusch, 1984). Gcn4 protein then binds to the promoters of amino acid biosynthetic genes and drives their transcription (Hinnebusch, 2005).

Amino acid starvation results in an initiation block, which decreases translation, but the converse happens in the case of *GCN4*, which is due to its unique 5´UTR, which contains uORFs. These uORFs enable an increase in translation of *GCN4* transcript under amino acid starved conditions or in a Gcd$^-$ (general control derepresed) phenotype, which result in constitutive derepression of amino acid genes as a result of constitutive levels of Gcn4 protein under non-starved conditions. Conversely mutation resulting in a Gcn$^-$ (general control non-inducible) phenotype results in constitutive repression even in the presence of amino acid starvation (Hinnebusch, 1984).

GCN4 has an unusually long 5´UTR at 577 bases which contains four upstream open reading frames, which are only 3-4 amino acids in length (Figure 1.11) (Hinnebusch et al., 1988). If the start codons of the uORFs are mutated out there is an increase in the production of Gcn4 under normal conditions by 40 fold, resulting in a Gcd$^-$ phenotype; the levels of Gcn4 produced are even greater than the levels seen during times of amino acid starvation, as none of the ribosomes fall off (Hinnebusch, 1984).

ATTAAAAAATTAAAGATCATTGAAAA*ATG*GCTTGC*TAA*ACCGATTATATTTTGTTTTTAAAGTAGATT
ATTATTAGAAAATTATTAAGAGAATT*ATG*TGT*TAA*ATTTATTGAAAGAGAAAATTTATTTTCCCTTAT
TAATTAAAGTCCTTTACTTTTTTTGAAAACTGTCAGTTTTTTGAAGAGTTATTTGTTTTGTTACCAAT
TGCTATC*ATG*TACCCG*TAG*AATTTTATTCAAG*ATG*TTTCCG*TAA*CGGTTACCTTTCTGTCAAATTATC
CAGGTTTACTCGCCAATAAAAATTTCCCTATACTATCATTAATTAAATCATTATTATTACTAAAGTTT
TGTTTACCAATTTGTCTGCTCAAGAAAATAAATTAAATACAAATAAA<u>ATG</u>TCCGAATATCAGCCAAGT
TTATTTGCTTTAAATCCAATGGGTTTCTCACCATTGGATGGT

***Figure 1.11: Sequence of the 5´UTR of* GCN4.** GCN4 *contains four uORFs, which are between 3 and 4 codons in length, the start and stop codons of the uORFs are highlighted in red.*

Moreover Alan Hinnbusch's group found that if uORF2 and uORF3 are removed from the *GCN4* transcript, this almost leads to wild-type regulation, enabling easier analysis of the *GCN4* transcript (Williams et al., 1988). A set of experiments by Alan Hinnebusch's group showed that uORF4, when present on its own, was a strong negative regulator of the expression of Gcn4 under amino acid starved conditions or in a Gcd$^-$ phenotype. It was shown to reduce *GCN4* translation 50 fold compared to a transcript with all uORF deleted (Miller and Hinnebusch, 1989), thus resulting in a Gcn$^-$ phenotype. uORF1 on the other hand was shown to be a weak negative regulator, which only decreases Gcn4 levels two fold compared with a transcript with no uORFs. At the same time though uORF1 is a

positive regulator of *GCN4* translation, as when present with uORF4 this results in Gcn4 protein production, determined by the reporter protein β-galactosidase, compared with uORF4 on its own. uORF3 is also a negative regulator, whilst uORF2 is a weak negative regulator, similar to uORF1 (Williams et al., 1988).

During normal and amino acid starved conditions, uORF1 is translated in both instances. When termination of translation occurs of the first uORF it is known that half of the ribosomes dissociate whilst the other half remain attached to the mRNA; this is due to the AT rich sequence surrounding the termination codon. If the uORF1 termination sequence is exchanged for the negative regulator uORF4 termination sequence which is GC rich, it results in the majority of ribosomes disassociating from the mRNA, due to greater stability in base pairing than between A and T in mRNA (Grant and Hinnebusch, 1994; Hinnebusch, 2005); the remaining ribosomes continue to scan. Under normal conditions the TC is at high concentrations and joins the scanning 40S between the space of uORF1 and UORF4, which enables the 40S to recognise AUG codons and translate uORF2-4, depending upon when the TC rejoins; thus Gcn4 levels are very low (Figure 1.12).

During times of stress, such as, amino acid starvation, there are fewer TC. As a result, the first uORF is translated, but this time 50% of the remaining scanning 40S scan through uORF2-4 as the TC levels are low due to eIF2α phosphorylation. The TC then associate with the PIC before the ORF of *GCN4*, as there is a long stretch of bases (148 nucleotides) between uORF4 and the *GCN4* ORF. As a result under amino acid starvation or in a Gcd^- phenotype *GCN4* is translated. The other 50%, which remained attached to the mRNA transcript, after the translation of uORF1 reassemble the PIC before uORF2-4 during amino acid starvation and as a result dissociate from the mRNA (Figure 1.12) (Hinnbusch 2005).

Gcn4 protein levels are also regulated by degradation. Gcn4 is rapidly degraded if made under normal conditions in 3-4 min by the protein kinase Pho85 causing phosphorylation of Gcn4, which then causes ubiquitylation, by Cdc34 and degradation. Under amino acid starved conditions degradation is 5 times slower (Hinnebusch, 2005) (See appendix A.1.5. for further detail on the factors that affect *GCN4* translation).

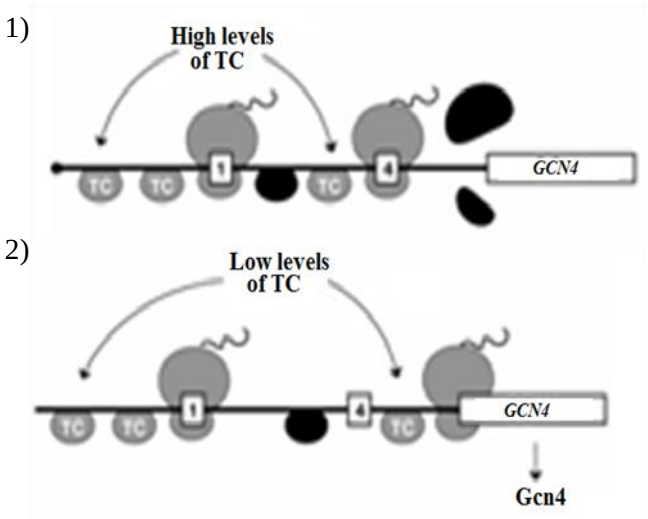


***Figure 1.12: Representation of translational regulation of* GCN4.** *1) During normal conditions, where TC levels are high and as a result* GCN4 *is not translated and 2) During amino acid starved conditions, where TC levels are low and as a result the negative uORFs are bypassed resulting in* GCN4 *translation . Adapted from (Hinnebusch, 2005).*

1.3.5.6. Translocation of membrane proteins

Membrane and secretory proteins are processed by the secretory pathway. These proteins either enter the endoplasmic reticulum (ER) co- or post-translationally. If they are inserted during translation, translation initiates in the cytosol, which results in the translation of a hydrophobic, 20 amino acid long N-terminal signal sequence. The signal sequence is removed upon translocation through the ER membrane. The signal sequence is bound by the signal recognition particle (SRP), which causes inhibition of elongation and directs the ribonucleoprotein to the ER. The SRP interacts with the SRP receptor (SR), at which point the ribonucleoprotein is transferred to the Sec61 translocon, during which GTP hydrolysis occurs and the SRP is released from the SR. Translation through the translocon can then occur (Figure 1.13). If the protein is a secretory protein it is threaded through the translocon, however if it is a membrane protein, the α-helices are inserted into the lipid bilayer laterally by the Sec61 complex. Membrane proteins that have several transmembrane domains are inserted into the membrane correctly with the use of multiple stop transfer sequences and signal anchor sequences (Wilkinson et al., 1997; Martínez-Gil et al., 2011).

Membrane proteins are usually inserted into the ER membrane co-translationally, but some membrane proteins and secretory proteins can be inserted into the ER membrane or lumen after translation. This requires, Hsp40 and Hsp70 to bind the newly formed protein to prevent it from folding so that it can be translocated into the ER by Sec61 with the help of Sec62 and Sec63 complex (Wilkinson et al., 1997; Martínez-Gil et al., 2011). When proteins are incorrectly folded in the ER and targeted for degradation, the Sec61 translocon is used to remove the protein (Zimmermann et al., 2011).

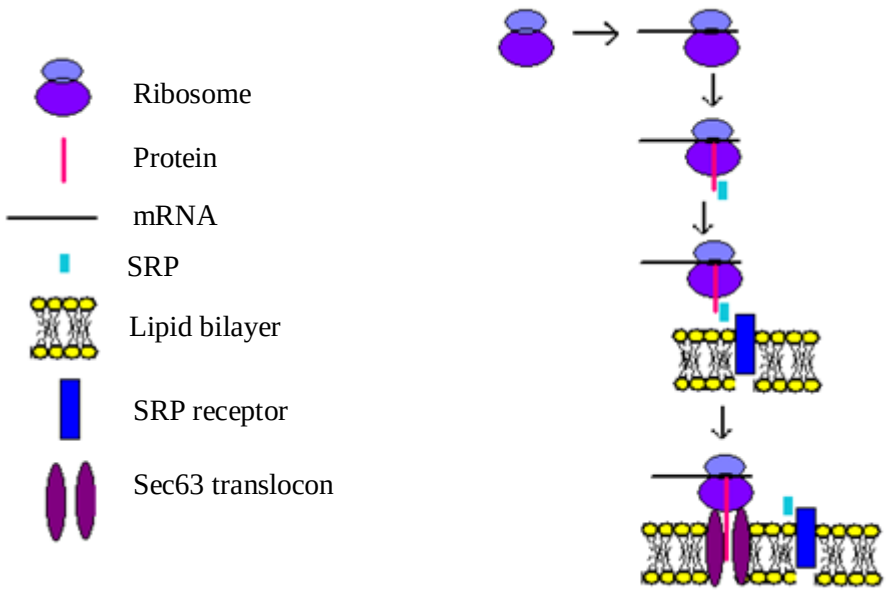


Figure 1.13: Representation of the secretory pathway. *Membrane proteins and secretory proteins are often processed co-translationally as they are inserted into the ER, this process is aided by the translocon Sec61, adapted from (Wilkinson et al., 1997).*

1.4. Project aims

S. cerevisiae is a flexible experimental organism because of the speed and low cost with which it can be grown to high cell yields, the knowledge that it is a GRAS organism and the ease with which its translational and metabolic pathways can be manipulated. In this thesis, these two pathways were explored in the context of their biotechnological application to ageing research: (i) understanding translational processes during the high-yielding production of membrane protein drug targets and (ii) the manipulation of yeast metabolism to study the molecule L-carnosine, which has been proposed to have anti-ageing properties. The overall aims of this thesis were therefore:

- To understand the mechanisms behind an increase in recombinant Fps1 yields in the high yielding strains yTHC*BMS1*, *srb5Δ*, *spt3Δ* and *gcn5Δ*. Previous evidence from studies on yTHC*BMS1* (with 0.5µg/mL doxycycline) suggest a change in the translational state of the cell, as yTHC*BMS1* was demonstrated to have a decrease in the levels of 40S subunit (Bonander et al., 2009). Since the other high yielding strains have similar levels of *BMS1* transcript, it is reasonable to hypothesise that that they might have similar characteristics. Thus, the first aim of the project was to examine the translational mechanisms responsible for high yields of Fps1.
- To establish that the action of L-carnosine is metabolism dependent using *S. cerevisiae* as a model organism, by taking advantage of its changed metabolism when grown on the fermentable carbon source glucose and the non-fermentable carbon source, glycerol. Thus, the second aim of the project was to examine, the mechanism of this metabolism dependence.

Chapter 2: Materials and Methods

2.1. Reagents and buffers

2.1.1. TFB I

30mM KOAc (ANH)

50mM $MnCl_2(4H_2O)$

100mM KCl (ANH)

10mM $CaCl_2(2H_2O)$

15%(v/v) glycerol

Solutions were made in double distilled sterile water, filter sterilised and stored at 4°C

2.1.2. TFBII

10mM MOPS(3-[N-Morpholin]propanesulfonic acid) pH7

75mM $CaCl_2$

10mM KCl

15% (v/v) glycerol

Solutions were made in double distilled sterile water, filter sterilised and stored at 4°C

2.1.3. Breaking Buffer pH7.4

50mM Na_2HPO_4

50mM NaH_2PO_4

2mM EDTA, pH7.4

100mM NaCl

5% Glycerol

Solutions were made in double distilled sterile water and autoclaved

2.1.4. Buffer A, pH7

20mM HEPES

50mM NaCl

10% glycerol

Solutions were made in double distilled sterile water and autoclaved

2.1.5. 5×Laemmli sample buffer

1.25mL 0.5M Tris-HCl pH 6.8

1mL 100% glycerol

2mL 10% SDS

0.5mL β-mercaptoethanol

10μL bromophenol blue

Solutions were made in double distilled sterile water to a final volume of 8mL

2.1.6. SDS Tris buffer (1L)

100mL 10×SDS Tris buffer (Geneflow)

900mL distilled water

2.1.7. Western Tris buffer (1L)

100mL 10×Tris buffer (Geneflow)

200mL methanol

700mL water

2.1.8. Phosphate buffer saline (PBS; 1L)

5 PBS tablets (Sigma) were dissolved in 1L of double distilled water

2.1.9. PBS-Tween 20 (PBST; 1L)

1L PBS

2mL Tween 20 (0.2%)

2.1.10. Lysis buffer

10mM Tris-HCl pH7.5

0.1M NaCl

30mM $MgCl_2$

50µg/mL cycloheximide (Molekula Dorset, UK)

200µg/mL heparin (Molekula)

0.2% DEP (Molekula)

Solutions were made to the required amount by the addition of cold double distilled sterile water on the day of use and kept on ice

2.1.11. Gradient buffer

50mM NH_4Cl

50mM Tris-OAc pH7

12mM $MgCl_2$

Solutions were made to the required amount with double distilled sterile water

2.1.12. Breaking buffer for β-galactosidase assay

100mM Tris-HCl pH8

1mM Dithiothreitol (DTT)

20% glycerol

Solutions were made up to the required amount with double distilled sterile water

2.1.13. Z buffer

60mM $Na_2HPO_4 \cdot 7H_2O$

40mM $NaH_2PO4 \cdot H_2O$

10mM KCl

1mM $MgSO_4 \cdot 7H_2O$ pH 7

Solutions were made up to the required amount with double distilled sterile water

2.1.14. 1M Potassium phosphate buffer, pH8 (1L)

940mL IM K_2HPO_4

60mL IM KH_2PO_4

2.1.15. L-Carnosine stock solutions

400mM L-carnosine (Molekula) was prepared by the addition of double distilled sterile water and pH adjusted to 6. This was then filter sterilised into an autoclaved bottle and stored at 4°C for no more than a month.

The same technique was used to make up stocks of β-alanine, L-histidine (Molekula) and D-carnosine (A generous gift of Flamma Chemicals, Milan, Italy).

2.1.16. Doxycycline

A 5mg/mL stock of doxycycline was made using double distilled water and filter sterilised into a falcon tube, stored at -20°C until required**.**

2.2. Equipment

All equipment used was standard laboratory equipment, and is referred to in the methodology. The centrifuges used are listed below:

Beckman Coulter Optima™ L-80XP Ultracentrifuge (Maximum revolutions per minute (rpm): 80,000)

Beckman Coulter Optima™ TLX Ultracentrifuge (Maximum rpm: 120,000)

Beckman Coulter Allegra 25R Centrifuge (Maximum rpm: 15,000)

Fisher Scientific Accuspin™ MicroR, benchtop (Maximum rpm: 13,000)

2.3. Microbial strains and culturing conditions

2.3.1. Yeast

Saccharomyces cerevisiae BY4741 (*MATα*, *ura3Δ0*, *leu2Δ0*, *met15Δ0*, *his3Δ1*) haploid strain is the parental strain of the deletion mutants *tor1*Δ, *tpk1*Δ, *gpa2*Δ, *gpr1*Δ, *hxk2*Δ, *rpl31a*Δ and *sch9*Δ *gcn5*Δ, *spt3*Δ *srb5*Δ *tpm1*Δ, *tpm2*Δ *mdm20*Δ *rpl22a*Δ, *rpl31a*Δ, *ssf1*Δ and *nop12*Δ (from the EUROSCARF collection: http://web.uni-frankfurt.de/fb15/mikro/euroscarf). yTHC*BMS1* has had its endogenous

promoter replaced with the tetracycline repressible promoter (Open Biosystems). *Pichia pastoris* strain X33 (*Mut+*, *His+*) was used which is the wild-type strain from Invitrogen.

Yeast cells from frozen glycerol stocks were grown on YPD plates for 2 days, incubated at 30°C. 5 mL of appropriate medium was then inoculated with a single yeast colony, grown to logarithmic phase and used to inoculate baffled shake-flasks containing at a culture to flask volume ratio of no more than 1 in 5. Cultures were incubated at 30 °C, 220 rpm until the required OD_{600} was obtained. OD_{600} was determined by diluting samples appropriately to be in the linear range of the spectrophotometer (IMPLEN OD600), often a 1 in 10 dilution. Growth rates of yeast were determined using Excel and the equations defining a logarithmic curve, using the equation below:

$$y=ce^{\mu x}$$

Where c is a constant, e is the exponential and μ is the specific growth rate.

The doubling time of the yeast were then calculated from the specific growth rate:

$$\text{Doubling time}= \ln(2)/\mu$$

To calculate the amount of inoculum needed to start an experiment the equation below was used:

$$\text{Inoculum}= ((OD_{600} \text{ required})/ (2^{(\text{Time}/ \text{ Doubling time})} \times OD_{600} \text{ currently})) \times \text{Volume in mL}$$

Where time represents the length of the experiment in h. OD_{600} represents the OD of the pre-inoculation, OD_{600} required represents the final OD_{600} required in the finished experiment. Volume in mL represents the volume of the experiment.

2.3.1.1. Yeast transformation

5mL YPD, inoculated with a single yeast colony, was cultured at 30°C, 220rpm overnight. 5mL YPD was then inoculated with the overnight culture at an OD_{600} ~ 0.25 and grown to OD_{600} ~1. Cells were harvested at 5,000rpm for 3min, suspended in sterile water, harvested again at 5,000rpm for 3min, suspended in 500μL 100mM lithium acetate (LiOAc) and transferred to a 1.5mL eppendorf tube**.** Cells were harvested at 13,000rpm for 15s, the supernatant removed and the cells suspended again in 500μL 100mM LiOAc. Salmon sperm DNA (ssDNA) was boiled for 5min and immediately chilled on ice. Yeast cells were then harvested at 13,000rpm for 15s, the supernatant removed and the transformation solution added sequentially (with the polyethyleneglycol (PEG) first to protect the cells from the highly concentrated LiOAc: 240μL PEG 3350 (50%w/v)**,** 36μL 1M LiOAc, 25μL ssDNA (2.0mg/mL), 50μL water and plasmid DNA (0.5μg of in ~1μL). The mixture was vortexed vigorously, incubated at 30°C for 30min and heat shocked in a water bath or hot plate for 20min at 42°C. Cells were harvested at 6,000rpm for 15s and the transformation mixture was removed with a pipette**.** 0.5-1mL sterile water was used to suspend the pellet gently with a pipette; 100μL cells were plated on selective plates.

2.3.1.2. Glycerol stocks

50% glycerol was made by adding 50mL water to 50mL glycerol and the solution was autoclaved. 1mL overnight yeast culture was put in cryovial with 1mL 50% glycerol, mixed, snap frozen in liquid nitrogen and stored at -80°C.

2.3.2. Bacteria

E. coli strain DH5α was used for vector amplification, while *E. coli* strain XL2-Blue (ultracompetent cells) from Agilent was used during molecular biology experiments

2.3.2.1. Preparation of competent DH5α cells

DH5α *E. coli* were grown on LB plates and incubated over night at 37°C. A single colony was cultured in 20mL LB medium overnight at 37°C, 220rpm. The next day, 100mL LB medium was inoculated with 2mL of the overnight culture and cultured until an OD_{600} of about 0.5-0.9 was reached (~2 h). This was then diluted with 500mL of LB in the same vessel and allowed to grow to OD_{600} 0.6 (about 40 min). The flask was put on ice-water and gently shaken for about 30 min to ensure rapid, even cooling. All subsequent steps were done at 4°C. A Beckman Coulter Allegra 25R Centrifuge was cooled to 4°C, the cold culture centrifuged at 4,200rpm for 10 min and supernatant removed. The pellet was suspended, by gentle pipetting, in 100mL cold TFB I (section 2.1.1) and incubated on ice for 1 h. Cells were collected by centrifugation at 3,000rpm for 10min at 4°C; after removing the supernatant, cells were drained and suspended by gentle pipetting in 20mL cold TFBII (section 2.1.2) and chilled on ice for 30 min. Cells were then divided into 200µL aliquots in ice-cold eppendorf tube, snap frozen in liquid nitrogen and stored at -80°C.

2.3.2.2. Transformation of DH5α cells

200µL competent DH5α were defrosted on ice, ~0.5µg plasmid DNA added to each eppendorf tube and the contents gently mixed and incubated on ice for 30 min. The tube was then incubated at 42°C for 90 s and transferred to ice for 2 min. 800µL warm (37°C) LB was added to the tube and incubated for 45 min at 37°C. 100µL was then cultured on a selective LB plate.

2.3.2.3. Transformation of ultracompetent XL2-Blue cells

Ultracompetent XL2-Blue cells were transformed with plasmid DNA according to the manufacturer's instructions (Agilent Technologies). 200µL of the transformation mixture was plated on LB agar plates containing the appropriate antibiotic (ampicillin 100µg/mL). For the pUC18 control transformation, 5µL was plated. Plates were incubated at 37°C overnight.

For pYX222-5´Δ1-43-*FPS1*-HA_3, pYX222-5´Δ1-215-*FPS1*-HA_3 and B1805-*HIS*, 10µL ligation mixture (ligation mixture; see section 2.4.6) was added to 100µL of cells. For control, digested vectors pYX222 and B1805, 10µL was added to 20µL of cells. For the control pUC18, 1µL was added to 10µL of cells and for the control with no vector added, 10µL of cells were used.

2.3.3. Growth media and components

2.3.3.1. Ampicillin

100mg/mL stocks were sterilised by syringe filter sterilisation into eppendorf tubes, and stored at -20°C until required. Stocks were diluted to a final concentration of 100µg/mL in the required media once cooled

2.3.3.2. 40% glucose (1L)

400g glucose per L double distilled water was filter sterilised into an autoclaved bottle.
The same protocol was followed for the sugars mannose, galactose, fructose, xylose, sorbitol and ribose

2.3.3.3. 40% glycerol (100mL)

40mL glycerol
60mL double distilled water
The solution was autoclaved prior to use

2.3.3.4. Yeast Peptone Dextrose (YPD) +/- Agar (1L)

10g (1%) Yeast extract
20g (2%) Peptone
20g (2%) Dextrose (Glucose)
20g (2%) Agar if making plates
Yeast extract, peptone (and agar for plates) were mixed with water and autoclaved. After autoclaving 2% glucose was added from a 40% filter sterilised stock.

2.3.3.5. Complete synthetic medium (CSM) +/- Agar (1L)

1.7g Yeast nitrogen base (YNB) without amino acids
5g Ammonium sulphate
20g Agar if making plates
The components were mixed with water and autoclaved. After autoclaving, 100mL autoclaved 10× drop out (DO) solution (see below); 100mL autoclaved 1M MES pH6 and 50mL of 40% filter sterilised glucose (2%) were added.

2.3.3.6. 2×CBS medium (1L)

10g Ammonium sulphate
6g Potassium dihydrogen phosphate
1g Magnessium sulphate heptahydrate
The components were mixed with water and autoclaved. After autoclaving, the following components were added:

50mL 40% filter sterilised glucose (final concentration of 2%)
100mL autoclaved 1M MES pH6
200mL autoclaved 10× DO solution (final concentration of 2×)
2mL filter sterilised vitamin solution
2mL autoclaved trace element solution
The medium was stored at 4°C prior to use

2.3.3.7. Trace element solution

3.75g EDTA and 1.125g Zinc sulphate heptahydrate were dissolved in 190mL water and the pH adjusted to pH6 with 1M NaOH.
The following components were then added sequentially whilst maintaining pH6
0.25g Magnessium chloride tetahydrate
0.075g Colbalt (II) chloride hexahydrate
0.075g Copper (II) sulphate pentahydrate
0.1g Sodium Molybdenum dehydrate
1.125g Calcium chloride dehydrate
0.75g Iron (II) sulphate heptahydrate
0.25g Boric acid
0.025g Potassium iodide
The pH was then adjusted to pH4 with 1M HCl and the final volume adjusted to 250mL with double distilled water. The bottle was covered in foil, the solution autoclaved and then stored at 4°C

2.3.3.8. Vitamin solution

0.0125g biotin was dissolved in 2.5mL 0.1M NaOH. 190mL water was added and the pH adjusted to pH6.5 with 1M HCl
The following components were then added sequentially whilst maintaining pH 6.5
0.25g Calcium-D-panthothenate
0.25g Nicotinic acid
6.25g Myo-inositol
0.25g Thiamine Hydrochloride (HCl)
0.25g Pyridoxol HCl
0.05g D-amino Benzoic acid
The final volume was adjusted to 250mL with double distilled water whilst maintaining pH6.5, the solution was filter sterilise and then stored at 4°C for a maximum of 6 months

2.3.3.9. 10× Drop Out (DO)solution (1L)

DO solution was made by omitting the required amino acid from:
200mg L-Adenine hemisulphate salt
200mg L-Arginine HCl

200mg L-Histidine HCl monohydrate
300mg L-Isoleucine
1,000mg L-Leucine
300mg L-Lysine HCl
200mg L-Methionine
500mg L-Phenylalanine
2000mg L-Threonine
200mg L-Tryptophan
300mg L-Tyrosine
200mg L-Uracil
1,500mg L-Valine
in 1L with double distilled water. The solution was autoclaved and stored at 4°C

2.3.3.10. Luria-Bertani (LB) E. coli media (1L) +/-Agar

20g LB (Sigma)
20g agar if making plates
Solutions were made in double distilled sterile water to a final volume of 1L and autoclaved. Ampicillin (Sigma) was then added to the cooled medium to a final concentration of 100μg/mL

2.4. Molecular biology

2.4.1. Vectors

Vector maps and sequences are provided as appendix A.3.

pYX222: contains the constitutive *TPI1* promoter, a multiple cloning site, a Kozak sequence after the *TPI1* promoter, ampicillin selection marker for selection in bacteria and the histidine selection marker for selection in yeast.

pYX212: contains the constitutive *TPI1* promoter, a multiple cloning site, a Kozak sequence after the *TPI1* promoter, ampicillin selection marker for selection in bacteria and the uracil selection marker for selection in yeast.

pYX222-*FPS1*-HA_3 and pYX212-*FPS1*-HA_3:The *FPS1* gene was inserted into pYX222 with a 3´tag, HA_3, to enable immunodetection. The C-terminal threonine was replaced with the sequence SGRIFYPYDVPDYA GYPYDVPDYA GYPYDVPDYAAQCGR where the three HA sequences are underlined. An *FPS1* fragment 249bp upstream of the *FPS1* ORF and 127bp downstream were inserted into the *BamH*I and *Hind*III site of pYX222.

pYX222-5´Δ1-43-*FPS1*-HA_3: pYX222 was digested with *Eco*RI and *Hind*III, to remove the Kozak sequence which is present after *Eco*RI. A forward primer containing an *Eco*RI consensus sequence and a reverse primer with a *Hind*III consensus sequence were designed (see 2.4.2 Primers) to amplify the

FPS1 gene with a truncated 5 ´ UTR, 216bp upstream of the *FPS1* ORF and which is native to the *FPS1* sequence. This *FPS1* segment was amplified from pYX222-*FPS1*-HA_3. The resulting PCR product was digested and ligated into digested pYX222 to create a vector without the Kozak sequence.

pYX222-5´Δ1-215-*FPS1*-HA_3: pYX222 was digested with *Eco*RI and *Hind*III, to remove the Kozak sequence which is present after *Eco*RI. A forward primer containing an *Eco*RI consensus sequence and a reverse primer containing a *Hind*III consensus sequence were designed (see 2.4.2 Primers) to amplify the *FPS1* gene with a truncated 5´UTR, 1bp upstream of the *FPS1* ORF. This removed the 5´uORF in the native *FPS1* sequence. This *FPS1* segment was amplified from pYX222-*FPS1*-HA_3. The resulting PCR product was digested and ligated into pYX222 to create a vector without the Kozak sequence and without the 5´uORF.

pYX222-αSS-*GFP*: contains the constitutive *TPI1* promoter, a multiple cloning site, a Kozak sequence after the *TPI1* promoter, ampicillin selection marker for selection in bacteria and the histidine selection marker for selection in yeast. The alpha secretion signal sequence and the GFP gene were cloned into pYX222 at the *Hind*III and *Xma*I site.

B1805: The vector B1805 was a gift from Mark Ashe and Alan Hinnebusch and contains the *GCN4* sequence with its native promoter and 5´UTR. The reporter gene *LacZ* was inserted into the *BamH*I site of the *GCN4* ORF (Alan Hinnebusch 1985, in which the vector is referred to as p180). The vector has *URA3* and ampicillin selection.

B1805-*HIS*: *URA3* was removed from B1805 by restriction digestion with *Xma*I and *Kas*I. The *HIS3* selection marker was amplified from pYX222. A forward primer containing an *Xma*I consensus sequence and a reverse primer containing a *Kas*I consensus sequence were designed (see 2.4.2 Primers). The products were ligated together.

2.4.2. Primers

PCR and sequencing primers were designed using Netprimer (http://www.premierbiosoft.com/netprimer/) to determine hairpin and dimer formation. Primers were designed to be approximately17 nucleotides long, ending in GC and with a preferred GC content of 40%, although this was not always possible due to sequence constraints. Six bases were added to the end of each primer to aid restriction enzyme digestion. Restriction enzyme consensus sequences are underlined. To aid in the design and correct sequence of the reverse primer the molecular tool kit from the website http://www.vivo.colostate.edu/molkit/ was used. Primers were purchased from Invitrogen.

pYX222-5′Δ1-43-*FPS1*-HA_3

Forward primer

5′ ccccccGAATTCGCGGGGAACAGTGTGAATC 3′ (T_m=58°C, GC=58%)

Reverse primer

5′ ccccccAAGCTTCTTTTTCTTGTCTGTTTTCTCAGCG3′ (T_m= 62°C, GC= 40%)

pYX222-5′Δ1-215-*FPS1*-HA_3

Forward primer

5′ ccccccGAATTCCATGAGTAATCCTCAAAAAGCTCTAAAC 3′ (T_m=61°C, GC=35%)

Reverse primer

5’ ccccccAAGCTTCTTTTTCTTGTCTGTTTTCTCAGCG3′ (T_m= 62°C, GC= 40%)

B1805-*HIS*

Forward primer

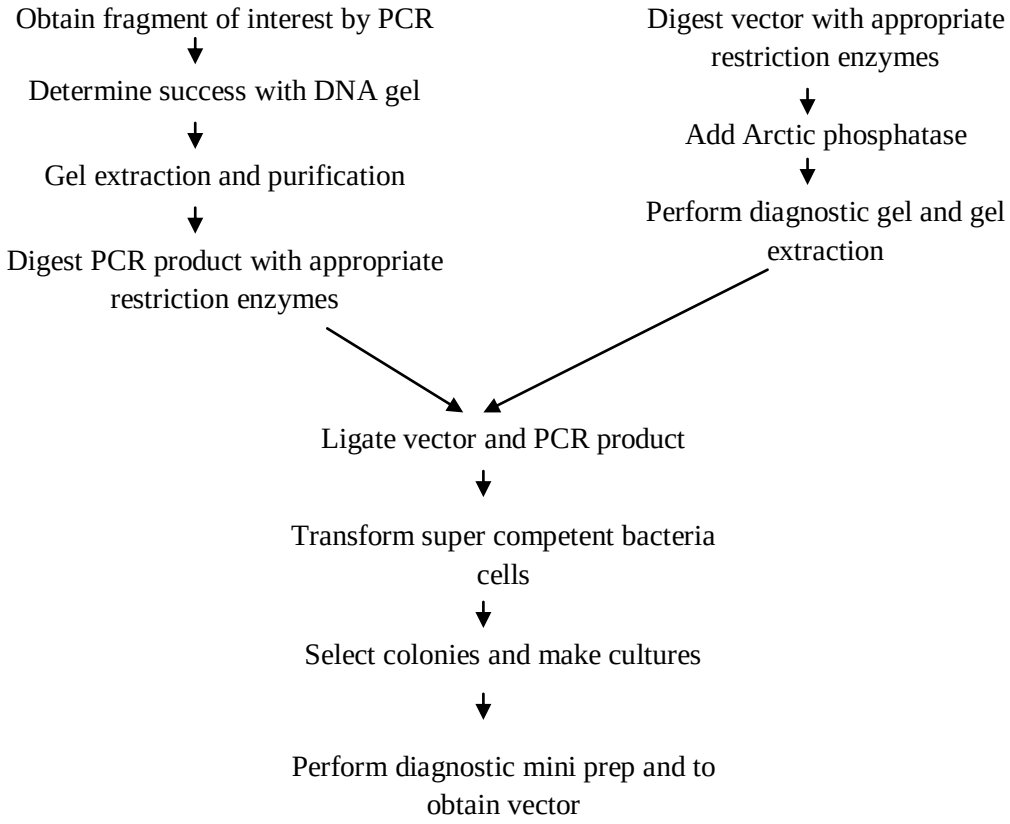
5′ ccccccGGCGCCGTATTTTCTCCTTACGCATC3′ (T_m=49°C, GC%=40)

Reverse primer

5′ggggggCCCGGGGGAGCAGACAAGCCCGTC3′ (T_m=58°C, GC%=66)

2.4.3. Polymerase chain reaction (PCR)

The overall strategy was:



The reaction comprised:

10µL 5× Phusion buffer, either High fidelity buffer or GC buffer (For templates rich in GC content) (final concentration was 1×)

1µL (200µM) 10mM dNTPs

2.5µL (0.5µM) 10µM forward primer

2.5µL (0.5µM) 10µM reverse primer

0.5µL (1U) Phusion polymerase (NEB)

3µL (15ng) Template DNA

1.5µL DMSO (100%) added if necessary (final concentration was 3%)

To 50µL with RNAase free water

A thermo cycler (PTC-200, MJ Research) was used and programmed accordingly:

1) 98°C, 2 min, denaturing

2) 98°C, 30 s

3) (45-55°C) 30 s, annealing

4) 72°C (3 min (1minute/kb)), extension

5) Repeat stages 2-4, 30 times

6) 72°C 10 min, extension

7) Hold at 4°C

For constructs pYX222-5´Δ1-43-*FPS1*-HA_3 and pYX222-5´Δ1-215-*FPS1*-HA_3 the annealing temperature was 55°C. For B1805-*HIS*, the annealing temperature was 45.5°C.

2.4.4. Restriction enzyme digestion

Restriction enzymes were purchased from New England Biolabs (NEB) and used according to the manufacturer's instructions, typically:

2µg DNA

0.5µL (10U) Restriction enzyme

0.5µL BSA

6µL appropriate restriction buffer

To 50µL with RNAase free water

The reaction was incubated at 37°C for 1 h followed by 65°C for 20 min to denature the enzyme

2.4.5. Dephosphorylation

To prevent any incompletely digested vector backbone from self- ligating, the following was added to a restriction digest mixture immediately after the denaturation step:

5µL Antarctic phosphatase buffer (NEB)

1µL Antarctic phosphatase

The reaction was incubated at 37°C for 30 min followed by 65°C for 5 min to denature the enzyme

2.4.6. Ligation

Ligation reactions comprised:

2.5µL T4 DNA ligase reaction buffer (10×)

1µL T4 ligase (NEB)

100ng vector

*x*ng insert, where *x* was calculated according to the equation: (ng of vector×size of insert in kb)/size of vector in kb)× molar ratio of (insert/vector) and the molar ratio used was 3:1 (insert:vector)

Make up to 25µL using RNAase free water

The reaction was incubated at room temperature for 3h or overnight followed by 65°C for 10 min to deactivate the enzyme.

2.4.7. Plasmid isolation

Following transformation, plasmids were isolated from single colonies on selective plates. A single colony was cultured in 5mL LB medium containing 100µg/mL ampicillin at 37°C and 220rpm, overnight. Cells were harvested at 5,000rpm for 3 min and the supernatant was discarded. Plasmids were extracted from the cell pellet following the protocol in the Thermo Scientific GeneJET Plasmid Miniprep Kit. Briefly, cells were resuspended in 250µL resuspension solution and transfered to an

eppendorf tube. 250µL lysis solution was added and mixed by inverting 5 times, until the solution cleared and became viscous (approximately 3 min). 350µL neutralization solution was added and mixed by inverting tube 5 times. The supernatant was collected by centrifugation at 13,000rpm for 5 min and transferred to a GeneJET spin column, which was centrifuged at 13,000rpm for 1 min, discarding the flow-through. 500µL wash solution was added to the GeneJET column and the column was centrifuged at 13,000rpm for 1 min, discarding the flow-through, followed by a second identical wash step. The column was transferred to eppendorf tube and 50µl elution buffer addede to the centre of the GeneJET spin column membrane to elute the plasmid DNA. After a 2 min incubation, the plasmid was eluted by centrifugation at 13,000rpm for 2 min. DNA concentration was quantified using a Nanodrop 100 spectrophotometer (Thermo Scientific) with the use of the ND2000 software. The solution stored at -20°C, or -80°C for long term storage.

2.4.8. Agarose gel electrophoresis (1% gel)

1g Hi-Res agarose was heated in 100mL 1×TAE buffer (from a 50×TAE stock) in a microwave to melt and then allowed to cool to room temperature. 5µL of Red Safe (Invitrogen) was added and the gel cast in a Multi Sub Midi gel tank (Geneflow). 1×TAE solution was added to the gel tank. To each well, 6µL sample was added (5µL sample and 1µL of 6×loading dye; Fermenatas). The DNA marker was GeneRuler 1kb ladder (Fermentas) was added and 100V applied to initiate electrophoresis.

2.4.9. Gel extraction

DNA was excised from the gel with a scalpel and was extracted using the Promega Wizard PCR clean up kit according to the protocol provided. DNA was stored at -20°C, or -80°C for long term storage.

2.4.10. DNA quantitation

DNA concentrations were determined using a Nanodrop 100 spectrophotometer (Thermo Scientific) and analysed with ND2000 software.

2.4.11. DNA sequencing

3.2pmol sequencing primer was added to 500ng template and made up to a final volume of 15µL. DNA sequencing was performed by Birmingham University and the data were analysed using Gentle software (http://gentle.magnusmanske.de/)

2.5. Determination of glucose concentration

Glucose concentration in yeast cultures was determined with a Roche Accu-Chek Active diabetes monitor.

2.6. Determination of cell viability

2µL culture was added to a microscope slide and mixed with 2µL trypan blue solution; 100 cells were counted at 100× magnification and the number of stained cells determined. Each determination was done in triplicate.

2.7. Gas chromatography

Yeast samples were taken throughout the growth period for analysis. Cells were pelleted at 13,000rpm for 1 min and supernatants stored at -20°C prior to ethanol analysis by gas chromatography using a CSi 200 series gas chromatograph and an ethanol standard curve.

2.8. Membrane preparation

Yeast were grown in shake flasks with 2×CBS media and harvested prior to the diauxic shift, as determined by measurement of glucose concentration. Cells were pelleted at 5,000rpm, 4°C for 3 min. The supernatant was removed and pellets frozen at -20°C until required. All stages were kept on ice and centrifuged at 4°C. Yeast pellets were defrosted if necessary, washed once, suspended in 1mL breaking buffer and transferred to a breaking tube to which 1mL glass beads was also added. Protease inhibitor cocktail IV (Calbiochem) was added at a dilution of 1:500 (usually 2µL). Cells were broken in a cold tissue lyser (Qiagen) at 50Hz for 10 min. Cell lysate was removed from the glass beads using a pipette and transferred to an eppendorf tube. The glass beads were washed with 500µL breaking buffer, which was also transferred to the eppendorf tube. Crude extract was centrifuged at 15,000rpm for 15 min and the resultant supernatant centrifuged again at 66,000 rpm for 1h to obtain membrane vesicles. Supernatants was discarded and the pellet was suspended overnight in 100µL Buffer A at 4°C. Membrane preparations were stored at -80°C.

2.9. Protein concentration determination: BCA assay

Protein concentrations from membrane preparations and cell lysates were determined using a BCA assay. Bovine serum albumin standard (Sigma) was used at a concentration between 0-10µg from a 1mg/mL stock. For the protein quantification solution, 1:50 4%(w/v) copper (II) sulphate solution (Sigma) to BCA solution (Sigma) was mixed and 200µL of this solution added to each well in a clear plastic 96 well plate. 1µL sample was added to the solution, repeated in triplicate, and the plate was read at 570nm using a plate reader. If sample was out of the range of the standard, the sample was diluted appropriately and quantification was redone.

2.10. SDS-PAGE and western blots

2.10.1. 10% separating gel

Per gel:
1.9mL 30% polyacrylamide
1.5mL 1.5M Tris-HCl, pH 8.8
60µL 10% SDS
2.15mL water
20µL 20%ammonium persulphate
4µL TEMED

2.10.2. 4% stacking gel

Per gel:

0.35mL 30% polyacrylamide

1.5mL 1.5M Tris-HCl, pH 6.8

25µL 10% SDS

1.55mL water

10µL 20% ammonium persulphate

2.5µL TEMED

2.10.3. SDS-PAGE

To make the gels, glass plates were cleaned with 70% ethanol and inserted into clamps. The 8% gel was poured between the glass to 75% of the height. Water-saturated butanol (75% butanol, 25% water) was layered on top and the gel allowed to set. The water-saturated butanol was then removed, the stacking gel poured and a comb inserted before allowing the gel to set. To run the experiment, a known concentration of protein was added to an eppendorf tube, to which was added a 1 in 5 dilution of 5× Laemmli sample buffer and Buffer A to a final volume of 25µL. The sample was heated at 95°C for 10 min in a heat block. Gels well placed into a gel tank with SDS Tris buffer. Denatured proteins were then loaded into each lane. 5µL of PageRuler^{TM} Plus pre-stained protein ladder (Fermentas) was loaded into one well to aid in band size determination. 100V was then applied for 1.5 h. The gel was then transferred into a plastic container with 10mL western buffer and allowed to rock for 10 min at 30rpm.

2.10.4. Western blots

For western blots, fibre pads, filter paper and nitrocellulose paper were all soaked in western buffer and a cassette made accordingly:

a. 1 fibre pad
b. 3 filter papers
c. gel
d. Nitrocellulose membrane
e. 3 filter pads
f. 1 fibre pad

The cassette was inserted into an electrical module in the correct orientation and the transfer initiated at 100V for 1 h. The nitrocellulose was stained with Ponceau red (Sigma) to visualise transferred proteins, destained in water and then blocked in phosphate buffered saline (PBS) containing 5% (w/v) milk for 1 h at room temperature. 1:5,000 dilution of primary monoclonal Anti-HA mouse antibody (Sigma) was then added and allowed to bind overnight at 4°C. The nitrocellulose was washed 3 times with PBST for 10 min, rocking at 30rpm. The secondary antibody, goat anti-mouse horseradish peroxidise conjugate (Sigma) was then added at 1:5,000 dilution to 5% blocking buffer for 2h at room temperature with rocking at 30rpm. After 3 washes in PBST for10 min, EZ-ECL Chemiluminescence

Detection Kit for HRP (Geneflow) was used following the manufacturer's instructions to enable visualisation with a UVI Chemi CAS 6000 (UVItech). Analysis of the blot was done using ImageJ (http://rsbweb.nih.gov/ij/), a free imaging analysis software programme. The background from each of the lanes was subtracted from the intensity from each of the bands. The intensities of the bands were then presented relative to the intensity of the standard to enable comparisons between blots.

An example of a western blot that has resulted in saturation is shown in Figure 2.1; gels such as this were not used for data analysis. Figure 2.2 shows an example of a western blot, which was used for data analysis; these western blots were achieved by adjusting the levels of protein in each well.

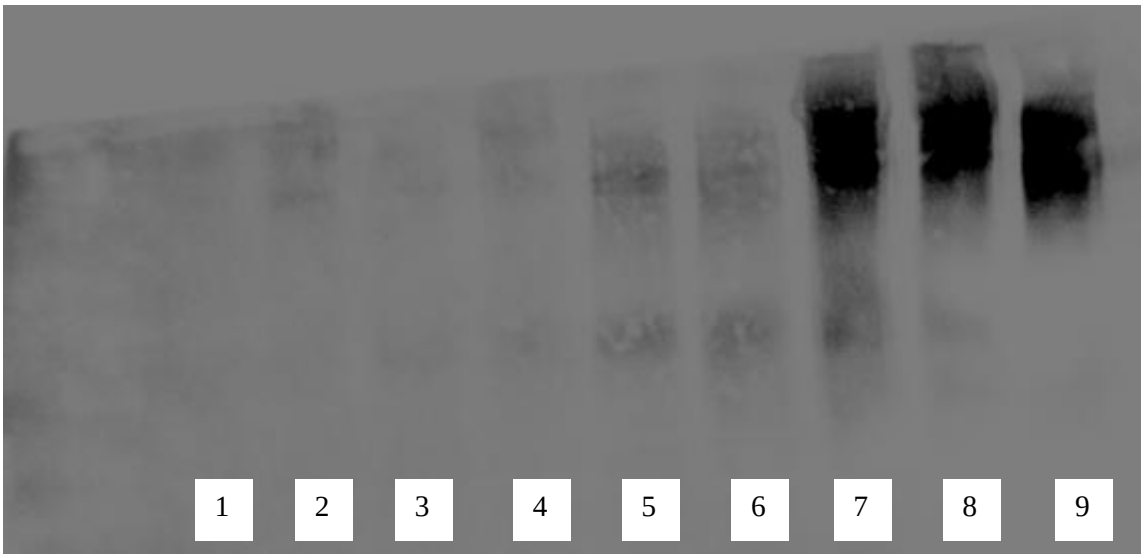


Figure 2.1: Western blot of wild-type yeast, *transformed with the three different vectors pYX222-*FPS1-*HA_3 (lanes 1-3), pYX222-5´Δ-1-43-*FPS1-*HA_3(lanes 4-6) and pYX222-5´Δ-1-215-*FPS1-*HA_3(lanes 7-9); where the same concentrations of total protein were loaded into each well.*

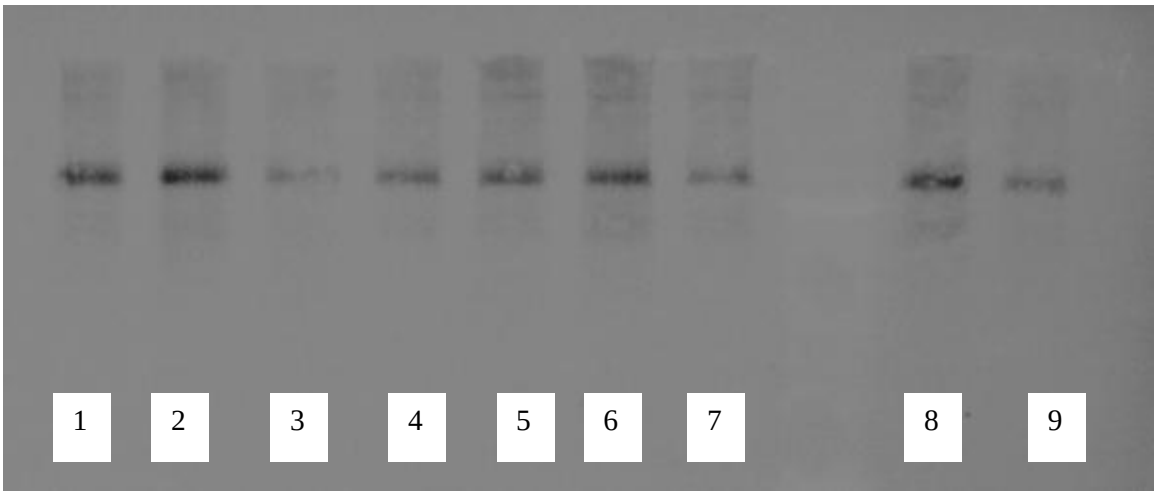


Figure 2.2: Western blot of* spt3Δ *yeast, *transformed with the three different vectors pYX222-*FPS1-*HA_3 (lanes 7-9), pYX222-5´Δ-1-43-*FPS1-*HA_3(lanes 4-6) and pYX222-5´Δ-1-215-*FPS1-*HA_3(lanes 1-3); where different concentrations of total protein were loaded into the wells.*

2.10.5. Ponceau red staining

To determine that protein had transferred to nitrocellulose and to analyse correct protein loading for a western blot, Ponceau red staining was used. 0.1g Ponceau red was added to 95mL water and 5mL concentrated acetic acid. The solution was added to the nitrocellulose after the transfer of protein, but prior to blocking. Excess staining was removed by washing in water and nitrocellulose was allowed to dry prior to photographing. To remove Ponceau red from the protein bands, 0.1M NaOH was added.

2.11. On-line flow microcalorimetry

Flow microcalorimetric data were collected for shake-flask cultures at 30°C, 220rpm, using a Thermal Activity Monitor 2277 with a flow vessel working volume of 0.6 cm^3 (Thermometric AB, Sweden). The system was sterilized by successively pumping through solutions of sterile deionized water, 70% ethanol (v/v), sodium hydroxide (0.5 M) and finally sterile deionized water, then allowing the flow lines to run dry before inserting into a culture. The pump rate was 20 rpm using a Watson Marlow 400 pump. The thermostatic water bath was maintained at 30°C. The software used to record data was Digitam v4.1. The calorimeter was calibrated at the same temperature, flow rate and amplifier setting (1,000 μW) to be used during experimental data collection in order to establish a steady baseline deflection. Electrical calibration was done in the culture medium. Once the baseline was established, the outside of the inlet and outlet tubes were sterilized with 70% ethanol and placed in the shake-flask.

2.12. Chronological lifespan (CLS) determination

The CLS experiment was developed by taking known yeast strains such as *tor1Δ* and *hxk2Δ*, which have an increased CLS compared to wild-type strains, as shown in Figure 2.3.

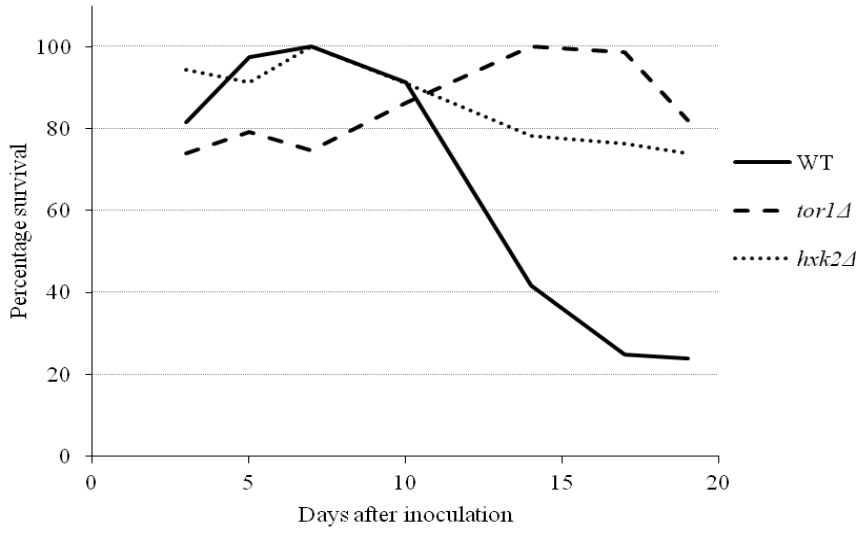


Figure 2.3: Determination of chronological lifespan (CLS). *To establish the CLS experiment two known longevity mutants were analyzed. Each experiment was performed in duplicate and an average recorded.*

A single colony was cultured overnight in 2×CBS (containing 4×DO rather than 2×DO so that amino acid levels were not a limiting factor) or YPG (2% glycerol). 125mL shake flasks were used containing 25mL medium that had been autoclaved before the inoculum was added. Shake flasks were then inoculated with 50µL of overnight culture and allowed to grow at 30°C, 220rpm. Every 2-3 days for ~4 weeks, a 100µL sample of culture was taken, diluted by 50,000 and 25µL of this plated on YPD plates. Yeast cells were allowed to grow for 2-3 days on plates and colony forming units (CFUs) were counted and recorded. Each experiment was repeated three times and 2 YPD plates were made to determine the average CFUs; the average of the three biological triplicates was taken. The percentage survival was calculated. For L-carnosine-containing experiments, L-carnosine was added once to the medium at the beginning of the experiment.

2.13. Polysome profiling

Cultures were grown in YPD to an OD_{600} between 0.5-1, 10mg of cycloheximide was added for every 100mL culture and incubated for a further 15 min at 30°C. All stages were kept at 4°C and RNAase free. Cultures were instantly cooled by pouring over ice and the cells were harvested at 5,000rpm for 5 min. Cells were washed in lysis buffer and collected by centrifugation at 5,000rpm for 5 min after removing the supernatant. The pellet was suspended in 250µL lysis buffer and transferred to a 2mL breaking tube containing 1mL glass beads. Cells were broken in a cold tissue lyser (Qiagen) at 50Hz for 3 min. The cell lysate was transferred to an eppendorf tube and the cell debris removed by centrifugation at 13,000rpm for 15 min. The supernatant was removed and stored at -80°C until required. The mRNA concentration (µg/mL) in the cell lysate was determined by measurement at 260nm with a Nanodrop (Thermoscientific).

10-50% sucrose gradients were made using gradient buffer. The first layer, 2mL 50% sucrose, was put into a Beckman-Coulter tube and frozen at -80°C for 1 h. The same procedure was followed for the 40% layer. The sucrose gradients were stored at -80°C. When required they were left at 4°C overnight to defrost. 150µg cell lysate was loaded on to the sucrose gradient and centrifuged at 38,000rpm for 2 h in a SW41 rotor (Beckman instruments). The gradients were collected continuously from the top using a Biocomp Gradient profiler at A_{254} to produce traces. The areas underneath the peaks were analysed using the programme ImageJ.

2.14. β-galactosidase assay for *GCN4-LacZ* determination

Gcn4 levels were determined with the use of the reporter gene *LacZ* (encoding β-galactosidase), which had been fused to *GCN4* in the vectors B1805 and B1805-*HIS*. Yeast were grown in 2×CBS-URA (or 2×CBS-HIS in the case of yTHC*BMS1*) until an OD_{600} of ~1. All subsequent stages were kept at 4°C. Cells were harvested at 5,000rpm for 3 min. The cells were washed in sterile water and pelleted at 5,000rpm for 3 min. The pellet was suspended in 500µL breaking buffer and transferred to a 2mL breaking tube containing 1mL glass beads and a 1:500 dilution of protease inhibitors cocktail IV(Calbiochem). The cells were then lysed in a cold tissue lyzer (Qiagen) at 50Hz for 3min. The cell lysate was transferred to an eppendorf tube and the cell debris was removed by centrifugation at

13,000rpm for 15min. A BCA assay was done to determine the protein concentration of the supernatant. 100µL cell lysate was added to an eppendorf tube with 900µL Z buffer and incubated at 28°C for 5 min. The reaction was started by adding 200µL o-nitrophenyl-β-D-galactoside (ONPG; stock 4mg/mL in Z buffer) and incubating at 28°C until a yellow colouration developed (typically 15-30 min). The reaction was stopped by adding 500µL Na_2CO_3, noting the time. Colour was recorded at 420nm on spectrophotometer. Using the equation $(OD_{420}\times1.7)/(0.0045\times\text{protein(mg/mL)}\times\text{extract volume(mL)}\times\text{time(min)})$ to obtain nmol/minute/mg protein, the data were converted to relative units to aid in data analysis.

2.15. ATP assay

A BacTiter-Glo™ Microbial cell viability assay kit was used to determine ATP levels; this kit measures ATP levels as an indicator of viability as luciferin in the reaction mixtures is converted to oxyluciferin and light by firefly luciferase in the presence of ATP. The level of light produced is proportional to the level of ATP. In the assay kit, the BacTiter-Glo reagent is provided which both lyses the cells and performs the reaction. This reaction mixture is aliquotted and stored at-80°C until required. It is then thawed and brought to room temperature before use. Cells were grown to an OD_{600} of 1, centrifuged at 13,000 for 1 min and the supernatant discarded. The cells were washed in double distilled water, centrifuged again, the supernatant removed and the pellet suspended in double distilled water to an OD_{600} of 0.1; this gave around 1×10^6 cells in 1mL. To a 96 well white plate, 90µL water was added followed by 10µL diluted cells. 100µL BacTiter-Glo reagent was added and allowed to incubate at room temperature for 5 min to enable cell lysis. An ATP standard curve was used to quantify ATP levels using concentrations 0.1nM to 100nM made by serial dilution. After incubation the plate was read using a Berthold detection luminometer and the programme Simplicity 4.2. Relative light units (RLU) were recorded.

2.16. GFP assay

Yeast cells were grown to just before the diauxic shift, as monitored by glucose concentration. All subsequent stages were kept on ice and centrifuges at 4°C. Cells were harvested and centrifuged at 5,000rpm for 3 min. In these experiments, GFP was secreted into the supernatant, and hence the supernatants were decanted into falcon tubes and stored at -20°C until required. 100µL supernatant/ cell lysate was added to black 96 well plates followed by 50µL 1M potassium phosphate buffer, pH8 thus ensuring that the pH was above 7 as GFP is sensitive to pH and the experiment must be done at pH7 or above. A GFP standard was used (Vector Laboratories); the standard curve ranged from 5mg/mL to 20mg/mL. The relative flouresence units (RFU) were determined using a Spectramax Gemini plate reader with λ_{exe} of 397nm and λ_{em} of 506nm.The plate reader was kept at 25°C. Experiments were done in triplicate.

Chapter 3: Understanding translation in high yielding yeast strains

As discussed in Chapter 1, it has been shown previously that the yeast strains yTHC*BMS1*, *spt3Δ, gcn5Δ* and *srb5Δ* are high yielding for Fps1 compared to wild-type cells (Bonander et al., 2005). As such, they are referred to as "the high yielding strains" throughout this thesis. The aim of the research described in this chapter was to determine the mechanisms behind their high-yielding phenotype.

3.1. Fps1 yield is increased in shake flask cultures of the high yielding strains, yTHC*BMS1*, *spt3Δ*, *gcn5Δ* and *srb5Δ*

To confirm the high-yielding phenotypes of the yeast strains yTHC*BMS1*, *spt3Δ, gcn5Δ* and *srb5Δ*, from previous experiments in bioreactors (Bonander et al., 2009) were reproduced in shake flask cultures. Yeast cells were transformed with the pYX222-*FPS1*-HA_3 vector using the lithium acetate method. Three separate colonies for each high yielding strain were chosen after transformation and pre-inoculations were made in 2×CBS without histidine. 50mL cultures were then made from the pre-inoculations and yeast were allowed to grow until just before the diauxic shift, as this was shown previously to be the point at which maximal Fps1 yields can be achieved (Bonander et al., 2005). Harvesting just before the diauxic shift was determined with the use of a Roche diabetes monitor, where cultures were harvested when the glucose concentrations were around 10-30mM, which roughly correlated to an OD_{600} of ~4. A membrane preparation was performed to isolate membrane bound Fps1, as crude cell lysates do not correlate with membrane bound Fps1 levels (Bonander et al., 2005). Western blots were then performed to determine the relative yields of Fps1 in each of the strains. As yields of Fps1 varied greatly between each strain, western blots were optimised to avoid saturation of bands by varying the protein concentrations in each well (see section 2.10.4). Once the images for the western blots had been acquired, band intensities were analysed using ImageJ and the data were reported relative to the yield in wild-type cells.

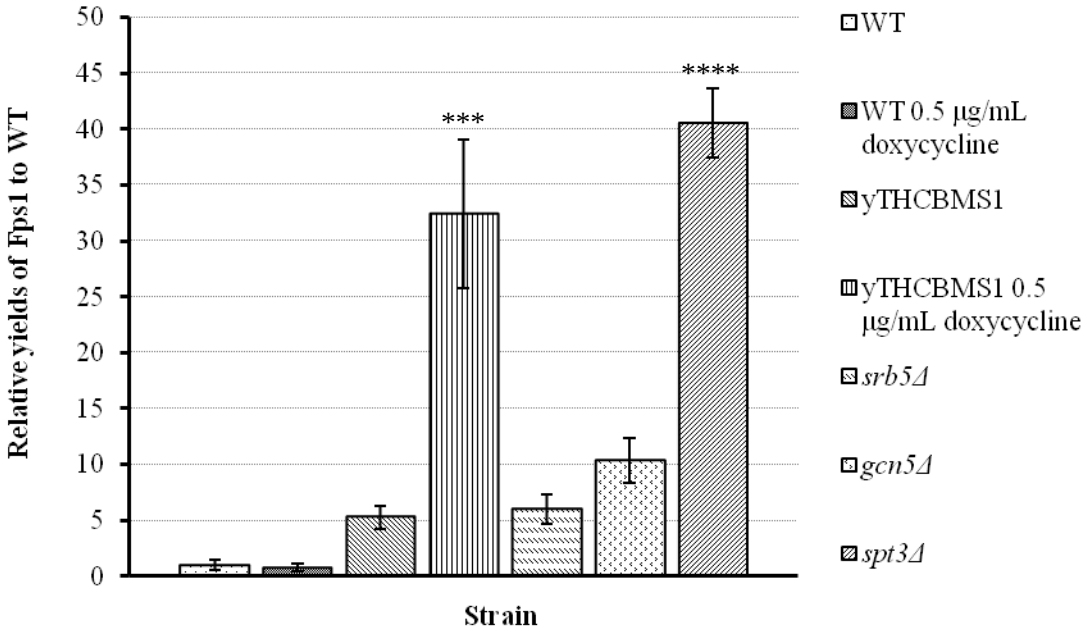


Figure 3.1: Relative yields of Fps1 in the high yielding strains. *Yeast were grown in shake flasks in 2×CBS until an OD_{600} ~4. Biological triplicates were performed for each strain. The data are relative yields from western blot data where the error bars represent standard error of the mean (SEM). Data was analysed using a one-way ANOVA (P<0.0001). Asterisks show the significance of the relative yields of Fps1 compared to wild-type, as determined by a Dunnett's multiple comparison test, where ***=P ≤ 0.001 and ****= P ≤ 0.0001.*

The data in Figure 3.1 confirm that all strains produce higher yields of Fps1 than wild-type cells. *spt3Δ* and yTHC*BMS*1 both had significant increases in Fps1 production compared to wild-type, at 40 fold and 32 fold increase respectively. The *srb5Δ* strain gives a 5 fold increase in Fps1 production, whilst *gcn5Δ* results in a 10 fold increase. Fig 3.1 also shows that yTHC*BMS1* (with 0.5µg/mL doxycycline) has the largest variation in Fps1 yields, shown by the size of the error bars. Figure 3.1 shows that yTHC*BMS1* in the absence of doxycycline gives a 5 fold increase in Fps1 yield. As expected, wild-type cells with doxycycline do not give an increase in Fps1 yield compared to wild-type cells without doxycycline.

3.2. yTHC*BMS1* exhibits an initiation block

It is known from the literature that defects in Bms1 protein result in a decrease in the 40S ribosomal subunit, due to a decrease in the levels of 18S rRNA. Bonander showed previously that in the presence of doxycycline yTHC*BMS1* has a decrease in the levels of 18S rRNA and that the levels continue to decrease as doxycycline concentrations are increased, whereas the levels of 25S remain the same (Bonander et al., 2009). As doxycycline addition to yTHC*BMS1* has an effect on 40S ribosomal subunit levels, polysome profiles were determined.

Polysome profiles were obtained by growing the untransformed yTHC*BMS1* strain in YPD. Yeast cells were harvested at an OD_{600} of ~1 and a polysome preparation was performed, as described in section 2.13. Each experiment was performed in replicate, as reported in Table 3.1; a single polysome profile is shown in Figure 3.2 to represent the data. The ratio of the areas of the monosome:polysome peaks is widely used as a measure of translational activity (Gross and Kinzy, 2007). In particular, an initiation block is associated with a high monosome:polysome ratio. To aid in the identification of each peak, the polysome profile of *tor1Δ* was compared to that of wild-type cells as *tor1Δ* is well known to exhibit an initiation block (Powers and Walter, 1999).

Table 3.1: Strains with known initiation blocks were selected as potential high-yielding strains. *Polysome profiles were determined as described in section 2.13 using* tor1Δ *as a positive control. Profiles were determined in replicates, as shown, and the ratio of peak areas of monosome (80S):polysome were reported. The corresponding standard error of the mean is shown in parentheses. The larger the value of the ratio, the greater the initiation block. Data was analysed using a one-way ANOVA (P<0.0001). Asterisks show the significance of the relative yields of Fps1 compared to wild-type, as determined by a Dunnett's multiple comparison test, where *=P ≤ 0.05 and **= P ≤ 0.01. The data in brackets represents SEM.*

Strain/ condition	Average 80S/ polysomes
wild-type (with 0.5µg/mL doxycycline) n=2	0.12 (0.01)
mdm20Δ n=2	0.14 (0.01)
wild-type n=3	0.15 (0.01)
gcn5Δ n=2	0.16 (0.02)
tpm1Δ n=3	0.17 (0.01)
tpm2Δ n=3	0.17 (0.01)
spt3Δ n=3	0.18 (0.03)
ssf1Δ n=2	0.20 (0.00)
nop12Δ n=3	0.21 (0.01)
tor1Δ n=1	0.21
yTHC*BMS1* n=3	0.24 (0.03)
rpl31aΔ n=3	0.27 (0.03)
rpl22aΔ n=3	0.31 (0.04)
srb5Δ n=3	0.39 (0.07) *
yTHC*BMS1* (with 0.5µg/mL doxycycline) n=3	0.43 (0.14) **

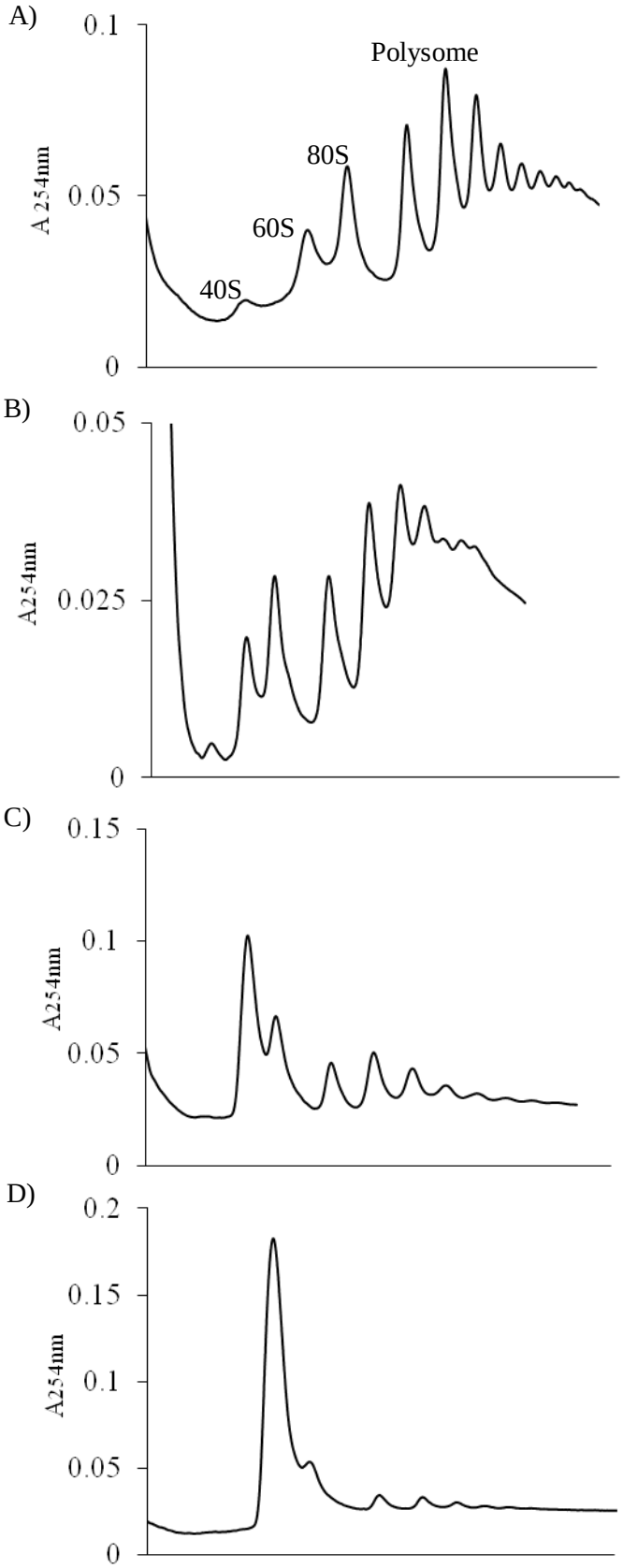


Figure 3.2: Representative polysome profiles for wild-type, wild-type with 0.5µg/mL doxycycline, yTHC*BMS1 *and yTHC*BMS1 *with 0.5µg/mL doxycycline. *A) Wild-type yeast [n=3, 0.15(0.01)], B) wild-type yeast with 0.5µg/mL doxycycline [n=2, 0.12 (0.01)], C) yTHCBMS1 [n=3, 0.24 (0.03)], D) yTHCBMS1 (with 0.5µg/mL doxycycline) [n=3, 0.43 (0.14)]. The data in square brackets are the monosome:polysome ratio and the corresponding standard error of the mean (SEM).*

Figure 3.2A shows a typical polysome profile for wild-type yeast cells, where free 40S and 60S can be seen. The 80S peak represents both free 80S and 80S bound to mRNA. The polysome peaks represent more than one 80S bound to mRNA. Figure 3.2B shows that addition of doxycycline did not affect the polysome profile.

The polysome profile of yTHC*BMS1* (Figure 3.2C) is different to that of wild-type cells. In this strain, the native promoter of *BMS1* has been replaced with a tetracycyline-repressible promoter (Mnaimneh et al., 2004) which is fully activated in the absence of repressor. As previously shown by Bonander and colleagues (Bonander et al., 2009) under these conditions, *BMS1* transcript levels are approximately 10 times those of wild-type cells. Furthermore, the yTHC*BMS1* strain exhibited a reduced growth rate compared to wild-type, ~$0.32h^{-1}$ compared to ~$0.37h^{-1}$ respectively. The polysome profile of yTHC*BMS1* shows an initiation block, marked by a decrease in polysome peaks, and the expected reduction in 40S levels (Bonander et al., 2009). As a result of the decrease in the 40S, there is an imbalance between the 40S and 60S subunit ratios, which in turn results in an apparent increase in the 60S peak, even though there is no increase in 60S subunit levels. There is also a decrease in polysome peaks; when there is a decrease in 40S subunit, there is a decrease in the levels of pre-initiation complex (PIC; see section 1.3.5.2). Addition of 0.5µg/mL resulted in a more severe phenotype (Figure 3.2 D), where the polysome peaks were reduced further, probably due to a further reduction in the availability of the 40S subunit.

3.3. *spt3Δ, gcn5Δ* and *srb5Δ* also have altered polysome profiles

As yTHC*BMS1* exhibits an initiation block and there is an increase in *BMS1* transcript levels in *spt3Δ, gcn5Δ* and *srb5Δ*, the polysome profiles of these strains were also determined; as for yTHC*BMS1* the strains were cultured in YPD and harvested at an OD_{600} ~1.

Figure 3.3A shows that he *spt3Δ* strain exhibits an initiation block, marked by a decrease in the polysome peaks compared to the monosome peak. The *srb5Δ* strain has a very severe initiation block (Figure 3.3B) similar to that of yTHC*BMS1*. However, unlike yTHC*BMS1* with and without doxycycline there does not appear to be a decrease in the 40S ribosomal subunit. In contrast, *gcn5Δ* appears to have an elongation block together with an initiation block (Figure 3.3C) based on the fact that the polysome peaks are of similar height to the monosome peak (communication with Dr Mark Ashe, University of Manchester). Similar to *spt3Δ, srb5Δ* also appears to also have a decrease in total translational levels.

It is interesting to note that all high yielding strains have different polysome profiles even though they have similar levels of *BMS1* transcript. This suggests that other factors within these strains are responsible for their phenotypes.

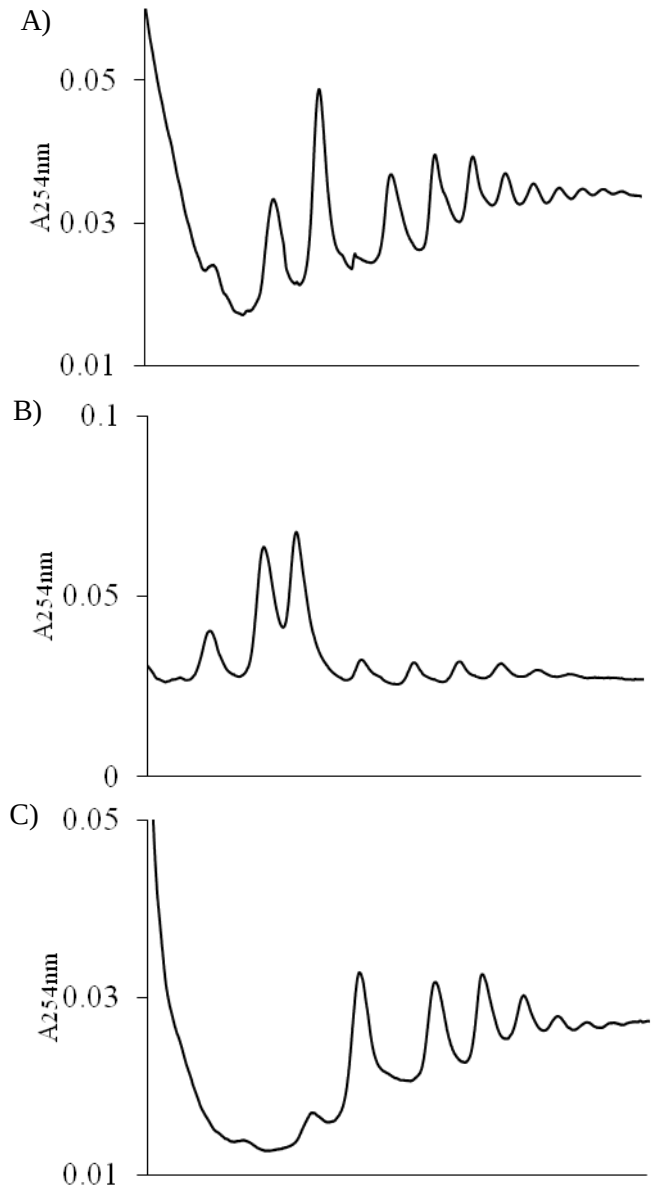


***Figure 3.3: Representative polysome profiles for* spt3Δ, srb5Δ *and* gcn5Δ.** *A)* spt3Δ *[n=3, 0.18(0.03)], B)* srb5Δ *[n=3, 0.39 (0.07)] and C)* gcn5Δ *[n=2, 0.16 (0.02)] strains. Data in square brackets are the monosome:polysome ratios and the corresponding standard error of the mean (SEM).*

3.4. Phosphorylation of eIF2α is increased in the high yielding strains

Since the high yielding strains were all found to have initiation blocks to differing extents (Figures 3.2 and 3.3), the levels of eIF2α phosphorylation were determined. eIF2α is a subunit of eIF2, which is part of the ternary complex (TC; see section 1.3.5.2). The TC associates with the 40S ribosomal subunit to make the 43S PIC. During times of stress, eIF2α is phosphorylated; the resultant phosphorylated form then competitively inhibits eIF2B, preventing the exchange of eIF2·GDP for eIF2·GTP. This decreases TC levels and as a result down-regulates translation by causing an initiation block.

Figure 3.4 shows that eIF2α is only phosphorylated in wild-type cells under amino acid starvation conditions (starvation for 10 min). In contrast, the high yielding strains all have constitutively high levels of eIF2α phosphorylation. This is likely to contribute to the initiation blocks in these strains. However, as stated above, since all the polysome profiles look different, other mechanisms are also likely to be involved.

***Figure 3.4: Phosphorylation of eIF2α in stressed and non-stressed cells, for wild-type, yTHC*BMS1 *with 0.5µg/mL doxycycline,* spt3Δ, gcn5Δ *and* srb5Δ.** *The upper panel shows eIF2α levels, whilst the lower panel shows the amount of phosphorylated eIF2α (eIF2α-P) probed with an antibody specific for eIF2α-P; (-) represents cells subjected to amino acid starvation for 10 min, whilst (+) represents non-starved cells cultured in medium containing all amino acids. yTHC*BMS1 *was cultured in the presence of 0.5µg/mL doxycycline (unpublished data generated by Dr Richard Darby).*

3.5. Fps1 yield can be increased in other strains that have a polysome profile defect

As there appeared to be a correlation between an altered polysome profile and an increase in Fps1 yield, yeast deletion strains with a known change in their polysome profiles were chosen as potential hosts. Strains with known initiation blocks were selected and their polysome profiles confirmed (Table 3.1).

3.5.1. Ribosomal biogenesis mutants

The ribosomal biogenesis mutants *ssf1Δ* and *nop12 Δ* were selected since both Ssf1 and Nop12 are involved in pre-60S biogenesis (Wu et al., 2001). Ssf1 is located in the nucleolus and is functionally redundant with Ssf2; when both are deleted this causes lethality. Ssf1 has been shown to prevent premature cleavage of the 27S pre-rRNA. Nop12 is also located in the nucleolus and is involved in the production of the 25S pre-rRNA from the 35S pre-rRNA (Wu et al., 2001). When either *NOP12* or *SSF1* are deleted, this causes a decrease in the levels of 60S ribosomal subunit (Fatica et al., 2002; Steffen et al., 2008).

Mutations in ribosomal proteins are known to cause defects in pre-rRNA processing in both 40S and 60S maturation. This is because non-ribosomal proteins, which are involved in maturation, physically interact with ribosomal proteins to function (Martín-Marcos et al., 2007). Rpl31a and Rpl22a are both proteins of the large ribosomal subunit. Deletion of *RPL31A* has been shown to cause a decrease in the levels of 60S subunits, while deletion of *RPL22A* has a very similar phenotype to *rpl31aΔ*, in that it has been shown to cause an increase in replicative lifespan in yeast. Interestingly all of these chosen mutants all cause an increase in yeast replicative lifespan (Steffen et al., 2008).

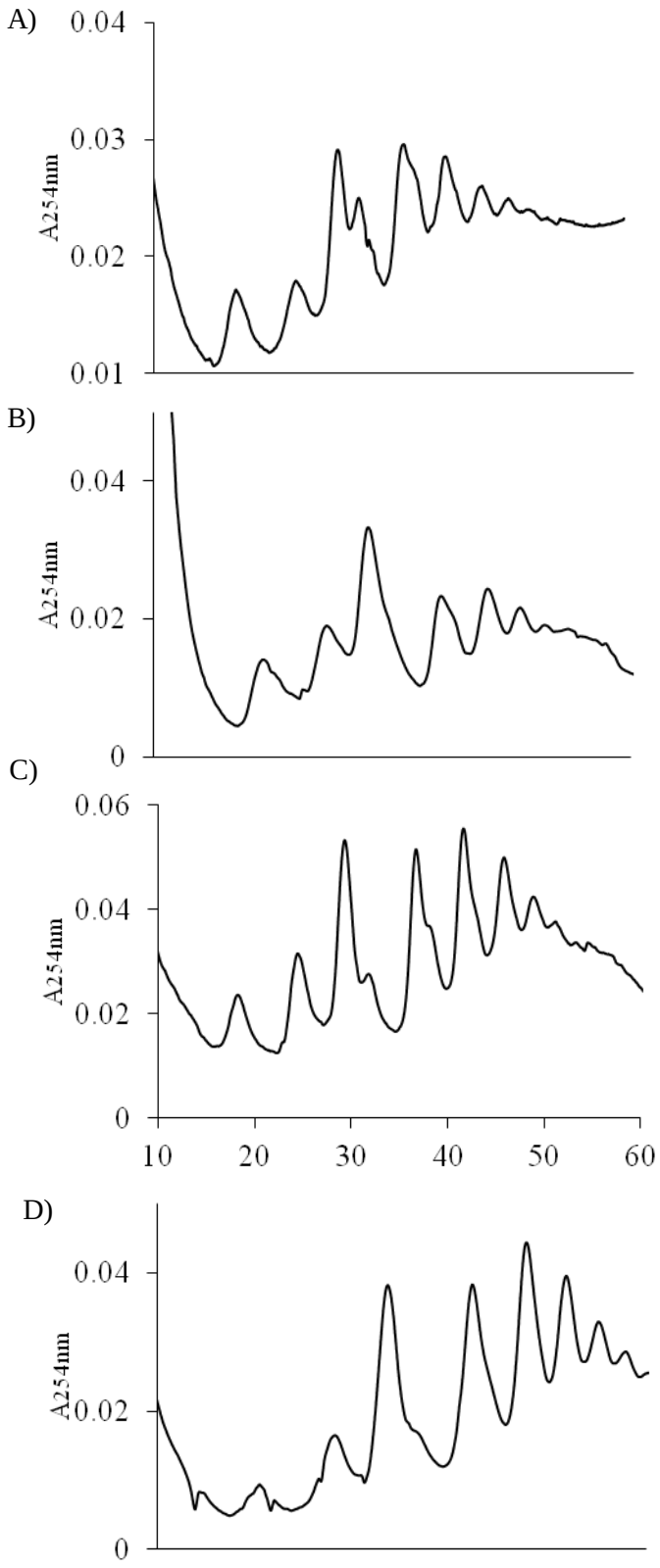


***Figure 3.5: Representative polysome profiles of* rpl31aΔ, rpl22aΔ, nop12Δ *and* ssf1Δ**. *A)* rpl31aΔ *[n=3, 0.27(0.03)], B)* rpl22aΔ *[n=3, 0.31 (0.04)], C)* nop12Δ *[n=3, 0.21 (0.01)] and D)* ssf1Δ *[n=2, 0.20 (0.00)]. Data in square brackets are the monosome:polysome ratios and the corresponding standard error of the mean (SEM).*

The polysome profiles for the selected strains in Figure 3.5 show initiation blocks, as expected. In addition, all profiles showed halfmer formation; halfmers are small peaks after monosome and polysome peaks that represent binding of the PIC (which does not contain the 60S subunit) to mRNA. As for ribosomes (80S), there can be more than one PIC associated with an mRNA transcript (Eisinger et al., 1997). Mutations that affect subunit binding may also exhibit halfmer formation (Eisinger et al., 1997). Halfmer formation has previously been reported for *rpl16bΔ*, another ribosomal protein mutant strain (Foiani et al., 1991), but not for the ribosomal biogenesis and ribosomal protein mutants shown in Figure 3.5 (Steffen et al., 2008); this may have been due to the low resolution of the polysome profiles presented in that study (Steffen et al., 2008).

Figures 3.5A-C shows that *rpl31aΔ*, *rpl22aΔ* and *nop12Δ* have a decrease in the 60S subunit compared to wild-type cells (Figure 3.2A). It is less clear from the polysome profile in Figure 3.5D whether this is also true for *ssf1Δ*, as previously reported in the literature (Steffen et al., 2008). In this study, high salt concentrations were not used in the polysome preparation experiment in contrast to the study by Steffen and colleagues (Steffen et al., 2008). High salt concentrations aid in the disassociation of 40S and 60S when they are associated as free 80S (Parnell and Bass, 2009).

3.5.2. Actin binding mutants

The yeast actin binding mutants, *tpm1Δ, tpm2Δ* and *mdm20Δ*, were chosen as they have been previously shown to have initiation blocks. It is thought that the actin cytoskeleton acts as a scaffold during translation, as a result mutations in actin and deletion strains in actin binding proteins cause a change in translation (Gross and Kinzy, 2007).

Actin is essential and its gene has been highly conserved throughout evolution. There is only a single gene encoding actin in yeast, which is 88% identical to actin from mammalian muscle (Ng and Abelson, 1980). Actin is involved in cellular structure, polarity, cellular organisation and translation. It forms two types of structures; cables, which are involved in polarising events, and yeast patches that are located near the cell membrane and are involved in exocytosis. Disruption to the actin cytoskeleton often results in a decrease in actin cables and an increase in patches (Gross and Kinzy, 2007; Ng and Abelson, 1980).

Actin binding proteins control the structure of actin and actin dynamics. The actin binding proteins, Tpm1 and Tpm2, are tropomyocin isoforms, whereas Mdm20 is the mitochondrial distribution and morphology protein. Deletion of these genes result in a decrease in actin cable formation and an increase in patch formation (Figure 3.6; (Gross and Kinzy, 2007)).

A) B)

***Figure 3.6: The structure of the actin cytoskeleton in wild-type yeast and* tpm1Δ.** *The actin cytoskeleton of yeast cells was stained with rhodamine phalloidin. A) Wild-type yeast with actin cables during bud formation B) representative image of actin binding mutants (*tpm1Δ*) which have very few cables. Images by Dr Stephane Gross, Aston University.*

Interestingly initiation (eIF) and elongation (eEF) factors involved in translation have been shown to affect the structure of the actin cytoskeleton. For example, eEF1A has been shown to disrupt the actin cytoskeleton when mutated (Gross and Kinzy, 2005). Moreover, cellular stresses such as heat shock, osmotic stress and glucose starvation cause actin depolarisation, while rapamycin addition also causes slow depolarisation (Uesono et al., 2004).

Polysome profiles were determined for these mutants as before. The strains *tpm1Δ* and *tpm2Δ* were shown to exhibit an initiation block, marked by a decrease in polysome peaks. Unexpectedly *mdm20Δ* did not have an initiation block (Figure 3.7).

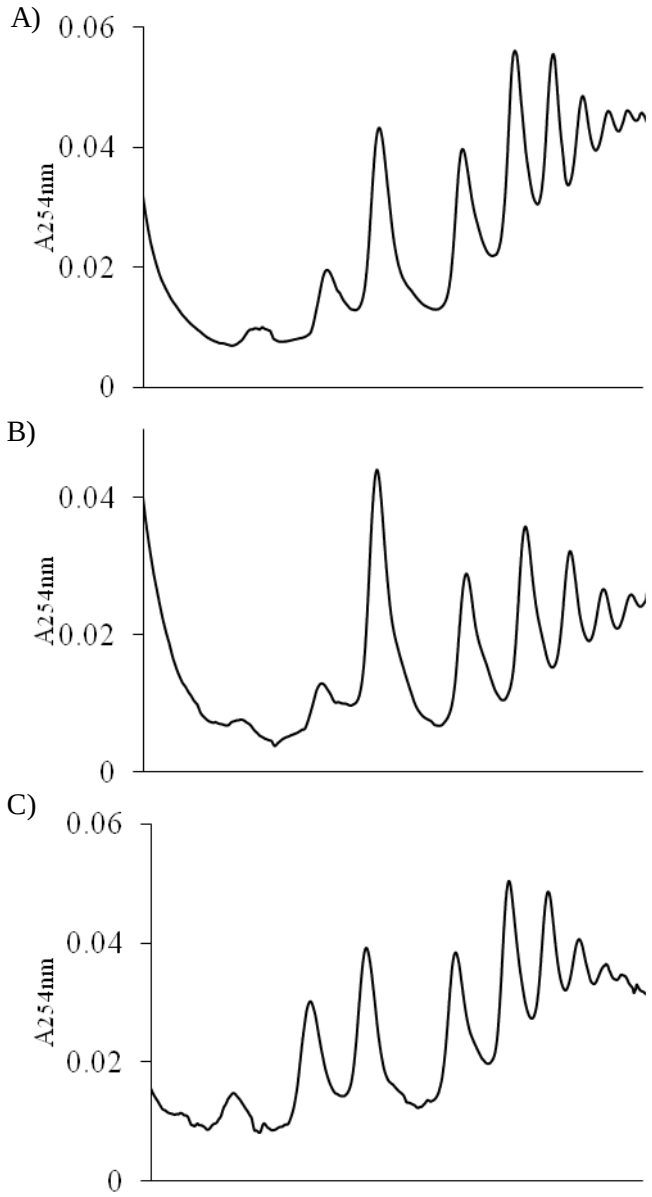


***Figure 3.7: Representative polysome profiles for* tpm1Δ, tpm2Δ *and* mdm20Δ.** *A)* tpm1Δ *[n=3, 0.17(0.01)], B)* tpm2Δ *[n=3, 0.17 (0.01)] and C)* mdm20Δ *[n=2, 0.14 (0.01)]. Data in square brackets are the monosome:polysome ratios and the corresponding standard error of the mean (SEM).*

3.5.3. Fps1 yields are increased in strains with an altered polysome profile

The yeast strains listed in Table 3.1 were transformed with pYX222-*FPS1*-HA_3 using the lithium acetate method. Three colonies per transformed strain were selected and cultured in 50mL 2×CBS without histidine. Yeast cells were harvested prior to the diauxic shift and membrane preparations were performed. The relative yields of Fps1 were determined from western blots, using the software ImageJ (http://rsbweb.nih.gov/ij/), and all data were reported relative to wild-type cells.

All deletion strains gave an increased Fps1 yield relative to wild-type cells (Figure 3.8). *tpm1Δ* and *tpm2Δ* gave the highest levels of Fps1, producing a 7 fold and a 4 fold increase respectively. *rpl31aΔ*

resulted in a 3 fold increase, which was the highest level achieved from the ribosomal biogenesis and ribosomal mutants. *tor1Δ* gave a 2.5 fold increase in Fps1 yield.

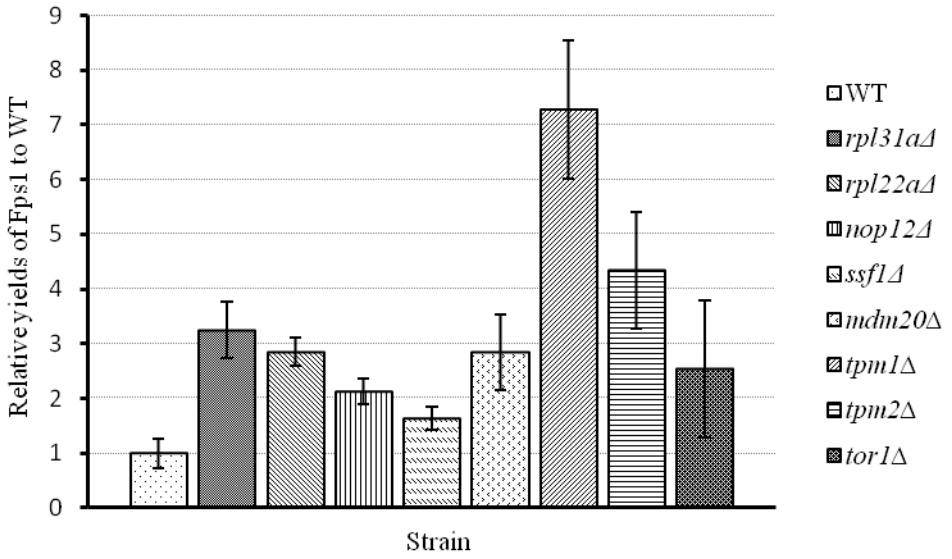


Figure 3.8: Relative yields of Fps1 in strains with a known change in polysome profile. *Yeast were grown in shake flasks in 2×CBS until an OD_{600} ~4. Biological triplicates were performed for each strain. The data are relative yields from western blot data where the error bars represent standard error of the mean (SEM).*

3.6. Gcn4 production is increased in all high-yielding strains

The levels of stress resistance transcription factors, such as Gcn4, are increased when initiation blocks occur (Hinnebusch, 2005). For example, it has been shown that the ribosomal mutants in Table 3.1 also have an increase in Gcn4 levels (Martín-Marcos et al., 2007; Steffen et al., 2008); these mutants have a decrease in the 60S subunit meaning that the PIC can scan through start codons. As *GCN4* is regulated by the presence of four untranslated open reading frames (uORFs); (Foiani et al., 1991), the ability of the PIC to scan through these control sequences results in the translation of the *GCN4* ORF (Foiani et al., 1991).

For example, deletion of *RPL16B* or *RPL33A* has been shown to cause a decrease in the levels of the 60S subunit ratio compared to wild-type cells, resulting in halfmer formation and an increase in Gcn4 production due to start codon read through, known as leaky scanning (Foiani et al., 1991; Martín-Marcos et al., 2007). *RPL16B* deletion was shown to cause an increase in Gcn4 by four fold compare to wild-type cells under non-amino acid starved conditions. Interestingly under amino acid starved conditions, the deletion strain *rpl16bΔ* resulted in a decrease by about a third in Gcn4 production compared with wild-type cells. The increase in Gcn4 in the case of *rpl33aΔ* has been associated with derepression of amino acid biosynthetic genes (Martín-Marcos et al., 2007).

Figure 3.9 shows relative yields of Gcn4 assayed using the reporter protein β-galactosidase. The gene for β-galactosidase, *LacZ* was inserted into the open reading frame of *GCN4* at the *BamH*I restriction

site in the vector B1805 (see Appendix A.3. for vector map and sequence). Thus β-galactosidase production was under the control of the *GCN4* promoter and the *GCN4* 5´UTR. The vector B1805 was the kind gift of Dr Mark Ashe, University of Manchester and had been previously constructed by Alan Hinnebusch (Hinnebusch, 1985). As this vector enabled selection on medium lacking uracil, the vector B1805-*HIS* was constructed to enable selection of yTHC*BMS1* transformants on medium lacking histidine; during the construction of yTHC*BMS1*, *URA3* had been reinserted into the genome (Mnaimneh et al., 2004). To construct B1805-*HIS*, the *URA3* gene was removed by restriction digest and the *HIS3* gene from pYX222 was inserted. Yeast were transformed with the resultant vector using the lithium acetate method and grown on 2×CBS without uracil or histidine, depending on the selection needed. The yeast cells were harvested at OD_{600} ~1 and cell lysates were collected and stored at -20°C until required. To monitor levels of β-galactosidase, O-nitrophenyl-β-D-galactoside (ONPG) was used and all data were reported relative to wild-type.

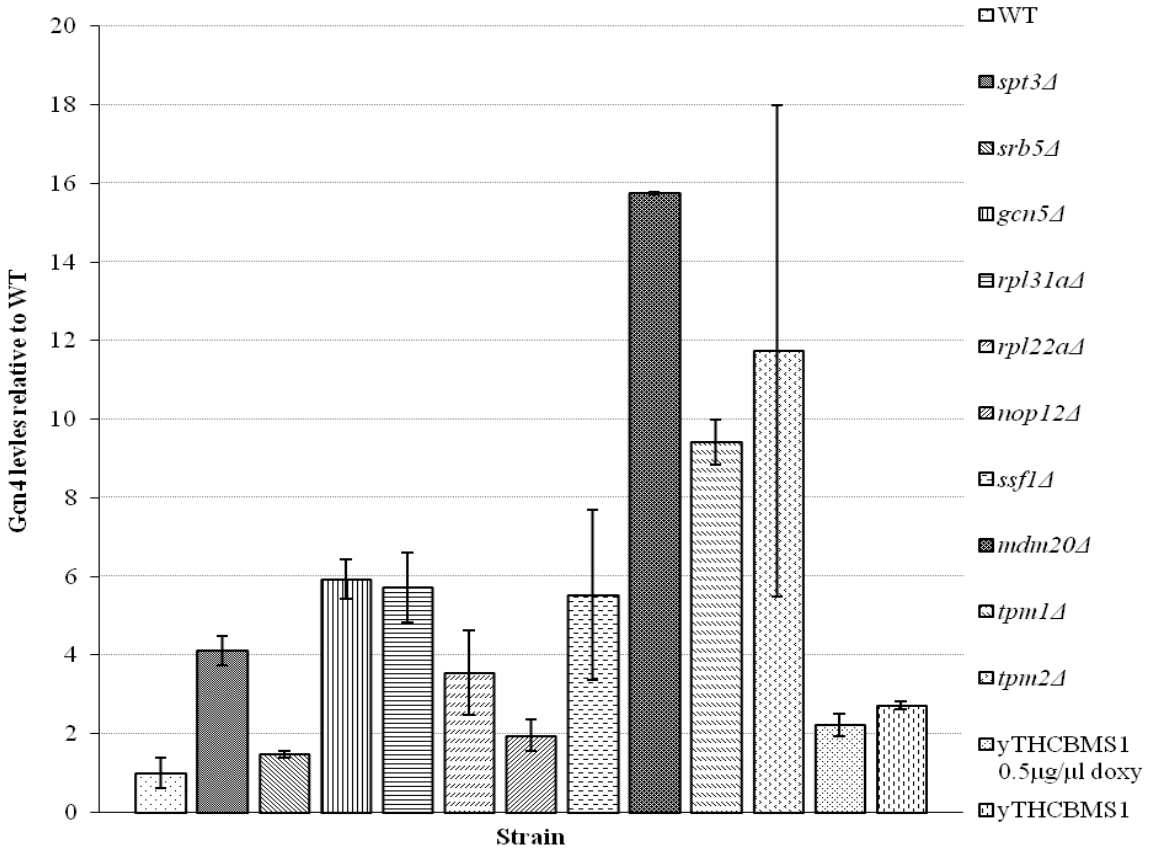


Figure 3.9: Relative yields of Gcn4 in high-yielding strains. *Gnc4 yields were measured using β-galactosidase as a reporter protein, the levels of which were determined with ONPG. All strains were transformed with B1805, except yTHC*BMS1 *which was transformed with B1805-HIS. Error bars represent SEM and n=3.*

Gcn4 levels were shown to be increased to differing extents relative to wild-type in all strains (Figure 3.9). *mdm20Δ* had the highest levels of Gcn4, which was surprising as its polysome profile did not show an initiation block. However, Gross had previously showed that *mdm20Δ* did have an initiation

block (Gross and Kinzy, 2007). The next highest level achieved was in *tpm2Δ* and *tpm1Δ*, which both have initiation blocks. yTHC*BMS1* (with and without 0.5µg/mL doxycycline) and *spt3Δ*, which are the highest producers for Fps1, resulted in a 2 fold and 4 fold increase in Gcn4 levels, respectively. This suggests that there is an association between increased Fps1 yields and increased Gcn4 levels, although it is not a strong, positive correlation. The ribosomal protein and ribosomal biogenesis strains also caused an increase in Gcn4 levels between 2 and 6 fold compared to wild-type levels; this was expected on the basis of previous studies (Foiani et al., 1991; Martín-Marcos et al., 2007; Steffen et al., 2008).

3.7. The 5´UTR of *FPS1* contains start codons with in-frame stop codons

It is well known from the work by Kozak (Kozak, 2001, 1987) and Hinnebusch (Hinnebusch, 2005) that uORFs in the 5´UTRs of transcripts prevent the translation of ORFs and play a part in the regulation of gene expression. It is also known that having start codons upstream and out of frame of an ORF start codon decreases protein translation (Kozak, 1984).

The sequence upstream of the *FPS1* ORF and downstream of the *TPI1* promoter was analysed for the presence of start codons. To confirm the length of the 5´UTR component of the vectors pYX222-*FPS1*-HA_3 and pYX212-*FPS1*-HA_3, transcriptional start site information for *TPI1* was obtained from the literature (Tuller et al., 2009). The sequence of the *TPI* promoter in this expression system is:

TAACTACAAAAAACACATACAG

The 5´UTR sequence of *FPS1* was consequently defined, below, where red represents the sequence of the *TPI* promoter, blue represents sequence from the pYX222/pYX212 backbone, green represents the sequence upstream of the *FPS1* gene and the underlined sequence represents the beginning of the *FPS1* ORF. This results in a 5´UTR of a length of 287nt:

TAACTACAAAAAACACATACAGGAATTCACCATGGATCTCATAGTGAGAAGGCGCAATTCAGTAGTTA
AAAGCGGGGAACAGTGTGAATCCGGAGACGGCAAGATTGCCCGGCCCTTTTTGCGGAAAAGATAAAAC
AAGATATATTGCACTTTTTCCACCAAGAAAAACAGGAAGTGGATTAAAAAATCAACAAAGTATAACGC
CTATTGTCCCAATAAGCGTCGGTTGTTCTTCTTTATTATTTTACCAAGTACGCTCGAGGGTACATTCT
AATGCATTAAAAGACATGAGTAATCCTCAAAAAGCTCTAAACGACTTTCTGTCCAGTGAATCTGTTCA
TACAC

Two start codons with in-frame stop codons were identified in the *FPS1* 5´UTR. Consequently, there are two uORFs in the *FPS1* 5´UTR as highlighted with a wavy line:

pYX222-*FPS1*-HA$_3$/ pYX212-*FPS1*-HA$_3$

TAACTACAAAAAACACATACAGGAATTCACCATGGATCTCATAGTGAGAAGGCGCAATTCAGTAGTTA
AAAGCGGGGAACAGTGTGAATCCGGAGACGGCAAGATTGCCCGGCCCTTTTTGCGGAAAAGATAAAAC
AAGATATATTGCACTTTTTCCACCAAGAAAAACAGGAAGTGGATTAAAAAATCAACAAAGTATAACGC
CTATTGTCCCAATAAGCGTCGGTTGTTCTTCTTTATTATTTTACCAAGTACGCTCGAGGGTACATTCT
AATGCATTAAAAGACATGAGTAATCCTCAAAAAGCTCTAAACGACTTTCTGTCCAGTGAATCTGTTCA
TACAC

The first uORF originates from the pYX222/ pYX212 backbone, since a Kozak sequence had been inserted before the multiple cloning site and after the *TPI* promoter, presumably to aid in the expression of the gene (Kozak, 1981). When *FPS1*, with 253 nucleotides upstream of the *FPS1* ORF, was inserted into the multiple cloning site of the vectors pYX222 and pYX212, this resulted in the insertion of a stop codon in-frame with the Kozak sequence, resulting in the creation of the first uORF.

The second uORF is native; using transcriptional start site information from Tuller et al., 2009 the length of the native 5´UTR of the *FPS1* transcript was determined and found to be 78 nucleotides long. The second uORF in the vector sequences pYX222-*FPS1*-HA$_3$ and pYX212-*FPS1*-HA$_3$ therefore occurs natively in the 5´UTR of *FPS1* when expressed from the yeast genome. Note that the vectors pYX222-*FPS1*-HA$_3$ and pYX2121-*FPS1*-HA$_3$ were those used in the Bonander et al., 2005 and Bonander et al., 2009 studies.

To examine the effect of these two uORFs, two vectors were created, one where the first uORF was removed (referred to as pYX222-5´Δ1-43-*FPS1*-HA$_3$) and the second where both uORFs were removed (pYX222-5´Δ1-215-*FPS1*-HA$_3$).

The vectors pYX222-5´Δ1-43-*FPS1*-HA$_3$ and pYX222-5´Δ1-215-*FPS1*-HA$_3$ were made by removing the Kozak sequence from the pYX222 vector by digestion with *Eco*RI and *Hind*III. The backbone was then dephosphorylated and purified. The required *FPS1* sequence was then obtained by creating appropriate primers incorporating the restriction sites *Eco*RI and *Hind*III, and the product obtained by PCR.

The new vectors therefore had the following 5´UTR sequences:

pYX222-5´Δ1-43-*FPS1*-HA$_3$

TAACTACAAAAAACACATACAGGAATTCGCGGGGAACAGTGTGAATCCGGAGACGGCAAGATTGCCCG
GCCCTTTTTGCGGAAAAGATAAAACAAGATATATTGCACTTTTTCCACCAAGAAAAACAGGAAGTGGA
TTAAAAAATCAACAAAGTATAACGCCTATTGTCCCAATAAGCGTCGGTTGTTCTTCTTTATTATTTTA
CCAAGTACGCTCGAGGGTACATTCTAATGCATTAAAAGACATGAGTAATCCTCAAAAAGCTCTAAACG
ACTT

pYX222-5´Δ1-215-*FPS1*-HA$_3$

TAACTACAAAAAACACATACAGGAATTCCATGAGTAATCCTCAAAAAGCTCTAAACGACTTTCTGTCC
A

3.7.1. Fps1 yield is increased in wild-type yeast when uORFs are deleted

To determine the impact of the uORFs on Fps1 yield, the new vectors were analysed in wild-type cells and two high yielding strains, yTHC*BMS1* (with 0.5 µg/mL doxycycline) and *spt3Δ*. Yeast cells were transformed using the lithium acetate method, cultures were grown to the end of the diauxic shift in 2×CBS without histidine and the cells were harvested. Membrane preparations and western blots were performed as described in section 2.10 and the western blot data were analysed using ImageJ. It is important to note that due to the large differences in Fps1 yields from the different yeast and vector systems, saturation of the western blots often occurred. Consequently, experiments were repeated and the protein levels loaded into the wells were adjusted to prevent saturation.

***Table 3.2: Yields of Fps1 resulting from different strains and vector combinations**. Data are reported relative to wild-type cells expressing Fps1 from the vectorpYX222-*FPS1*-*HA$_3$*. Values in parentheses are the standard error of the mean calculated from biological triplicates. Analysis of western blot data was done using ImageJ.*

Strains and Vectors	Fps1 yield relative to wild-type cells transformed with pYX222-*FPS1*-HA$_3$
WT pYX222-*FPS1*-HA$_3$	1.0 (0.5)
WT pYX222-5´Δ1-43-*FPS1*-HA$_3$	9.2 (2.5)
WT pYX222-5´Δ1-215-*FPS1*-HA$_3$	541.8 (75.0)
yTHC*BMS1* (with 0.5µg/mL doxycycline) pYX222-*FPS1*-HA$_3$	32.4 (6.7)
yTHC*BMS1* (with 0.5µg/mL doxycycline) pYX222-5´Δ1-43-*FPS1*-HA$_3$	106.5 (24.2)
yTHC*BMS1* (with 0.5µg/mL doxycycline) pYX222-5´Δ1-215-*FPS1*-HA$_3$	355.8 (110.3)
*spt3*Δ pYX222-*FPS1*-HA$_3$	40.6 (8.7)
*spt3*Δ pYX222-5´Δ1-43-*FPS1*-HA$_3$	60.7 (12.3)
*spt3*Δ pYX222-5´Δ1-215-*FPS1*-HA$_3$	209.9 (49.6)

Table 3.2 shows the relative yields of Fps1 compared to wild-type cells transformed with the vector pYX222-*FPS1*-HA$_3$. In the absence of the first uORF from the Fps1 transcript (Δ1-43), the Fps1 yield increased 9-fold in wild-type yeast. As expected when both uORFs were deleted from the transcript (Δ1-215), the yield of Fps1 increased to 542-fold relative to the wild-type control. These data suggest that the uORFs in the 5´UTR of the *FPS1* transcript limit the translation of *FPS1* in wild-type cells.

As shown in Figure 3.1, yTHC*BMS1* (with 0.5µg/mL doxycycline) increases Fps1 production by 32-fold compared to wild-type cells when both uORFs are present in the *FPS1* transcript. When

expressing *FPS1* from the vector pYX222-5′Δ1-43-*FPS1*-HA_3, Fps1 yield was increased-107 fold compared to the wild-type control with two uORFs in the transcript. Furthermore, when there were no uORFs in the *FPS1* transcript, Fps1 yield increased 356-fold. For *spt3Δ* the yield of Fps1 expression was initially 41-fold higher than the wild-type control. Yields increased to 61-fold and 210-fold higher than the wild-type control when the first uORF and both uORFs were deleted, respectively. These data strongly suggest the high yielding strains are able to circumvent the uORFs to enable translation of *FPS1*.

*3.7.2. Expression of Fps1 from the vector pYX222-5′Δ1-215-***FPS1-*HA*$_3$ *causes a reduction in growth rate*

Table 3.3 shows that the expression of Fps1 from the vector, pYX222-5′Δ1-215-*FPS1*-HA_3, caused a reduction in culture growth rates. This suggests that the production of high levels of protein is correlated with decreased growth rates, potentially due to the corresponding metabolic burden. Growth rates were slightly increased in all three strains when expressing Fps1 from the vector pYX222-5′Δ1-43-*FPS1*-HA_3. This vector system represents the native version of the *FPS1* transcript found in yeast. Growth rates for the original pYX222-*FPS1*-HA_3 vector were slightly lower, which could be due to the production of uORF1 (resulting in a protein of 34 amino acids) representing a metabolic burden.

Table 3.3: Growth rates of wild-type and the high yielding strains yTHC*BMS1 *(with 0.5µg/mL doxycycline) and* spt3Δ *when expressing* FPS1 *from different vectors*. Yeast cells were grown in 2×CBS-HIS. OD_{600} readings were recorded to generate growth rate data. Values in brackets represent the standard error of the mean.***

Strains and Vectors	Growth rate (h^{-1})
WT pYX222-*FPS1*-HA_3	0.398 (0.003)
WT pYX222-5′Δ1-43-*FPS1*- HA_3	0.401 (0.003)
WT pYX222-5′Δ1-215-*FPS1*- HA_3	0.358 (0.002)
yTHC*BMS1* (with 0.5µg/mL doxycycline pYX222-*FPS1*- HA_3)	0.352 (0.000)
yTHC*BMS1* (with 0.5µg/mL doxycycline pYX222-5′Δ1-43-*FPS1*- HA_3)	0.369 (0.002)
yTHC*BMS1* (with 0.5µg/mL doxycycline pYX222-5′Δ1-215-*FPS1*- HA_3)	0.308 (0.005)
*spt3*Δ pYX222-*FPS1*- HA_3	0.275 (0.001)
*spt3*Δ pYX222-5′Δ1-43-*FPS1*- HA_3	0.279 (0.003)
*spt3*Δ pYX222-5′Δ1-215-*FPS1*- HA_3	0.256 (0.003)

3.8. GFP yield is increased in the yTHC*BMS1* strain (with 10µg/mL doxycycline), but not significantly in the other high yielding strains

GFP is found natively in the jelly fish *Aequorea victoria*, is 238 amino acids long and contains a chromophore, which when excited at 395nm emits a green light at 509nm. GFP is often used as a reporter protein to monitor gene expression and protein localisation and can also be used to investigate protein interactions. The chromophore of GFP has been mutated to generate different fluorescent

proteins such as RFP and YFP which can be used in microscopy when determining the localisation of different proteins (Prasher, 1995; Sirerol-Piquer et al., 2012). Bonander demonstrated a doubling in GFP yield in the yTHC*BMS1* strain (with 10µg/mL doxycycline); on addition of 0.5µg/mL doxycycline there was no increase compared to wild-type cells (Bonander et al., 2009).

To confirm this finding in shake flasks, strains were transformed with the vector pYX222-alphaSS-*GFP* (see Appendix A.3.), using the lithium acetate method. The vector contained the Kozak sequence from the pYX222 backbone, but the start codon was in-frame with the alpha secretion signal start codon resulting in a protein with a short N-terminal addition. Cultures were grown until the end of the diauxic shift and supernatants collected and stored at -20°C until required. The yield of GFP was analysed using a Gemini plate reader.

The data in Figure 3.10 show the relative yields of GFP produced in wild-type cells and the high yielding strains. There was no significant increase in GFP levels in yTHC*BMS1* (with 0.5µg/mL doxycycline), yTHC*BMS1* (without doxycycline), *spt3Δ, gcn5Δ* or *srb5Δ*. When the 5´UTR of GFP was analysed it was found not to contain any uORFs. In contrast, for Fps1 all strains including yTHC*BMS1* (without doxycycline) resulted in at least a 5-fold increase in Fps1 yield.

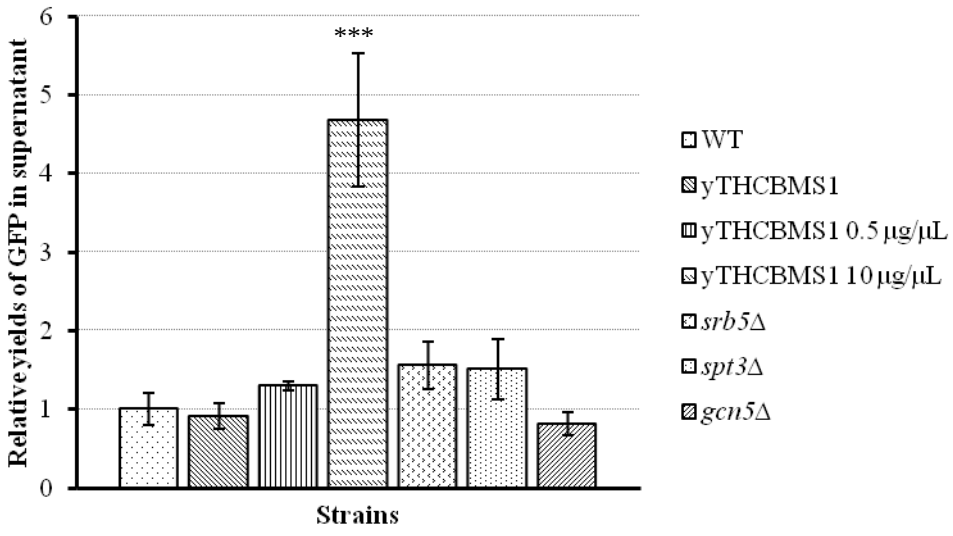


Figure 3.10: Relative yields of GFP in the high yielding strains*. Yeast cells were transformed with pYX222-alphaSS-GFP, grown until just before the diauxic shift in 2×CBS-HIS and supernatants harvested. All experiments were performed in triplicate. Data were analyzed using a one-way ANOVA (P <0.001). Asterisks show the significance of the data determined by a Dunnett's multiple comparison test, where *** = P ≤ 0.001.*

In the presence of 10µg/mL doxycycline, the yTHC*BMS1* strain gave a 4.5-fold increase in GFP concentrations in the supernatant; moreover it was previously shown that the levels of GFP steadily increase when higher concentrations of doxycycline are added between 0.5 - 10µg/mL of doxycycline (Bonander et al., 2009). One factor which may be influencing an increase in GFP when 10µg/mL of

doxycycline is added is the growth rate (Table 3.4), which is reduced from $0.33h^{-1}$ in yTHC*BMS1* to $0.24h^{-1}$ in yTHC*BMS1* with 0.5µg/mL doxycycline to $0.18h^{-1}$ in yTHC*BMS1* (with 10µg/mL doxycycline). This reduction in growth rate may be due to the further reduction in the 40S ribosomal subunit, which has been shown to be undetectable when 10µg/mL doxycycline is added to yTHC*BMS1* (Bonander et al., 2009). However, the high yielding strain, *gcn5Δ*, also has a slower growth rate than yTHC*BMS1* (with 0.5µg/mL doxycycline) and does not give an increased yield of GFP. It is therefore likely that other mechanisms are responsible for increasing GFP yield in yTHC*BMS1* (with 10µg/mL doxycycline).

Table 3.4: Growth rates of wild-type and the high yielding strains when transformed with pYX222-alphaSS-GFP. *Yeast cells were grown in 2×CBS without histidine, OD_{600} readings were recorded and growth rate data obtained from the growth curves. Where n=1.*

Strain transformed with pYX222-alphaSS-GFP	Growth rate (h^{-1})
WT	0.35
srb5Δ	0.21
spt3Δ	0.27
gcn5Δ	0.21
yTHC*BMS1*	0.33
yTHC*BMS1* (with 0.5µg/ml doxycycline)	0.24
yTHC*BMS1* (10µg/ml doxycycline)	0.18

3.9. Summary

The high yielding strains yTHC*BMS1* (with 0.5µg/mL doxycycline), *srb5Δ, gcn5Δ* and *spt3Δ* have been shown to increase Fps1 yields in shake flasks up to 40-fold compared to wild-type cells. These high yielding strains were also found to have a change in their translational states; most of them had initiation blocks and all strains exhibited constitutive phosphorylation of eIF2α under normal conditions. Further strains were selected with known initiation blocks and were shown to increase Fps1 yields. All strains tested had increased Gcn4 levels compared to wild-type cells. These data prompted an examination of the 5´UTR of *FPS1*, which was found to contain two uORFs, one which was created during cloning and a second which is native to the *FPS1* transcript. When these uORFs were removed, this resulted in a 550-fold increase in Fps1 yield in wild-type yeast. The high yielding strains *spt3Δ* and yTHC*BMS1* (with 0.5µg/mL doxycycline) gave a 210-fold and 360-fold increase in Fps1 production, respectively, compared to the wild-type control with both uORFs intact. These yields are lower than those in wild-type cells. The high yielding strains are proposed to function either by reading through the uORFs or re-initiating translation at the *FPS1* ORF. Data for GFP indicated that only yTHC*BMS1* with 10µg/mL doxycycline increased yields, suggesting that the yTHC*BMS1* may have some generic benefits as a tuneable cell factory for recombinant protein production. The results of this chapter are published as three articles; an original research article currently in submission, a review (Bawa et al., 2011) and a book chapter (Darby et al., 2012).

Chapter 4: The metabolism-dependent effects of L-carnosine on *Saccharomyces cerevisiae*

As discussed in Chapter 1, L-carnosine has both anti-senescent and anti-cancer properties. The aim of the research described in this chapter was to establish a metabolism-dependent mechanism for its actions.

4.1. L-Carnosine addition does not increase the chronological lifespan of glucose-grown *S. cerevisiae*

The initial aim of this project was to determine whether L-carnosine increases the CLS of yeast cells, since it can increase the lifespan of mammalian (McFarland and Holliday, 1994) and bacterial cells (Pepper et al., 2010). Initial experiments were therefore performed to establish a chronological life span assay; this was done by using the known longevity mutants, *tor1Δ* and *hxk2Δ*, as controls to replicate previously published results (Kaeberlein et al., 2005).

The CLS experiment was performed in biological triplicates (each in technical duplicate) with or without 10mM L-carnosine. Yeast were grown in 2×CBS with 2% glucose and 4×DO (rather than 2×DO) with all amino acids, so that amino acids were not a factor in determining lifespan. Samples were removed every 2-3 days and diluted 50,000 fold so that colony forming units (CFUs) could be counted by eye on YPD plates after 2-3 days of growth at 30°C.

Unexpectedly, as shown in Figure 4.1, yeast grown in the presence of L-carnosine resulted in very few CFUs, suggesting a lack of growth or even cell death. This was surprising as the L-carnosine cultures had high optical density readings and visualisation through a microscope confirmed similar numbers of cells compared with controls. However, the morphology of the yeast cells grown in L-carnosine was different. Due to the lack of CFUs, the plates were incubated for 1 week to confirm that the CFUs were not growing slowly; this experiment indicated that the addition of L-carnosine to glucose-grown cells results in cell death. Consequently, the effect of L-carnosine on yeast lifespan could not be pursued.

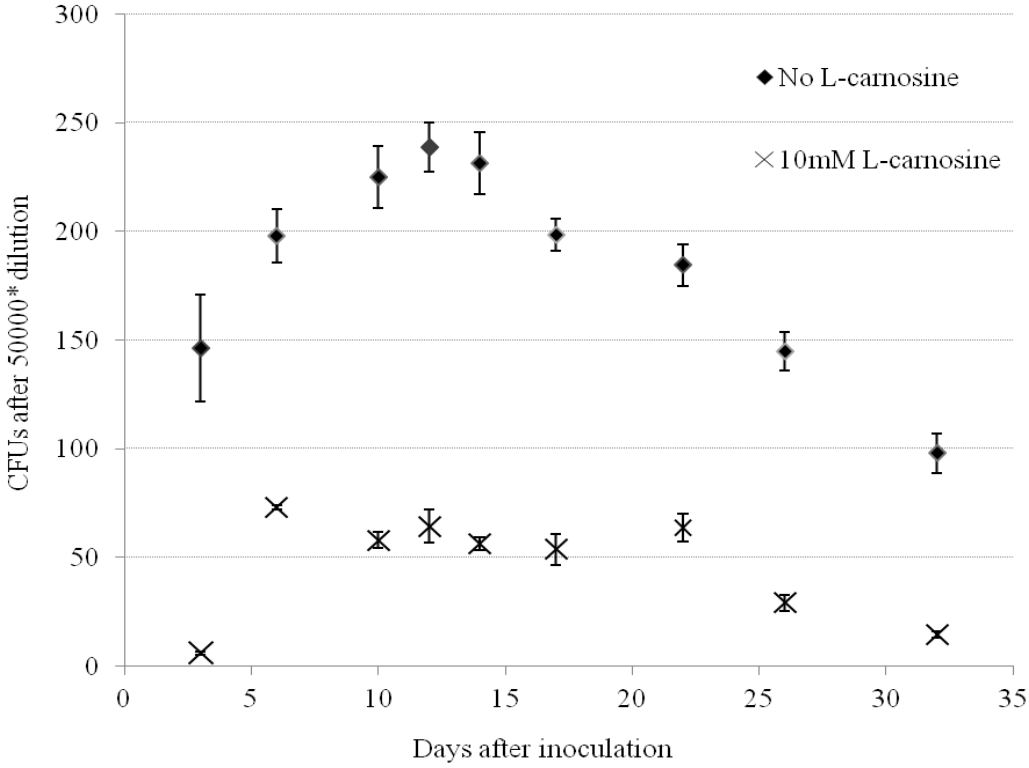


Figure 4.1: Colony forming units (CFUs) of yeast grown with or without 10mM L-carnosine. *Yeast were grown on 2×CBS with 4×DO, where n=3. Error bars represent the standard error of the mean (SEM).*

4.2. L-Carnosine addition decreases the specific growth rate of glucose-grown *S. cerevisiae*

Since there was a decrease in CFUs when *S. cerevisiae* cells were grown in the presence of L-carnosine and with glucose as the carbon source, growth rate, glucose consumption rate and ethanol production and consumption rates were monitored. As described in Chapter 1, when yeast cells are grown in liquid culture on a fermentable carbon source, such as 2% glucose, they exhibit a characteristic growth profile. During exponential growth, respiration is inhibited (the Crabtree effect). Once the glucose has been consumed, this is followed by a diauxic shift, after which the ethanol generated in the glucose consumption phase is metabolized aerobically.

Yeast cells were grown in liquid cultures of 2×CBS medium, with or without 10-30mM L-carnosine. The optical densities of the cultures were recorded throughout growth and supernatants were collected and stored at -20°C for subsequent analysis: glucose readings were made using a diabetic monitor and ethanol analysis was done by gas chromatography, as described in sections 2.5 and 2.7.

Table 4.1 shows that L-carnosine addition to yeast cultures decreased their specific growth rate in a dose-dependent manner, slowing growth to 65% of the control value at a concentration of 30 mM; the growth rates of cultures containing 20 mM and 30 mM L-carnosine were calculated at the beginning of the logarithmic growth phase as the growth rates were so slow. Consequently the majority of subsequent experiments were performed in the presence of 10 mM L-carnosine in complex (YP) medium, as growth rates on YP medium are higher. The reduction in growth rate was consistent with a decreased rate of glucose consumption and decreased ethanol production and consumption rates,

compared with control cultures (Figure 4.2). Following the diauxic shift, L-carnosine-treated cells did not reach the same optical densities as untreated control cells, even though similar levels of ethanol were produced. It is also interesting to note that the smell of cultures containing L-carnosine was different from control cultures. It was therefore thought that cultures containing L-carnosine were not producing ethanol, however as Figure 4.2 shows they do produce ethanol; the molecule or molecules which changed the smell are still unknown. One possibility is that the change in smell could be due to the absence of another aromatic compound, or more than one, of which many are produced during yeast fermentation, such as, fusel alcohols. These aromatic compounds give alcoholic drinks their distinct properties (Mills, 1968).

As L-carnosine has an effect on growth rates, the effect of its enantiomer D-carnosine (β-alanyl-D-histidine) and the components of L-carnosine, L-histidine and β-alanine, were investigated. As Table 4.1 indicates, yeast grown in the presence of 10 mM D-carnosine or 10 mM β-alanine and /or L-histidine did not exhibit a reduced growth rate. On addition of 30 mM β-alanine and 30 mM L-histidine, there was a slowing of growth comparable to that caused by addition of 10 mM L-carnosine, but no cell death.

Table 4.1: The effect of L-carnosine, D-carnosine , L-histidine and β-alanine on the specific growth rate and viability of yeast cultures grown on fermentable (glucose) and non-fermentable (glycerol) carbon sources*. Yeast cells were grown in shake-flasks at 30°C in 2×CBS medium supplemented with 2% glucose or in YP supplemented with 2% glycerol as well as L-carnosine, D-carnosine, L-histidine or β-alanine (0–30 mM). Replicate cultures were performed as indicated and specific growth rates (μ; derived from OD_{600} growth curves) and viability (%; determined by trypan blue dye exclusion) were determined. Data were analyzed using a one-way ANOVA (P <0.0001). Asterisks show the significance of the specific growth rate data for each culture condition compared to the control, as determined by a Dunnett's multiple comparison test, where * = P ≤ 0.05 and ** = P ≤ 0.01. The standard error of the mean (SEM) is given in parentheses. A dash (–) indicates that the experimental conditions indicated were not investigated.*

	μ (h^{-1})		**Viability (%)**	
L-Carnosine, D-carnosine, L-histidine or β-alanine concentration	***+2% glucose***	***+2% glycerol***	***+2% glucose***	***+2% glycerol***
Control (0 mM L-carnosine); n=3	0.41 (0.00)	0.20 (0.01)	99.56 (0.12)	97.60 (1.04)
10 mM L-carnosine; n=3	0.36** (0.01)	0.22* (0.01)	90.09* (0.28)	99.14 (0.63)
20 mM L-carnosine; n=5	0.28** (0.01)	–	86.31** (0.94)	–
30 mM L-carnosine; n=5	0.27** (0.02)	0.25** (0.01)	82.71** (1.67)	97.52 (1.92)
10 mM D-carnosine; n=3	0.44 (0.00)	0.30**(0.00)	99.35 (0.33)	97.31 (0.34)
10 mM L-histidine; n=6	0.40 (0.02)	–	99.94 (0.04)	–
10 mM β-alanine; n=3	0.41 (0.00)	–	99.78 (0.12)	–
10 mM L-histidine, 10 mM β-alanine; n=2	0.42 (0.00)	–	100 (0.00)	–
30 mM L-histidine, 30 mM β-alanine; n=3	0.37** (0.01)	0.23* (0.00)	99.81 (0.24)	98.45 (1.55)

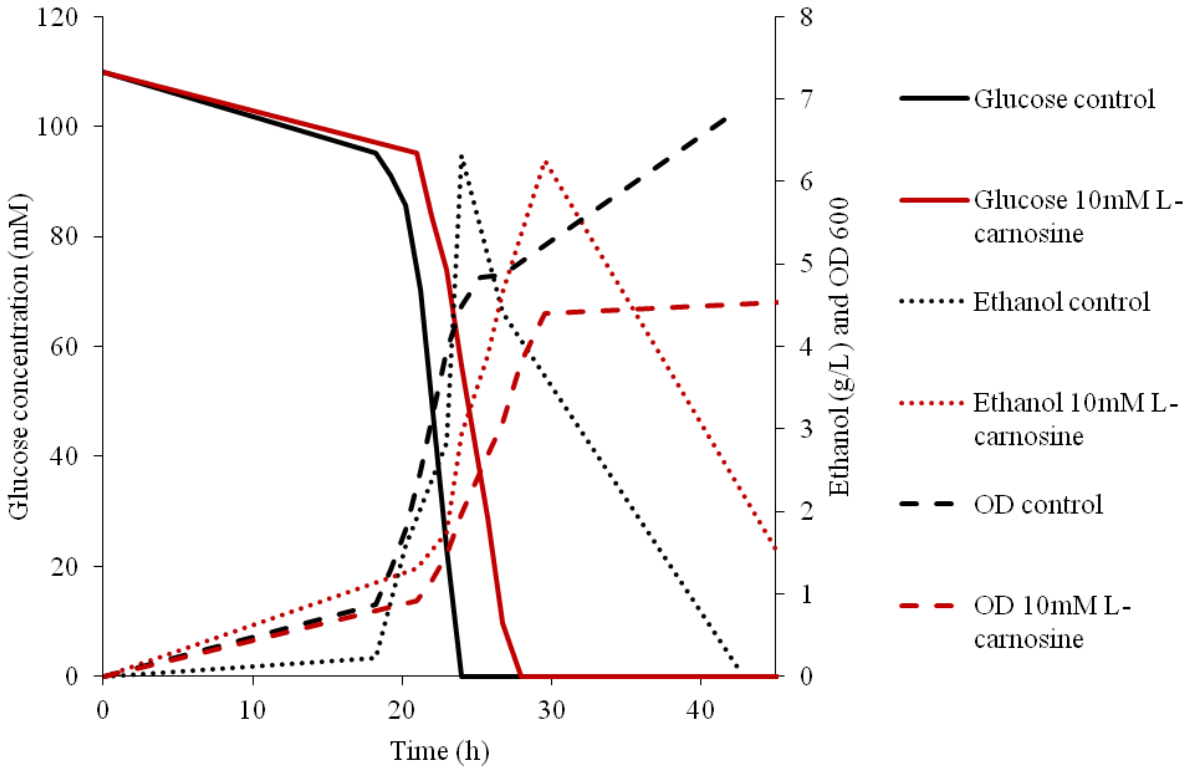


Figure 4.2: Representative growth curve, glucose consumption and ethanol production and consumption curves for yeast grown with or without 10mM L-carnosine in 2×CBS*. Black lines represent growth with no L-carnosine, whilst red lines represent growth with 10mM L-carnosine. Lines represent glucose concentrations, dots ethanol concentrations and dashes optical density measured at 600nm.*

4.3. The addition of L-carnosine changes the morphology of yeast cells

It was noticed that there was a change in the morphology of yeast cells when 10mM L-carnosine was added to yeast cultures. To obtain data for analysis, images were taken using a camera (Zeiss AxiocamHRC) mounted on a light microscope (Zeiss Axioskop). Images were taken at 40× and 100× zoom. The width and length of cells that had been cultured with and without L-carnosine were recorded using ImageJ, n=149 for 10mM L-carnosine and n=153 for no L-carnosine.

The average length of cells when L-carnosine had been added to the culture was 7.03µm (SEM: 0.11) and the width 4.29µm (SEM: 0.06). The average length of cells when there was no L-carnosine added was 6.80µm (SEM: 0.12) whist the width was 4.42µm (SEM: 0.07). This gives a percentage increase in length of 3.36% and a 2.97% decrease in width of cells when L-carnosine is added to cultures. Statistical analysis was performed on the data after the areas were calculated (using the equation for the area of an elipse: $\pi \times L/2 \times W/2$); the data did not have a normal distribution thus a non-parametric t-test was performed. No significant differences were found between the medians of the data. A Levene's test was then performed to compare the distribution of the data; this revealed a significant difference between the variance of the data ($P = 0.001$). Histograms (Figure 4.3) were made of the elipse area of the yeast cells to show the differences in the size distribution. Thus when L-carnosine is added to yeast cells, this results in a greater variation in cell size than in control cells. As Figure 4.4 shows, yeast cells

grown in the presence of L-carnosine appear more irregular in shape, with irregular vacuoles and, in some cases, cells appear darker.

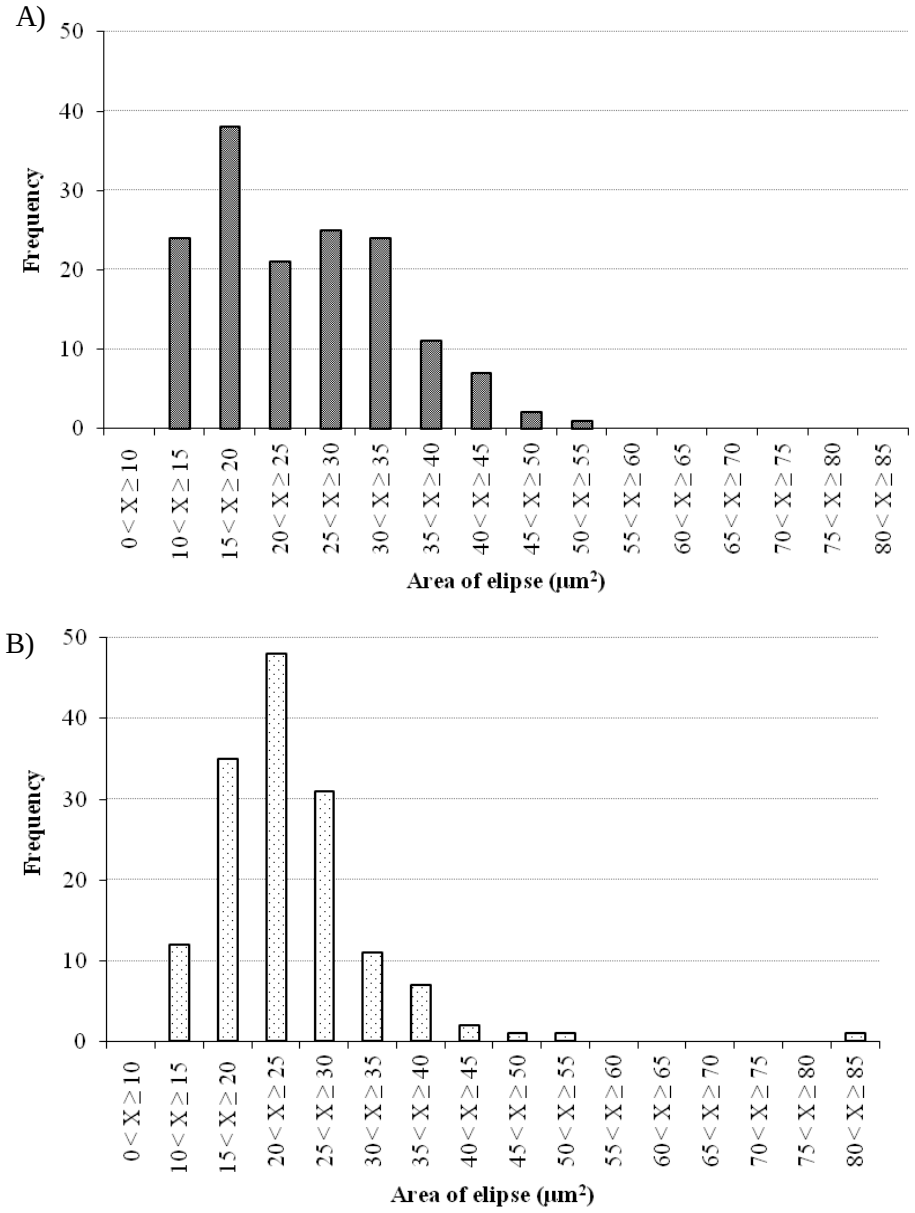


Figure 4.3: Histograms showing size distribution of yeast cells grown on the fermentable carbon source, 2% glucose*. Yeast cells cultured in shake-flasks at 30°C in 2×CBS medium supplemented with 2% glucose. A) Histogram of yeast cells grown in the absence of L-carnosine. B) Histogram showing size distribution of yeast cells grown in the presence of 10 mM L-carnosine.*

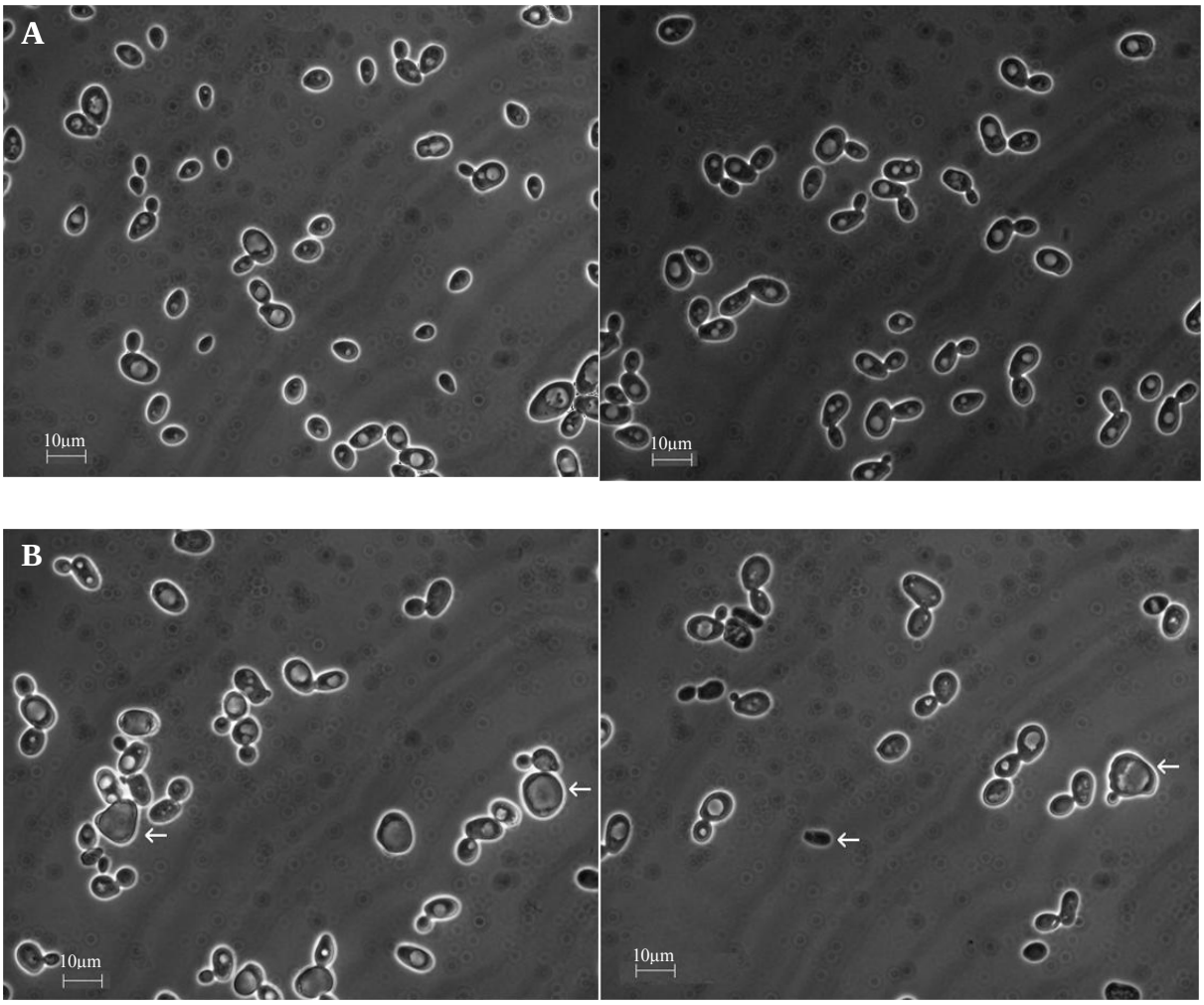


Figure 4.4: L-Carnosine addition alters the morphology of yeast cells grown on the fermentable carbon source, 2% glucose*. Yeast cells cultured in shake-flasks at 30°C in 2×CBS medium supplemented with 2% glucose (A) were compared with those where the growth medium also contained 10 mM L-carnosine (B). Cells were visualized by light microscopy after reaching stationary phase, 48 h post inoculation. A proportion of the L-carnosine-treated cells (indicated with a white arrow; B) appear more irregular in shape than control cells. The bar is 10 µm in length.*

4.4. L-Carnosine addition reduces the viability of glucose-grown cells

Since cells grown in the presence of L-carnosine had a reduction in growth rate, formed fewer CFUs and had altered cellular morphology, the viability of yeast cultures grown overnight on 2×CBS with 2% glucose, with and without L-carnosine, was determined microscopically by trypan blue dye exclusion. The number of blue cells in a total population of 100 cells was determined to give percentage viability. The number of biological replicates performed is reported in Table 4.1.

The addition of L-carnosine to cultures was observed to cause an increase in the proportion of blue-stained cells, which is an indicator of cell death. There was an increase in cell death in a dose dependent manner, reducing the viability from 99.6% in control cells to 82.7% when 30mM L-carnosine is added to cultures (Table 4.1).

The viability of cultures was also determined when grown in the presence of 10mM D-carnosine or 10-30mM β-alanine and/or L-histidine. Under these conditions, yeast cells did not exhibit a reduction in viability compared to control cells (Table 4.1).

To determine whether L-carnosine had an effect on yeast cells once they had entered stationary phase, yeast cells were grown for 7 days in shake flasks (to guarantee that they had entered stationary phase) in 2×CBS with 2% glucose, after which 10mM L-carnosine was added to the cultures. The viability of the cultures immediately before addition of L-carnosine was 99.7% (SEM: 0.29), where n=3, Once L-carnosine had been added the viability had not changed; after 2h it was at 99.07% (SEM: 0.93) and 24h 100% (SEM: 0.00).

4.5. L-Carnosine addition affects the metabolism of glucose-grown cells

As L-carnosine addition causes a change in growth rate, the metabolic activity of cultures was determined by on-line flow microcalorimetry using a Thermal Activity Monitor (TAM) (Bonander et al., 2009). This was done by running tubing from the TAM to shake flask cultures in a shaker incubator and back into the TAM (Figure 4.5). The heat output rate from the yeast cultures was converted to microwatts by the TAM and a digital reading was recorded.

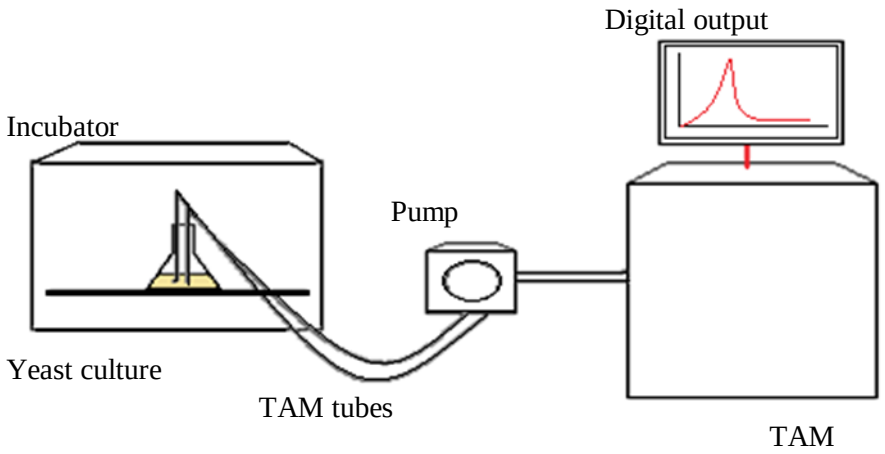


Figure 4.5: Representation of Thermal Activity Monitor (TAM) set up. *Yeast cultures are grown in an incubator at 30° at 220 rpm. Culture is pumped from the shake flask into the TAM, where the heat output of the cultures is detected and recorded in µW, where a digital output is recorded and analysed in excel.*

Figure 4.6, shows that the addition of L-carnosine profoundly affected the heat output profiles of the cultures in a dose-dependent manner. As L-carnosine concentrations were increased, the calorimetry profiles appeared to become monophasic rather than biphasic, and develop broader peaks, where the peak was more delayed. This is probably due to a decrease in growth rate as a result of cell death; heat output is proportional to the number of metabolically active cells. Moreover, the secondary peak associated with ethanol consumption was either not present in the L-carnosine profiles or became very large and broad. This was unexpected as ethanol production was shown to be taking place (Figure 4.2),

Even though ethanol consumption is delayed in L-carnosine growing yeast, the slower consumption would not explain the huge elongation of the second peak.

The heat output rate calculated from the glucose phase of the microcalorimetry traces mirrored that of the specific growth rates in Table 4.1, being 0.37 h^{-1} (SEM: 0.00; n=4) for control cells with a dose-dependent reduction to 0.28 h^{-1} (0.02; n=4), 0.21 h^{-1} (0.03; n=2) and 0.14 h^{-1} (0.02; n=2) for cells grown in the presence of 10, 20 and 30 mM L-carnosine, respectively. Close examination of the microcalorimetry traces revealed that in the presence of L-carnosine, growth did not fit strictly to an exponential curve prior to the diauxic shift. This is probably due to cell death. Together with the observation that L-carnosine addition causes a subset of yeast cells to die in culture (Table 4.1), this might explain the numerical differences between the two sets of rate data; the OD_{600} growth curves will necessarily yield higher growth rates than the heat output curves as the former measures the density of all cells, whilst the latter only measures metabolically active ones.

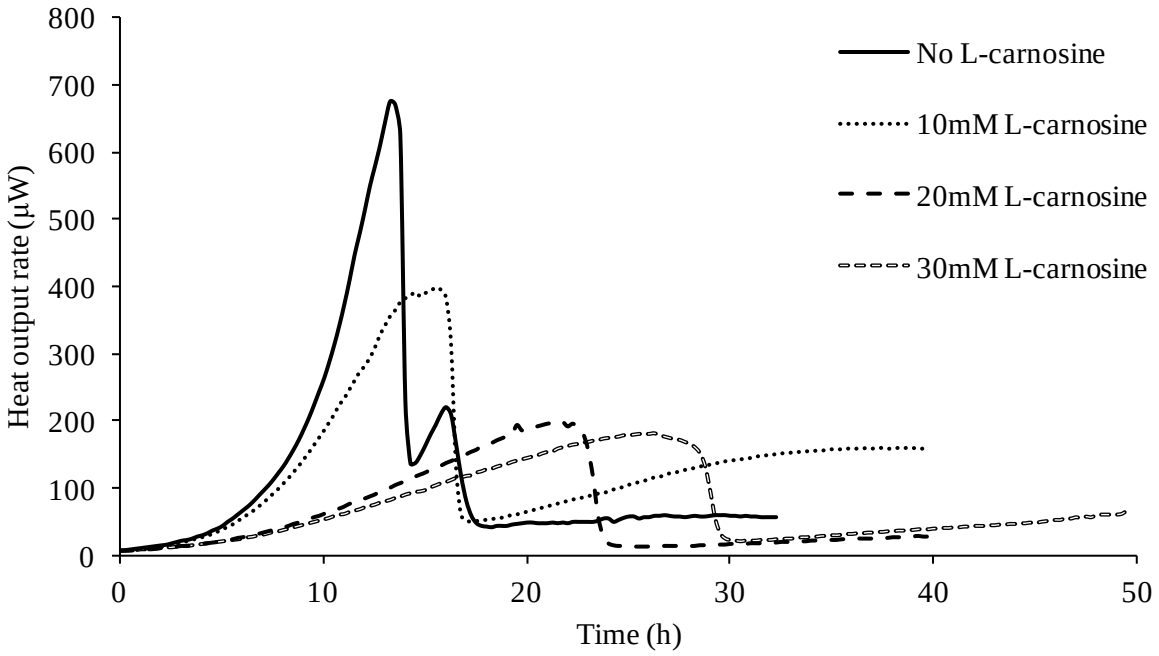


Figure 4.6: L-Carnosine profoundly affects the metabolism of yeast cells grown on the fermentable carbon source, 2% glucose*. Representative data are shown from triplicate determination of yeast cells cultured in shake-flasks at 30°C in 2×CBS medium supplemented with 2% glucose in the presence of 0–30 mM L-carnosine, as indicated. The heat output rate was measured by on-line flow microcalorimetry. Control cells exhibit a classic biphasic metabolic profile on glucose; on addition of L-carnosine the profile is altered and a delayed broad peak is observed.*

To determine whether L-carnosine had an immediate effect on yeast cultures, 10mM L-carnosine was added midway through growth during a TAM experiment, at an OD_{600} of ~ 0.5 (around 100µW). Even when the addition of 10mM L-carnosine was delayed until mid-exponential growth, the resultant heat output rate began to resemble that of cells grown initially in 10mM L-carnosine (Figure 4.7), suggesting that L-carnosine exerts its effects immediately.

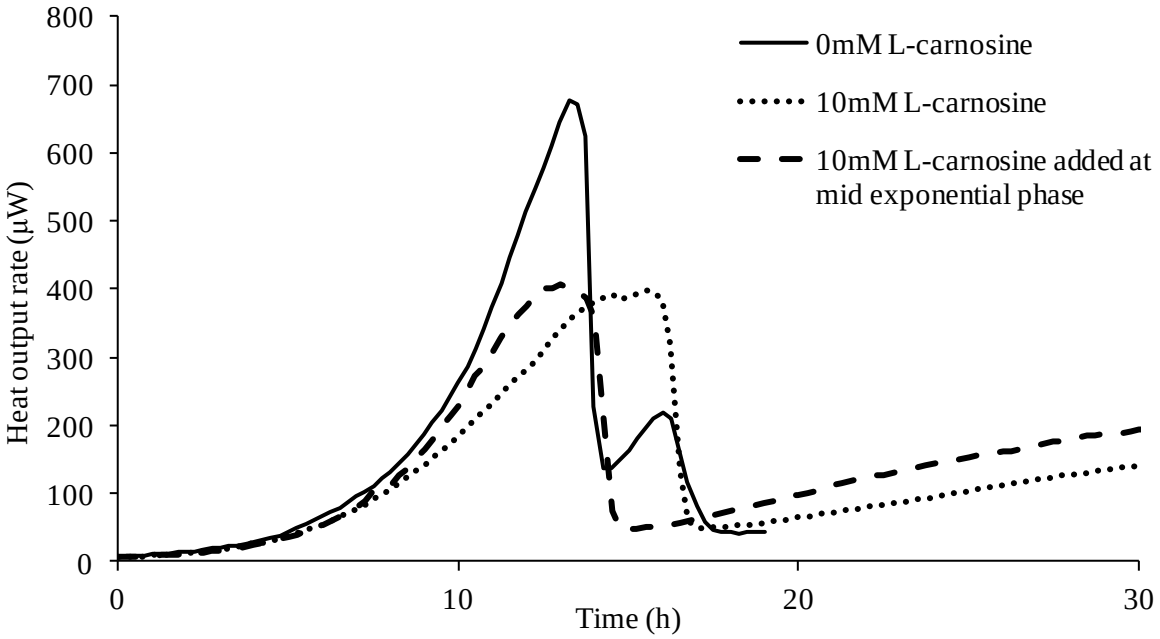


Figure 4.7: L-Carnosine addition midway through logarithmic growth has an immediate effect on metabolic output. *Representative data are shown from triplicate determinations of yeast cells cultured in shake-flasks at 30°C in 2×CBS medium supplemented with 2% glucose in the absence or presence of 10 mM L-carnosine or addition of L-carnosine midway through logarithmic growth.*

4.6. L-Carnosine addition increases the specific growth rate of cells grown on the non-fermentable carbon source, 2% glycerol, and does not reduce their viability

The addition of L-carnosine to cultures of *S. cerevisiae* resulted in cellular death in a dose-dependent manner. It is also known that L-carnosine addition can kill cancer cells, such as HeLa cells, as shown originally by Holliday and McFarland (Holliday and McFarland, 1996) and later confirmed by other research groups (Renner et al., 2010). The majority of tumour cells have a predominantly anaerobic metabolism, known as the Warburg effect. Yeast cells, similar to cancer cells, also have a fermentative metabolism when grown on fermentable carbon sources such as glucose, due to the Crabtree effect, which is the inhibition of respiration.

As both tumour cells and yeast cells grown on a fermentable carbon source have similar metabolisms and L-carnosine causes cell death in both, L-carnosine was added to yeast grown on the non-fermentable carbon source glycerol, to investigate whether the effects of L-carnosine were dependent on metabolism. Glycerol is metabolised by being converted to glycerol-3-phosphate by glycerol kinase which requires ATP; glycerol-3-phosphate is then converted to dihydroxyacetone phosphate by FAD-dependent glycerol-3-phophate dehydrogenase, which is located at the mitochondrial outer membrane. This enzyme requires FAD which is reduced to $FADH_2$, which is used in the electron transport chain. It is interesting to note that glycerol production uses different enzymes (Figure 1.3) (Nevoigt and Stahl, 1997). Growth in 2×CBS substituted with 2% glycerol did not support the growth of yeast, as a result yeast were grown on complex YP medium containing 2% glycerol (YPG).

As Table 4.1 shows, the addition of 10-30mM L-carnosine to cultures grown on glycerol did not result in a reduction in cellular growth; instead the addition of L-carnosine stimulated growth rate by 25%. At the same time there was no change in cellular morphology. There was no reduction in cellular viability, as determined by trypan blue dye exclusion.

Similar to the results shown with growth on glucose-containing medium, 10mM D-carnosine or 30mM L-histidine and/or β-alanine did not cause cellular death and stimulated cellular growth (Table 4.1). As yeast growing on glycerol in the presence of L-carnosine does not result in cellular death, yeast were grown in the presence of both 2% glucose and 2% glycerol, however this resulted in similar levels of cellular death as seen in cells grown on 2% glucose only. The reason cellular death may have occurred in this experiment is that glucose represses enzymes required for glycerol metabolism, thus yeast would act as if they are growing on glucose only (Nevoigt and Stahl, 1997).

Microcalorimetry traces of growth on YPG with and without 10mM L-carnosine indicated that the addition of L-carnosine stimulated growth rates. Figure 4.8 shows that the addition of L-carnosine causes an increase in heat output rate from $0.23h^{-1}$ (SEM: 0.16; n=2) to $0.29h^{-1}$ (SEM: 0.15; n=4). Growth on 2% glycerol, unlike growth on 2% glucose, results in a monophasic profile as there is no ethanol production. The increase in growth rate upon addition of L-carnosine may be due to L-carnosine being used as a source of amino acids.

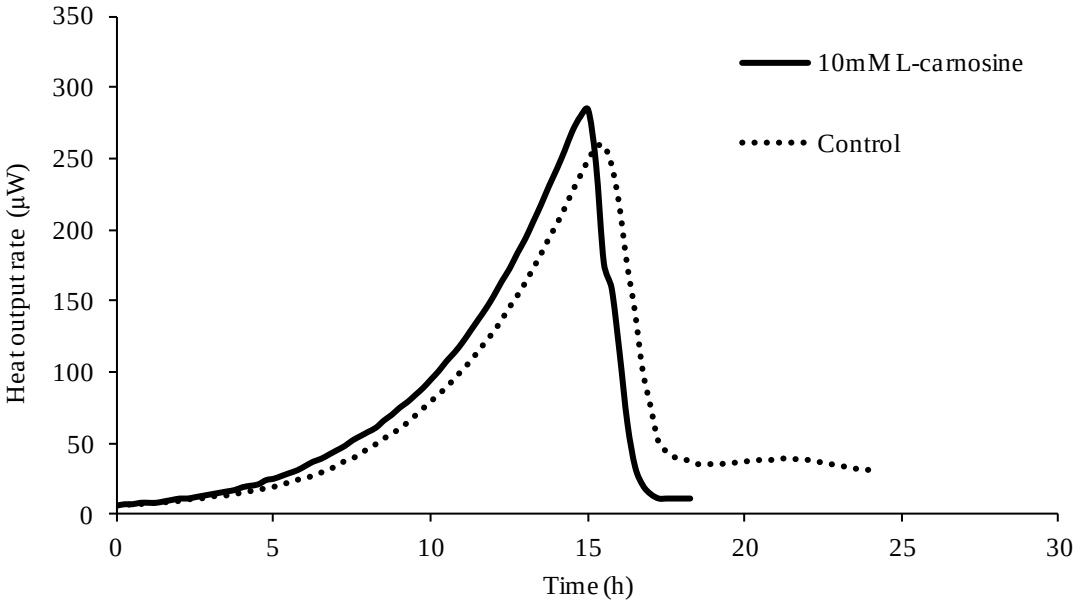


Figure 4.8: L-Carnosine affects the heat output rate of yeast cells grown on the non-fermentable carbon source, 2% glycerol. *Yeast cells were cultured in shake-flasks at 30°C in YP supplemented with 2% glycerol in the absence or presence of 10 mM L-carnosine, as indicated, and the heat output rate measured by on-line flow microcalorimetry. Control cells exhibit a classic monophasic metabolic profile on glycerol; on addition of L-carnosine the profile is altered only slightly as the onset of the peak is earlier. Data are representative of triplicate determinations.*

4.7. The metabolism-dependent effects of L-carnosine addition are observed for cells grown on a range of carbon sources

The addition of L-carnosine to glucose-grown cells results in a reduction in growth rate and causes cellular death, while the addition of L-carnosine to glycerol-grown cells does not cause cellular death, but supports growth. The effect of L-carnosine on a range of other fermentable and non-fermentable carbon sources (at 2%) was therefore determined. The fermentable carbon sources were glucose, mannose, galactose and fructose, whilst the non-fermentable carbon sources were glycerol, xylose, ribose and sorbitol.

The fermentable sugar, fructose is converted to fructose-6-phosphate by hexokinase and then enters glycolysis. Mannose is converted to mannose-6-phosphate by hexokinase and then fructose-6-phosphate by phosphomannose isomerise. Galactose has a slightly different metabolic fate (Figure 4.9); it is taken up by a specialised hexose transporter, galactose permease, Gal2. Galactose is then converted to galactose-1-phosphate by galactokinase, which is then converted to glucose-1-phosphate by galactose-1-phosphate uridylytransferase and glucose-uridine diphosphate (UDP), which is converted to galactose-UDP. Glucose-1-phosphate then enters glycolysis following its conversion to glucose-6-phosphate by phosphoglucomutase. Galactose-UDP is converted to glucose-UDP by UDP-glucose 4-epimerase to complete the cycle. It is interesting to note that in the presence of glucose, mannose and galactose metabolism is repressed (Van Maris et al., 2006).

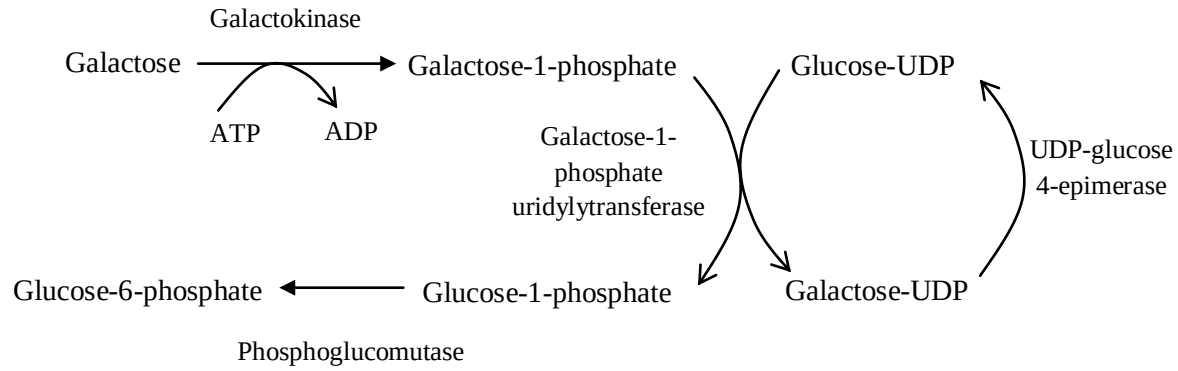


Figure 4.9: Diagram of galactose metabolism. *Galactose is taken up by the cell, where it converted to Galactose-1-phosphate by galactokinase, using UDP-glucose, galactose is transferred to UDP whereas glucose is transferred to the phosphate. This glucose-1-phosphate is then converted to glucose-6-phosphate by phosphoglucomutase.*

The non-fermentable sugars, ribose, sorbitol, xylose and glycerol cannot be fermented as their metabolisn results in the production of excess NADH and the depletion of NAD^+, this is important as NAD^+ is used as a cofactor during glycolysis, thus when there is a decrease in NAD^+, glycolysis cannot occur. Xylose is metabolised to xylitol by xylose reductase which requires NADPH as a cofactor; xylitol is then converted to xylulose by xylitol dehydrogenase (which requires NAD^+). This is metabolised to xylulose-5-phosphate by xylulose kinase, which requires ATP; xylulose-5-phosphate then enters the pentose phosphate pathway where it is metabolised to fructose-6-phosphate and glyceraldehyde-3-phosphate (Figure 4.10). Ribose also enters the pentose phosphate pathway *via* its metabolism to ribose-5-phosphate by the ATP-requiring enzyme ribose kinase (Jeffries, 2006). Sorbitol is metabolised to fructose by sorbitol dehydrogenase which requires NAD+ as a cofactor (Horton et al., 2006).

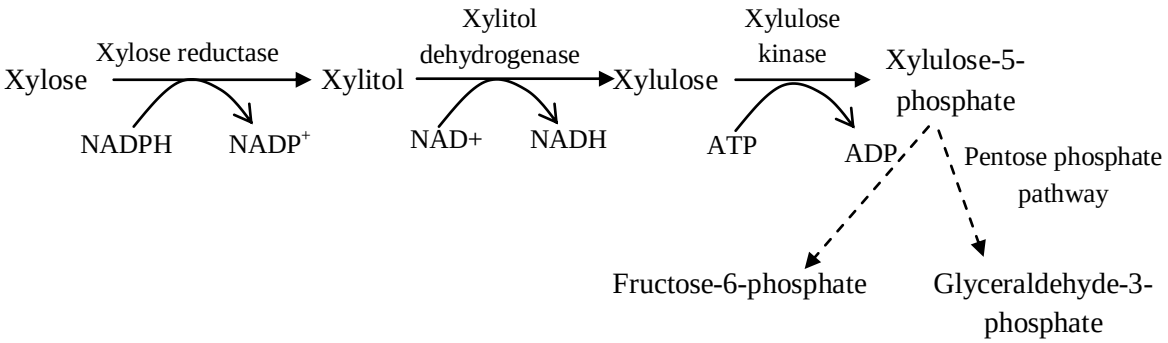


Figure 4.10: Diagram of xylose metabolism. *Xylose is converted to xylose-5-phosphate by a series of reactions, and xylose-5-phosphate then enters the pentose phosphate pathway and is converted to fructose-6-phosphate or glyceraldehyde-3-phosphate, which then enters glycolysis.*

Yeast cultures were grown overnight and the viability during late logarithmic growth was recorded by trypan blue dye exclusion. As Table 4.2 shows, cultures grown on glucose as carbon source, mannose,

galactose and fructose, with the addition of 10mM L-carnosine experienced a reduction in viability; however, this was not significantly different from the control with no L-carnosine. The viability of cultures grown on 2% glucose in YP medium was found to be lower at 84.85% compared to 90.09% when cells are grown on the synthetic medium 2×CBS with L-carnosine. Furthermore, it was noted that growth on glucose in YP medium with L-carnosine, still resulted in a change in morphology and a reduction in growth rate. The slight reduction in viability of the remaining fermentable carbon sources was much less compared to the reduction in viability seen in glucose growing cells, at around 97% for mannose, fructose and galactose. This may reflect the fact that these carbon sources, unlike glucose, result in slower rates of glycolysis and do not repress respiration to the same extent (De Deken, 1966). Furthermore, it was noted that there was only a small amount of morphological change between control and L-carnosine conditions. Yeast cells cultured on the non-fermentable carbon sources, sorbitol, xylose and ribose, with 10mM L-carnosine, behaved similarly to those cultured on glycerol, where the viability was not changed.

Table 4.2: The effect of L-carnosine on the viability of yeast cultures grown on other fermentable and non-fermentable carbon sources*. Yeast cells were grown in shake-flasks at 30°C in YP medium supplemented with the indicated carbon source with or without 10 mM L-carnosine. Viability (%; determined by trypan blue dye exclusion) was determined in triplicate. Data were analyzed using a one-way ANOVA (P <0.0001). Asterisks show the significance of the specific growth rate data for each culture condition compared to the control, as determined by a Dunnett's multiple comparison test, where **** = P ≤ 0.0001. The standard error of the mean (SEM) is given in parentheses.*

	Viability (%)	
Carbon source added to YP medium (2%)	***no L-carnosine***	***+ 10 mM L-carnosine***
Fermentable carbon sources		
Glucose	99.33 (0.33)	84.85 (1.89) ****
Mannose	100 (0.00)	97.83 (0.56)
Galactose	99.67 (0.33)	96.85 (0.56)
Fructose	99.38 (0.62)	97.68 (1.26)
Non-fermentable carbon sources		
Glycerol	97.56 (1.04)	99.14 (0.63)
Xylose	99.35 (0.32)	99.69 (0.31)
Ribose	99.35 (0.33)	100 (0.00)
Sorbitol	99.08 (0.53)	100 (0.00)

4.8. L-Carnosine addition does not reduce the viability of the respiratory yeast *Pichia pastoris*

Since it appears that the effects of L-carnosine are dependent upon the metabolic status of the cell, its effect on the Crabtree-negative yeast *Pichia pastoris* (Mattanovich et al., 2009) was determined. As *P. pastoris* is Crabtree-negative, it respires in the presence of glucose.

P. pastoris cultures were grown in YPD medium with or without 10mM L-carnosine. Viability was monitored during late logarithmic growth with trypan blue staining. The addition of 10mM L-carnosine

to *P. pastoris* was shown not to reduce the viability of the yeast cells. The viability of cultures in the absence of L-carnosine was 99.35% (SEM: 0.65; n=3). Viability of yeast grown in the presence of 10mM L-carnosine was 99.67% (SEM: 0.33; n=3). Moreover, the morphology of the cells was found to be the same.

4.9. Yeast strains with deletions in nutrient-sensing pathways are resistant to L-carnosine-induced cell death

As *S. cerevisiae* is resistant to L-carnosine when grown on a non-fermentable carbon source, a range of deletion strains involved in the nutrient-sensing pathway were chosen; it is known that the down-regulation of these pathways causes an increased dependence on respiratory metabolism (Bonawitz et al., 2007; Chen and Powers, 2006; Lavoie and Whiteway, 2008; Skinner and Lin, 2010). Nutrient-sensing pathways link nutrient availability with cell growth and metabolism. In yeast, these nutrient-sensing pathways include the target of rapamycin (TOR), the Sch9 protein kinase and the protein kinase A (PKA) pathways (Kaeberlein et al., 2005). Yeast strains with deletions in these pathways are often referred to as calorie restriction mimics, as down-regulation of the corresponding kinases promotes longevity in yeast (Kaeberlein et al., 2005). A selection of deletion strains was therefore chosen, and grown on YPD medium, to investigate the metabolism-dependent effects of L-carnosine addition to yeast cells (Table 4.3): In a *tor1Δ* strain, an increase in oxygen consumption, mitochondrial translation and proteins associated with respiration is observed (Bonawitz et al., 2007). Rpl31a is a downstream protein target of the TOR pathway (Kaeberlein et al., 2005). As part of the PKA pathway, the G protein-coupled receptor, Gpr1, activates the alpha subunit Gpa2; activation of PKA (which comprises 3 functionally redundant catalytic subunits, Tpk1-3, and a regulatory subunit Bcy1) in response to nutrient availability also causes an increase in the expression of genes associated with fermentation; down-regulation of this pathway is associated with an increase in respiration and a decrease in fermentation (Chen and Powers, 2006). The Sch9 pathway has very similar functions: deletion of pathway components causes an increased number of mitochondria, increased oxygen consumption and an increase in proteins involved in respiration (Lavoie and Whiteway, 2008). Hxk2, is a hexose kinase involved in the phosphorylation of glucose to glucose-6-phosphate.The *hxk2Δ* strain has respiratory characteristics (Lin et al., 2002).

The deletion strains showed, to differing extents, resistance to a decrease in viability when 10mM L-carnosine was added (Table 4.3) and most of them were not significantly different from wild-type cells with no L-carnosine added. The percentage viability of most of the deletion strains, when L-carnosine is added is similar to the reduction in viability seen in the fermentable carbon sources, mannose, galactose and fructose, emphasising that these strains have an increase in respiration which may explain their resistance to L-carnosine. The largest reduction in viability was that of *tpk1Δ* at 91.67%, which might be explained by the functional redundancy with Tpk2 and Tpk3.

Table 4.3: The effect of L-carnosine on the viability of yeast strains with deletions in the TOR, Sch9 and PKA nutrient sensing pathways*. Yeast cells were grown in shake-flasks at 30°C in YP medium supplemented with 2% glucose, with or without 10 mM L-carnosine. Viability (%; determined by trypan blue dye exclusion) was determined in triplicate. Data were analyzed using a one-way ANOVA (P <0.0001). Asterisks show the significance of the specific growth rate data for each culture condition compared to the control, as determined by a Dunnett's multiple comparison test, where **** = P ≤ 0.0001. The standard error of the mean (SEM) is given in parentheses.*

	Viability (%)	
Strain	***no L-carnosine***	***+ 10 mM L-carnosine***
Wild-type	99.33 (0.33)	84.85 (1.89) ****
tor1Δ	99.87 (0.20)	94.20 (1.30)
rpl31Δ	100 (0.00)	99.33 (0.67)
gpa2Δ	100 (0.00)	97.99 (0.22)
tpk1Δ	100 (0.00)	91.67 (2.25) ****
gpr1Δ	100 (0.00)	97.42 (1.21)
sch9Δ	100 (0.00)	96.44 (0.33)
hxk2Δ	100 (0.00)	98.22 (1.07)

4.10. L-Carnosine addition does not affect the chronological lifespan of glycerol-grown *S. cerevisiae*

Since L-carnosine addition did not reduce the viability of yeast grown on a non-fermentable carbon source, the CLS experiment previously done on glucose-grown cells (section 4.1) was repeated on glycerol-grown cells. As L-carnosine addition results in an increase in both RLS and CLS of mammalian cells, the CLS of yeast grown on YPG was determined to determine whether L-carnosine addition increases the CLS of yeast cells, similar to mammalian cells (McFarland and Holliday, 1994).The experiment was done in biological triplicates, with or without 10mM L-carnosine, and yeast were grown in YPG (2% glycerol). Samples were removed every 2-3 days and diluted 50,000 fold so that colony forming units (CFUs) could be counted by eye on YPD.

Figure 4.11 shows that the addition of L-carnosine to the medium did not result in a reduction in CFUs, which was expected as L-carnosine does not reduce the viability of glycerol-grown yeast. However, in contrast to mammalian cells L-carnosine addition did not result in an increased CLS compared to control cells. The reason for this difference could be human fibroblast cells live for over 100 days in culture, and in the case of some L-carnosine experiments, cells lived for over 400 days (McFarland and Holliday, 1994) whereas the yeast CLS was only one month. Another explanation could be that yeast cells respond differently to the addition of L-carnosine.

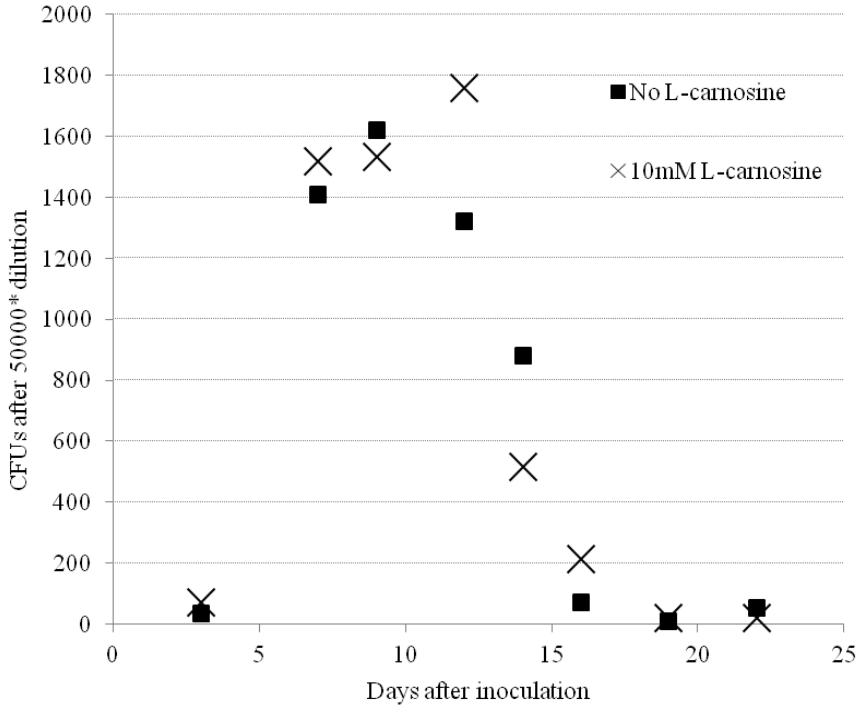


Figure 4.11: Colony forming units (CFUs) of yeast grown with or without 10mM L-carnosine. *Yeast cells were grown in YPG (2% glycerol) cultures for just over 20 days, with or without 10mM L-carnosine. Every 2-3 days a small amount of culture was removed and plated on to YPD plates, without L-carnosine and the CFUs counted. Data were generated by Clare Bromley, Aston University.*

4.11. The addition of L-carnosine to yeast cultures growing on YPG or YPD results in an increase in ATP levels.

Since there appears to be a metabolic rationale for the effects of L-carnosine on yeast cells, the levels of ATP in both glucose- and glycerol- grown cells were determined. Gaunitz and colleagues previously reported that the addition of L-carnosine to tumour cells resulted in cellular death due to a decrease in ATP production (Renner et al., 2010); this mechanism of action might therefore explain the metabolism-dependent effect of L-carnosine in *S. cerevisiae* for cells grown on glucose compared to those grown on glycerol. Similar to tumour cells, yeast cells growing on glucose rely on ATP generated from glycolysis.

For the detection of ATP levels in yeast cells, a Promega BacTiter-Glo^{TM} Microbial cell viability assay kit was used. Yeast cells were grown until an OD_{600} ~1 in either YPD or YPG, with or without 10-30mM L-carnosine. Yeast cells were washed and diluted to an OD_{600} of 0.1 in sterile water. 90µL of the diluted cells were then added to a 96 well opaque plate; there were ~90,000 cells in each well. 90µL of Bac-titer reagent from Promega was added to the cells and allowed to incubate for 5 min to enable cell lysis. An ATP standard curve was used to quantify ATP levels using the concentrations 0.1nM to 100nM. The ATP levels were detected with a luminometer and the relative light units (RLUs) recorded.

Figure 4.12 shows the unexpected data; rather than reducing ATP levels, the addition of L-carnosine increased ATP levels in a dose-dependent manner in both glucose- and glycerol- grown cells. This increase in ATP levels was not due to an interaction of the reagent with L-carnosine; when 10-30mM L-carnosine was added separately to the reagent, there was no difference between wells with or without L-carnosine. Figure 4.12 also shows that glycerol-grown cells produced more ATP than glucose- grown cells. This may be due to the difference in metabolism, as YPG enables respiration to take place, which produces more ATP (Ferreira, 2010). Furthermore, the increasing concentration of ATP in glycerol-grown cells may account for the increase in growth rate seen in these cells, in the presence of L-carnosine, however for the ATP analysis the OD_{600} was adjusted so that they were the same.

The viability of the yeast cells was assayed concurrently with the ATP experiment; L-carnosine was shown to decrease viability as expected. Since the same OD_{600} was loaded into each well, there were less viable cells in the wells where L-carnosine had been added. Differences in the ability of the kit to lyse control cells, compared to L-carnosine treated cells were investigated by monitoring the levels of ATP within 5 min of starting the reaction, and were recorded every 5 min for 30 min. It was clear that the RLUs in all wells, with different L-carnosine concentrations, were decreasing to the same extent after 5 min of starting the reaction.

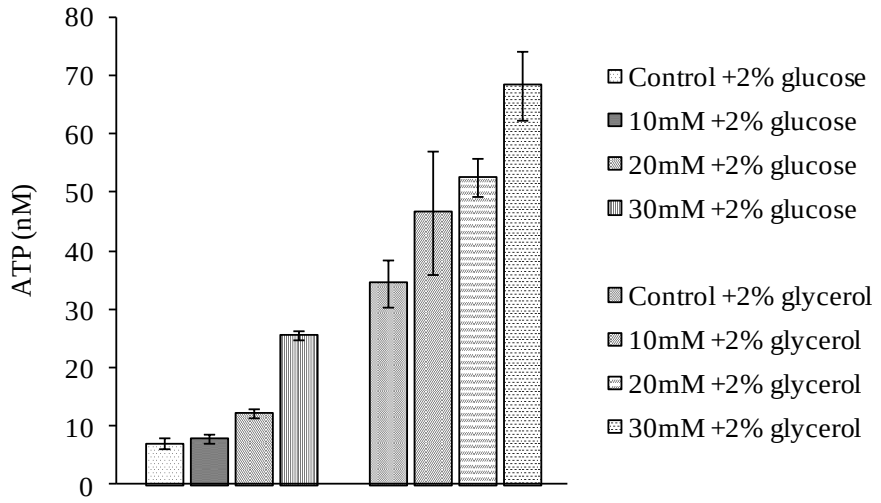


Figure 4.12: ATP levels are increased in both glucose and glycerol growing cells as increasing concentrations of L-carnosine are added*. Yeast cells were cultured in shake-flasks at 30°C in YP supplemented with 2% glucose or 2% glycerol in the absence or presence of 10-30 mM L-carnosine. ATP levels were recorded from ~90,000 cells, which were in 10µL of culture. This was added to 90µL water and 100µL BacTiter-Glo reagent, in a 96 well plate. Cell lysis occurred and 5 min later the levels of ATP were recorded. Each data point is the mean of biological triplicates, where the error bars represent standard error of the mean.*

4.12. L-carnosine addition causes an initiation block in glucose-grown cells but does not change the polysome profile of glycerol-grown cells

The ATP data (Figure 4.12) could not explain the effects of L-carnosine on glucose-grown cells. Polysome profiles of cultures with and without L-carnosine in glucose- and glycerol-containing media were therefore determined since L-carnosine has been reported to have an effect on translation (Son et al., 2008).Yeast cultures were grown in YPD or YPG with or without 10-40mM L-carnosine to an OD_{600} ~1, at which point cell extracts were collected and polysome profiles were determined as described in section 2.13. Figure 4.13 shows that as the concentration of L-carnosine was increased, the cells exhibited an initiation block that became more severe in a dose-dependent manner; this initiation block may be due to L-carnosine actively affecting the initiation of translation, but is more likely to be a consequence of cell death in the presence of L-carnosine; some cells were observed, *via* microscopy, to be viable, but had morphological changes indicating that the cells were stressed and dying; this would lead to an initiation block.

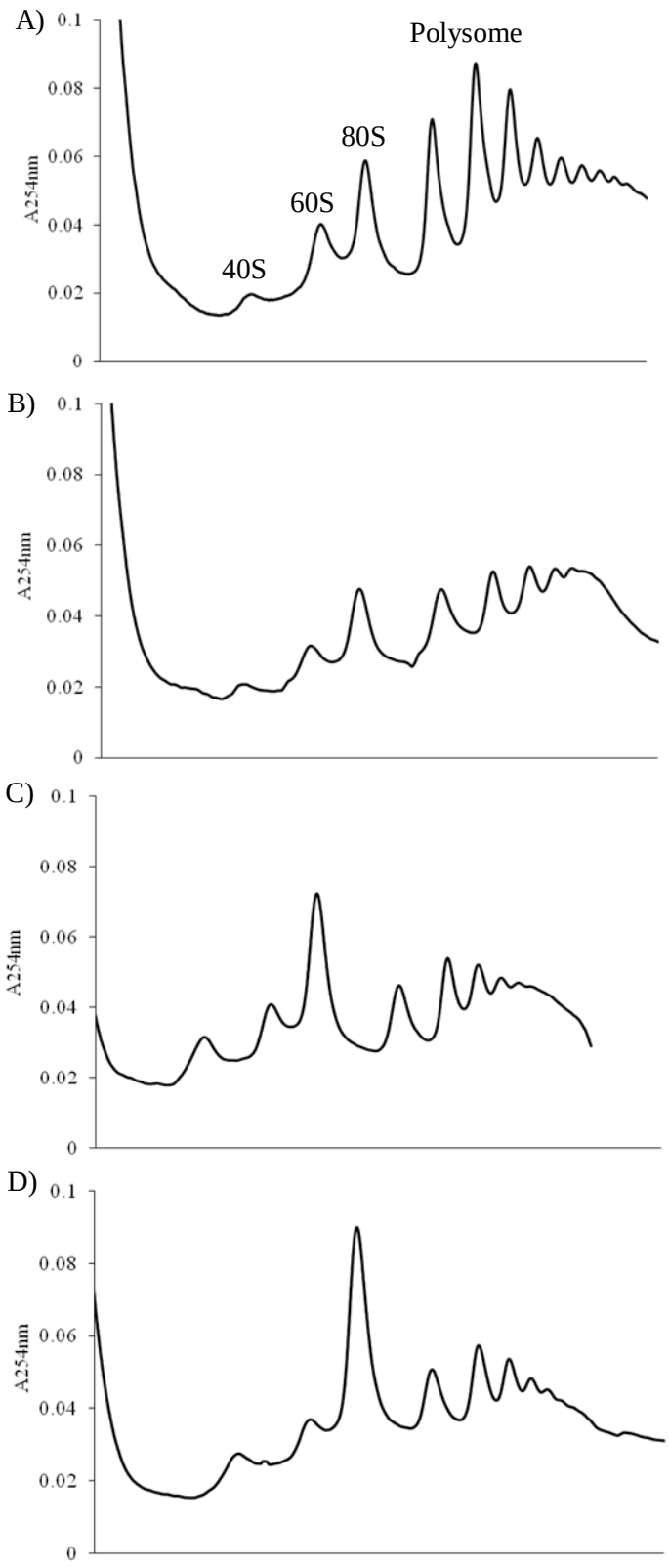


Figure 4.13: Representative polysome profiles showing the effect of L-carnosine on the initiation of translation in* S. cerevisiae *cells grown on glucose-containing medium*. A) Control cells; n=3 [0.14 (0.01)]. B) 10mM L-carnosine; n=1 [0.17]. C) 20mM L-carnosine; n=2 [0.30 (0.03)]. D) 40mM L-carnosine; n=2 [0.36(0.05)]. Data in square brackets are the monosome:polysome ratios and the corresponding standard error of the mean (SEM). The number of replicates is also stated.*

Polysome profiles of *S. cerevisiae* grown on glycerol, with no L-carnosine, showed an initiation block (Figure 4.14), which did not change with increasing concentrations of L-carnosine. Since L-carnosine does not appear to affect translation in glycerol-grown cells, it seems unlikely that, in yeast, carnosine has an effect on translation; the change in polysome profiles in glucose-grown cells is probably the result of cellular death.

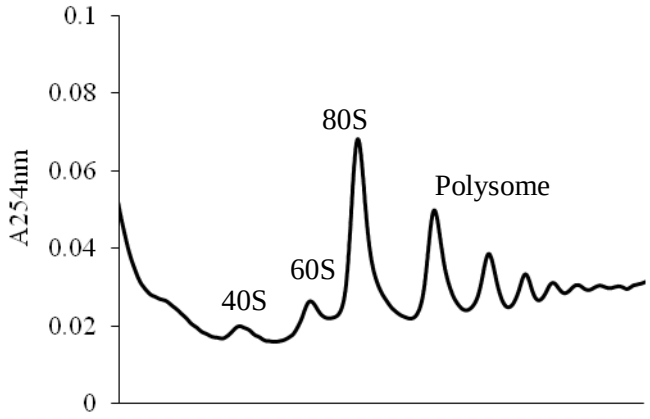


Figure 4.14: Representative polysome profile of yeast grown on glycerol with and without L-carnosine, graph shown is of yeast with no L-carnosine. *The data in square brackets are the monosome:polysome ratios and the corresponding standard error of the mean (SEM).Control cells n=3 [0.30 (0.04)]. 10mM L-carnosine n=3 [0.28 (0.02)]. C) 20mM L-carnosine n=2 [0.31 (0.02)]. D) 30mM L-carnosine n=3 [0.33(0.03)].*

4.13. Summary

The addition of L-carnosine to glucose-grown yeast cultures did not cause the expected increase in CLS; instead it caused a decrease in cell viability, as determined by trypan blue staining. This reduction in viability also coincided with a decrease in growth rate; both occurred in a dose-dependent manner. The components of L-carnosine, L-histidine and β-alanine, had no effect on cell viability, thus the mechanism of action was attributed specifically to L-carnosine. In support of this, addition of the optical isomer, D-carnosine, did not cause a reduction in viability when yeast were grown on glucose; this may be because D-carnosine cannot be taken up by yeast cells.

The addition of L-carnosine to yeast grown on glycerol was shown to cause an increase in growth rate in a dose-dependent manner; the addition of L-histidine and/or β-alanine to yeast grown on glycerol also caused an increase in growth rate. This suggests that the increase in growth rate may be attributed to the use of L-carnosine as an amino acid source.

The finding that the action of carnosine is metabolism-dependent was further tested on a panel of carbon sources: yeast grown on the fermentable carbon sources glucose exhibited a significant reduction in viability whilst viability was reduced when grown on other fermentable carbon sources, but not in a significant manner. Yeast grown on non-fermentable carbon sources did not have any reduction in viability. Furthermore, the Crabtree-negative yeast species, *P. pastoris*, and *S. cerevisiae*

strains with an increase in their respiratory metabolism (*tor1Δ* and *sch9Δ*) were shown to be resistant to L-carnosine-induced cell death. These data are consistent with previous literature reports demonstrating that L-carnosine addition decreases the growth rate of cancer cells (which have similar metabolic characteristics to glucose-grown yeast), but not somatic cells (Diaz-Ruiz et al., 2009; Holliday and McFarland, 1996), and explains the initial CLS data for glucose-grown yeast. Unexpectedly, however, when CLS experiments were repeated with glycerol as carbon source, no increase in lifespan was observed. This suggests that metabolism-dependence is an important component, but cannot provide a complete description, of L-carnosine's mechanism of action. ATP levels in yeast grown on glycerol or glucose in the presence of L-carnosine unexpectedly increased (as opposed to the decrease expected for glucose-grown cells) in a dose-dependent manner, again suggesting other pathways must be involved. Yeast cells grown on glucose were found to exhibit an initiation block, most likely a consequence of cell death, while there was no change in polysome profile for glycerol-grown cells following L-carnosine addition. This suggests that translational pathways are not involved. Overall, it can be concluded that L-carnosine has a metabolism-dependent effect, causing yeast cell death when yeast are reliant on glycolysis. However, the mechanism of action remains unknown and additional pathways are likely to be involved. The results of this chapter are published as two articles; an original research article (Cartwright et al., 2012) and a review (Hipkiss et al., 2013).

Chapter 5: Discussion

5.1. Understanding the mechanisms of high-yielding yeast strains

The aim of this research was to understand why the high yielding strains, *srb5Δ, gcn5Δ, spt3Δ* and yTHC*BMS1* (with 0.5µg/mL doxycycline) resulted in increased Fps1 yields compared to wild-type cells. The results in Chapter 3 showed that all of the high yielding strains had initiation blocks and that the initiation blocks may have been due to the combination of an increase in *BMS1* transcript levels and phosphorylation of eIF2α. However, since all polysome profiles looked different it was concluded that other factors must play a role in altering the translational states of *srb5Δ, gcn5Δ* and *spt3Δ*. Other strains with known initiation blocks were also confirmed to increase Fps1 yield. The levels of Gcn4 were analysed in all strains investigated in this study, as initiation blocks are associated with an increase in Gcn4. The levels of Gcn4 were shown to be increased in all the high yielding strains. As there was a relationship between an increase in Gcn4 and increased Fps1 yield, the 5´UTR of *FPS1* was analysed for uORFs; the translation of Gcn4 is regulated by the presence of 4 uORFs. The 5´UTR of *FPS1* was shown to have 2 uORFs.

When these uORFs were removed from the *FPS1* transcript, the yield of Fps1 produced by wild-type cells was increased 540-fold. The removal of the uORFs also increased Fps1 production in the high yielding strains, *spt3Δ* and yTHC*BMS1* (with 0.5µg/mL doxycycline), to 350- and 210-fold, respectively. However, the yields obtained were not as high as in wild-type cells. These data suggest that the high yielding strains were able to circumvent the uORFs in the *FPS1* transcript. It is possible that yTHC*BMS1* (with 0.5µg/mL doxycycline) and *spt3Δ* are able to read through the start codons of the uORFs when the TC is bound to the PIC, known also as leaky scanning (Foiani et al., 1991). However, it is possible that other mechanisms, or a mixture of mechanisms, are at work. For instance, as eIF2α is phosphorylated in the high yielding strains, this means the TC is not bound to the scanning 40S thus leaky scanning of uORF2 may occur, similar to the mechanism for an increase in Gcn4 during initiation blocks (Hinnebusch, 2005).

An interesting point to consider for the Fps1 data in this thesis and the work presented in (Bonander et al., 2009, 2005), is that the high yielding strains were developed from data obtained from western blot analysis, which does not provide information on functional yields. It is also known that the levels obtained from western blots do not correlate with the levels of functional GPCR production (Lundstrom et al., 2006). Thus, even though the high yielding strains increased Fps1 production from the *FPS1* transcript with 2 uORFs, this does not necessarily mean there was an increase in functional yields. Functional data for Fps1 could be obtained through glycerol flux experiments (Luyten et al., 1995), however the specificity required to be able to detect small changes in functional protein production may not be achievable.

The high yielding strains, yTHC*BMS1* (with 0.5 or 10µg/mL doxycycline) and *spt3Δ*, have been shown to increase the functional yield of proteins other than Fps1. The classic high yielding strains, *srb5Δ,*

gcn5Δ, spt3Δ and yTHC*BMS1* (with 0.5µg/mL doxycycline) did not increase GFP production, whereas the addition of 10µg/mL doxycycline to yTHC*BMS1* did increase the functional yields of GFP produced. This same condition has also been shown to increase the function yields for adenosine A_{2a} receptor by 2-fold; yTHC*BMS1* (with 0.5µg/mL doxycycline) did not result in a functional increase in adenosine A_{2a} receptor (Bonander et al., 2009). Moreover, Marvin Dilworth at Aston University has shown that *spt3Δ* reduces functional adenosine A_{2a} receptor yield by 60% relative to wild-type cells (unpublished results).

Conversely Dr Debasmita Sarkar at Aston University was able to increase functional horseradish peroxidise (HRP) yields in yTHC*BMS1* (with 0.5µg/mL doxycycline) 40 fold compared to wild-type cells. HRP is expressed in the same pYX222 vector as *FPS1* and there are no uORFs. *spt3Δ* was also shown to increase HRP production three fold. The reasons for the differences seen in the production of functional yields in the high yielding strains, *spt3Δ* and yTHC*BMS1* (with 0.5µg/mL doxycycline) with the different proteins are unknown. However, it is known that different proteins, even from similar families can be differentially expressed, using the same culture conditions (Tyo et al., 2012), which may also be occurring in the strains. One of the mechanisms that may be occurring, especially in the case of yTHC*BMS1* (with 10µg/mL doxycycline), is a reduction in protein synthesis: growth rate is reduced from 0.24 h^{-1} in yTHC*BMS1* (with 0.5µg/mL doxycycline) to 0.18h^{-1} in yTHC*BMS1* (with 10µg/mL doxycycline). This decrease in protein production may prevent the overloading of the yeast secretory pathway, enabling the correct folding of the protein and increasing functional yields; especially as the gene transcription is driven from the strong constitutive promoter *TPI* and the plasmid has a high copy number, which can be detrimental to functional protein production (Griffith et al., 2003). A classic example of the reduction of transcript levels increasing protein production occurs in the *E. coli* Walker strains (Wagner et al., 2008). This mechanism may also be occurring in the original high-yielding strains strains and resulting in an increase in HRP. However, this remains unproven.

5.1.1. The roles of Bms1, Srb5, Spt3 and Gcn5

An interesting point to consider is the levels of *BMS1* transcript in yTHC*BMS1* (with 0.5µg/mL doxycycline) were shown to be 6 times higher than in wild-type cells (Bonander et al., 2009). The assumption would be that there is a consequent increase in Bms1 protein. However, data described in Chapter 3 (Figure 3.2) and the previous study by Bonander in 2009 could be consistent with a decrease in Bms1 protein levels, as there is an initiation block and a clear reduction in the levels of 40S, which is also associated with mutant *BMS1* (Gelperin et al., 2001). Thus, there may be a lack of correlation between *BMS1* transcript levels and Bms1 protein levels. Alternatively, there may be an increase in Bms1 protein which results in a similar phenotype to when there is a defect in Bms1. Notably, doxycycline addition had no effect on the translational state of the cell as wild-type cells with the addition of 0.5µg/mL doxycycline resulted in no change in polysome profile.

The polysome profiles, in the case of *srb5Δ, gcn5Δ* and *spt3Δ* may be the result of the combination between constitutive levels of eIF2α phosphorylation (a stress response) and an increase in *BMS1*

transcript levels. However, as there are distinct variations between the polysomes, it is likely that the translational state of the cells are changed as, Srb5, Spt3 and Gcn5 are involved in transcription. Srb5 is part of the mediator complex, which is involved in the transcription of RNA polymerase II genes (Kremer and Gross, 2009) whereas, Gcn5 and Spt3 are involved in the SAGA complex, which is involved in the transcription of about 10% of the yeast genome (Jacobson and Pillus, 2009). Thus, there may be a change in cellular transcription in these deletion strains, affecting transcription of the genes involved in ribosomal biogenesis, ribosomal proteins or other factors which can alter translation. The effect of these transcription factors on translation have not been reported previously, to our knowledge.

5.1.2. The role of stress responses

The constitutive levels of phosphorylated eIF2α suggests that these strains are experiencing cellular stress, as eIF2α phosphorylation only occurs during environmental and cellular stresses, such as, amino acid starvation, the addition of hydrogen peroxide, osmotic shock and many more (Hinnebusch, 2005; Shenton et al., 2006). The cellular stress causing eIF2α-P is unknown.

The levels of stress resistance transcription factors, such as Gcn4, are also increased in stress conditions (Hinnebusch, 2005). In the ribosomal biogenesis and ribosomal protein mutants investigated in Chapter 3, increased Gcn4 production can be explained by the reduction in 60S in these strains (Steffen et al., 2008; Wu et al., 2001). This in turn results in halfmer formation as the PIC, with the bound TC, can read through the start codons of the *GCN4* uORFs since the 60S is not recruited due to a decrease in its levels. Thus the strains read through the start codons of the uORFs in *GCN4*, resulting in an increase in Gcn4 during non-stress conditions (Eisinger et al., 1997). Interestingly, the levels of Gcn4 in these strains may also be lower than expected as leaky scanning of the *GCN4* ORF itself can also take place. Moreover, as leaky scanning of uORF1 may occur, translation may initiate at an inhibitory uORF and also cause a reduction in Gcn4; this is seen during times of amino acid starvation, where wild-type cells produce more Gcn4 than *rpl16bΔ*, which also has decrease in 60S (Foiani et al., 1991). For strains with initiation blocks and phosphorylation of eIF2α, such as, *srb5Δ, gcn5Δ, spt3Δ* and yTHC*BMS1* (with 0.5µg/mL doxycycline), translation of uORF1 may occur. Phosphorylation of eIF2α suggests a decrease in TC thus leaky scanning of uORFs 2-4 may occur with the TC rejoining the scanning 40S between uORF4 and the *GCN4* ORF, enabling translation of *GCN4*.

Activation of the unfolded protein response (UPR), which is another major stress response, also causes an increase in Gcn4 expression (Boyce and Yuan, 2006). This is due to the phosphorylation of eIF2α during the UPR, which is dependent on the protein kinase Gcn2 (Boyce and Yuan, 2006). Moreover, Gcn4 is involved in the transcription of UPR genes (Patil et al., 2004).The UPR is activated by unfolded proteins in the secretory pathway and results in increases in chaperone activities, glycosylation, translocation, folding capacity of the ER (by increasing the size of the ER) and the ER associated degradation (ERAD) pathway. The ERAD pathway results in the removal of misfolded

proteins from the ER into the cytosol, where they are ubiquitinylated and degraded by the 26S proteasome (Mattanovich et al., 2004; Patil et al., 2004).

In yeast, the UPR is triggered by the oligormerisation of the ER transmembrane protein Ire1, which is located in the ER. Under normal conditions, Ire1 is found as a monomer, as it is bound by the chaperone protein BiP, which is a member of the Hsp70 family. When there is an increase in unfolded proteins, BiP joins an unfolded protein to prevent aggregation, thus Ire1 can oligomerize. At the same time, Ire1 can detect unfolded proteins, and Ire1 trans-autophosphorylates resulting in the activation of Ire1's cytoplasmic endoribonuclease activity. *HAC1* mRNA interacts with Ire1, through its 3´UTR. Ire1 once activated, removes the structured intron from the *HAC1* mRNA, with its endoribonuclease activity, which prevents the translation of *HAC1* since it interacts with the 5´UTR region and prevents translation. Once the intron is removed, *HAC1* mRNA is ligated by tRNA ligase, and then translated. Hac1 protein enters the nucleus and binds the UPR elements (UPRE) in promoters and drives transcription of UPR genes (Mattanovich et al., 2004; Kohno, 2010). As mentioned, Gcn4 can also drive the transcription of UPR genes, can result in the transcription of UPRE 2 without Hac1 and aids in the transcription of UPR genes with UPRE1 and 2 along with Hac1(Patil et al., 2004).

Activation of the UPR has been shown to increase protein production in yeast. For example co-expression of protein disulphide isomerise (Pdi1 involved in disulphide bond formation) was shown to increase human platelet derived growth factor, human lysozyme and single chain antibody fragment (Mattanovich et al., 2004). The expression of adenosine A_{2a} receptor was increased 1.8-fold when co-expressed with spliced *HAC1* (Guerfal et al., 2010). The overexpression of secretory and membrane proteins has been shown in, some cases, to trigger the UPR, due to a lack of folding capacity (Mattanovich et al., 2004). For instance, the overexpression of the secreted proteins, human trypsinogen and a single chain antibody, in *P. pastoris* was noted to cause an increase in BiP expression (Mattanovich et al., 2004).

It is possible that the increase in Fps1 yield is a result of an increase in Gcn4 production, which in turn actives the cells stress responses. Alternatively, there could be a correlation between the two proteins as they could share similar transcript properties. As shown in this work, the sequence of the 5´UTR of *FPS1* contains two uORFs, similar to *GCN4* transcript that contains four uORFs.

5.1.3. The role of uORFs in the regulation of FPS1 *translation*

The first uORF of *FPS1* was found to be the result of a Kozak sequence from the pYX222/pYX212 backbone, after the *TPI* promoter. This was probably originally inserted before the multiple cloning site to aid in the translation of a target protein. The *FPS1* transcript, along with 253 nucleotides upstream of the *FPS1* ORF, when inserted into the multiple cloning site, resulted in the addition of an inframe stop codon. This resulted in a uORF which was 34 codons long. The second uORF is native to the *FPS1* transcript, is 5 nucleotides away from the *FPS1* ORF and is 3 codons long; it has a start codon, sense codon and a stop codon, and is the minimum length of a uORF (Hood et al., 2009). This

second uORF is present in the native mRNA of *FPS1* in yeast, as the transcriptional start site of *FPS1* is 78 nucleotides upstream of the *FPS1* ORF (Tuller et al., 2009).

The data in Chapter 3 show that the presence of both uORFs was preventing the translation of the *FPS1* ORF in wild-type cells; upon their removal there was a 540-fold increase in Fps1 yield. The data strongly suggest that the high yielding strains are high yielding because they are able to initiate or reinitiate translation at the *FPS1* ORF and thus are able to circumvent the uORFs in the *FPS1* transcript. Taking a closer look at the data in Table 3.2 aids in the determination of whether the mechanism for high yielding Fps1 production in the high yielding strains is due to leaky scanning (where the scanning PIC reads through the start codons in the uORFs, even with the presence of the TC) or a re-initiation (where translation of uORF1 occurs but as there is phosphorylation of eIF2α, read through of uORF2 can occur, after which the TC is recruited enabling Fps1 production) mechanism when both uORFs are present in the *FPS1* transcript. For wild-type cells it can be said that on average, 1 in every 542 (~0.2%) scanning ribosomes are able to translate the *FPS1* ORF. Due to the Kozak sequence in uORF1 it is likely that most of the scanning ribosomes will translate uORF1. Ribosomes are unlikely to reinitiate translation after the translation of uORF1, as the codon length of uORF1 is 34 codons long, which is very close to the 35 codon length that has been shown to prevent re-initiation of translation at ORFs (Vilela and McCarthy, 2003). However, it is clear that some ribosomes are able to reinitiate or even scan through the Kozak sequence since Fps1 protein is produced.

As there are 140 nucleotides between uORF1 and uORF2, it is likely that the TC could re-associate with those scanning 40S subunits that remain attached after uORF1 translation (Kozak, 1987) in wild-type cells, and thus translation of uORF2 will occur; some ribosomes may then dissociate from the transcript. For those scanning 40S subunits that remain attached, there are only 5 nucleotides between uORF2 and the ORF of *FPS1*. Consequently, the potential for the recruitment of the TC before reaching the *FPS1* ORF is low. However, data obtained from wild-type yeast expressing *FPS1* from the pYX222-5′Δ1-43-*FPS1*-HA_3 vector suggests that 1 in every 60 (1.7%) ((9.2/541.8)× 100) scanning ribosomes are able to translate *FPS1*, even in the presence of uORF2. This may be *via* a re-initiation mechanism. However, it is more likely that leaky scanning of the uORF2 start codon occurs since the sequence surrounding the start codon of the second uORF does not have an A in the -3 position, and this is known to affect translation from the start codon in yeast by 2-3 fold (Vilela and McCarthy, 2003). Thus, leaky scanning of this start codon could be occurring. The leaky scanning of uORF2 can also explain how translation of *FPS1* occurs after translation or leaky scanning of uORF1. In addition, the start codon of *FPS1* does not have an A in position -3 and this could also decrease translation rates.

The levels of ribosomes reaching the *FPS1* ORF in yTHC*BMS1* (with 0.5µg/mL doxycycline) are very different from the situation in wild-type cells cells. From Table 3.2, it can be estimated that 1 in every 11 (9%) ((32.4/355.8) ×100) scanning ribosomes are able to translate the *FPS1* ORF, either by a leaky scanning or re-initation mechanism. As stated above, since uORF1 is 34 codons long and it is known

that a codon length of 35 prevents re-initiation of translation in yeast (Vilela and McCarthy, 2003), it is likely that the PIC of yTHC*BMS1*, with the TC, is able to scan through the Kozak sequence, the mechanism of which is unknown. However there could be a defect in subunit joining (Eisinger et al., 1997) or start codon recognition (Hinnebusch and Lorsch, 2012). Another mechanism that could be taking place, due to eIF2α phosphorylation in yTHC*BMS1* (with 0.5μg/mL doxycycline) is that uORF1 could be translated (similar to the situation for Gcn4); as there is a reduction in the TC, leaky scanning of uORF2 takes place. The TC is then able to rejoin the scanning ribosome within the 13 nucleotides between the start codon of uORF2 and the ORF of *FPS1*. However, long stretches between uORF and ORF are usually required for efficient re-initiation (Kozak, 1987). Furthermore, a leaky scanning mechanism by PIC with TC attached, is suggested by the data obtained from yTHC*BMS1* expressing *FPS1* from the vector pYX222-5′Δ1-43-*FPS1*-HA_3, where on average 3 in every 10 ribosomes (30%) ((106.5/355.8) × 100) are able to translate *FPS1*. This is a huge difference compared to 1.6% ((9.2/541.8) × 100) in wild-type cells. It is unlikely that re-initiation is occurring after uORF2 translation, as there is only 5 nucleotides between uORF2 and *FPS1* ORF; eIF2α has also been shown to be phosphorylated in both yTHC*BMS1* and *spt3Δ,* thus there is likely to be a decrease in the levels of TC and a decrease in re-initiation rates. Instead, the data suggest that start codon recognition is affected. Similar rationales can be used to explain increases in Fps1 production in the *spt3Δ* strain.

The leaky scanning mechanism is further supported by data for the ribosomal protein and ribosomal biogenesis mutants that cause an increase in Fps1 yield. As stated above, the ribosomal mutants increase Gcn4 levels as leaky scanning of start codons occurs due to a decrease in the levels of 60S; this could also be occurring in the *FPS1* transcripts. Moreover, due to the ability of these strains to read through start codons during times of stress, they result in lower levels of Gcn4 compared to wild-type cells due to leaky scanning of the *GCN4* start codon (Foiani et al., 1991). Leaky scanning of the *FPS1* ORF could also be occurring in the high yielding strains yTHC*BMS1* and *spt3Δ*. This would explain why the levels of Fps1 produced when there are no uORFs is lower than in wild-type cells.

The mechanism of how the remaining high yielding strains increase Fps1 yields remains uncertain. A leaky scanning mechanism by PIC, with TC bound, may be occurring, but the actin binding mutants, *srb5Δ* and *gcn5Δ* only result in a small increase in Fps1 yield suggesting that uORF1 may be translated. However, as there is a reduction in the levels of TC there may be a greater level of scanning 40S subunit that read through the uORF2 start codon, after which the TC associates within the 13 nucleotide gap and thus increases Fps1 production.

Differences in the levels between Fps1 and Gcn4 yields can be attributed to the differences in their 5′UTRs. All four of the uORFs in *GCN4* are small at 4 codons or less. Also all of the uORFs in *GCN4* have an A in position -3, and so does the ORF, which would encourage translation at the uORF by a PIC with a bound TC. Moreover, there are 148 nucleotides between uORF4 and the ORF of *GCN4* which enables re-initiation of translation (Hinnebusch, 2005). The first uORF of *FPS1* on the other

hand is 34 codons long and would almost certainly prevent re-initiation of translation, after being translated (Vilela and McCarthy, 2003). The second uORF is within 5 nucleotides of the *FPS1* ORF which would also affect re-initiation (Kozak, 1987).

5.1.4. What is the role of the FPS1 *uORF?*

FPS1 contains a native uORF in its transcript (Known as uORF2 in our system, uORF1 is not present) (Tuller et al., 2009). This uORF has been shown to limit Fps1 production to about 1.6%, compared to a transcript without uORFs. This similar reduction in translation rates would also be occurring in the native *FPS1* transcript. The function or affect upon Fps1 regulation by this uORF has not been recorded in the literature to our knowledge.

Fps1 is involved in the release of glycerol from the cell during anaerobic growth (Luyten et al., 1995) and hypo-osmotic shock (Tamás et al., 1999) and thus is involved in protecting the cell from stress. Alterations in protein levels have not been recorded, but it is established that Fps1 is regulated at the protein level by the opening and closing of the channel, via its N- and C-termini (Hedfalk et al., 2004; Mollapour and Piper, 2007). An interesting point is that osmotic stress results in eIF2α phosphorylation and results in an initiation block (Simpson and Ashe, 2012). However, due to the close proximity of the native uORF to the ORF of *FPS1* it is uncertain if this would have any effect on the levels of Fps1, as there is probably not a long enough stretch between the uORF and ORF to enable recruitment of the TC.

Alignment of the 5´UTR of *FPS1*, using the alignment facility of the *Saccharomyces* Genome Database (SGD; www. yeastgenome.org/cgi-bin/FUNGI), showed that *S. mikatae* and *S. paradoxus* also have a uORF in their 5´UTR of *FPS1*. This suggests that the uORF may play a function, possibly to decrease the levels of *FPS1* translation under normal conditions, as high levels of Fps1 may be detrimental to the cell; Fps1 has been shown to be involved in the uptake of arsenite and acetic acid (Thorsen et al., 2006).

5.2. The metabolism dependent effects of L-carnosine on *S. cerevisiae*

The original aim of this project was to determine the effect of L-carnosine on the lifespan of yeast as it has been shown to increase the lifespan of mammalian and bacterial cells. However, it was clear from the chronological lifespan assay that was performed that the addition of L-carnosine to the yeast medium resulted in a reduction of growth or cellular death as the number of colony forming units was reduced compared to control. As a result, we investigated the causes behind a reduction in colony forming units, by studying the effect of L-carnosine on the growth rate and viability of yeast. As *S. cerevisiae* metabolism can be changed from a respiro-fermentative metabolism, when grown on glucose, to a respiratory metabolism, when grown on non-fermentable carbon source, the effect of L-carnosine on growth and viability was again investigated. These results were then linked back to the effects of L-carnosine on cancer cells (Holliday and McFarland, 1996) and experiments were performed to understand the mechanisms.

5.2.1. L-carnosine does not increase the CLS of S. cerevisiae

L-carnosine has been previously shown to increase the lifespan of human fibroblast cells (McFarland and Holliday, 1994), bacterial cells (Pepper et al., 2010) and male fruit flies (Yuneva et al., 2002). The ability of L-carnosine to increase the lifespan of a range of different cells and organisms has been attributed to (i) its ROS scavenging properties, as it donates electrons to ROS (Kohen et al., 1988; Preston et al., 1998) and (ii) its ability to chelate metal ions, which in turn, prevents the Fenton reaction, and thus the formation of more reactive ROS (Kang, 2010; Kantha et al., 1996). However other CRC, such as, homocarnosine and anserine do not increase lifespan of human fibroblasts (McFarland and Holliday, 1994) and neither do antioxidants such as vitamin C or E (Hipkiss et al., 2001). The ability of L-carnosine to increase lifespan is therefore more likely to be due to its anti-glycation activities (Hipkiss et al., 1995); L-carnosine has been shown to act as a sacrificial peptide, during glycation. Thus, rather than a reactive sugar reacting with a lysine side chain of a functional protein, it instead reacts with L-carnosine. The reason the reactive sugar reacts with L-carnosine instead is that its structure mimics a lysine side chain next to a L-histidine, which is a favoured glycation site (Hipkiss et al., 1995). Moreover, L-carnosine reacts with AGEs and is thought to prevent further reactions and as a result can aid in their degradation (Brownson and Hipkiss, 2000). Though homocarnosine and anserine do have anti-glycation properties, L-carnosine is the most effective (Alhamdani et al., 2007). In addition, L-carnosine is able to reverse protein-glycation links, which could also account for its rejuvenation properties towards human fibroblasts (McFarland and Holliday, 1994).

As L-carnosine has been shown to increase lifespan of human cells, bacterial cells and male flies, the effect of L-carnosine on the CLS of *S. cerevisiae* was determined. The experiment was performed by growing yeast cells in culture, with or without L-carnosine in media containing glucose and plating yeast cells every 2-3 days, to enable the counting of CFUs. The results showed that there was a reduction in CFUs from cultures grown with 10mM L-carnosine, which was unexpected. The decrease in CFUs suggested there was a decrease in the number of cells or the number of viable cells. The optical densities for both cultures with and without L-carnosine were similar, suggesting that there was a reduction in viability. Thus, it appears that L-carnosine rather than increasing the CLS of yeast grown on glucose as a substrate, actually resulted in cellular death.

L-carnosine did not increase the lifespan of yeast grown on glycerol. One possible explanation for this is that L-carnosine typically extends the lifespan of cells or organisms that are under oxidative stress. For instance, bacterial cells were grown on glucose, which was shown to cause a decrease in their lifespan; the addition of L-carnosine to these cells was then shown to increase their lifespan (Pepper et al., 2010). Moreover, L-carnosine only extended the lifespan of male flies. Male flies are thought to have a decrease in oxidative stress resistance compared to female flies, which results in shorter lifespans. L-carnosine failed to extend the lifespan of female flies (Yuneva et al., 2002). Thus L-carnosine may fail to extend the lifespan of yeast grown on glycerol, as cellular death may not be the result of oxidative stress.

5.2.2. L-carnosine affects the viability of S. cerevisiae *in a metabolism-dependent manner*

L-carnosine has very opposite affects towards normal and cancerous cells. L-carnosine has been shown to cause an increase in RLS and CLS of normal cells. L-carnosine has also been shown by many groups to cause a decrease in the growth rate of cancer cells *in vivo* and *in vitro* (Holliday and McFarland, 1996; Iovine et al., 2012; Renner et al., 2010, 2008). The different effects of L-carnosine towards cancer cells and normal cells have been suggested to be due to a difference in metabolism in these two cell types. Cancer cells often rely on glycolysis for the production of energy; this is known as the Warburg effect, whereas normal cells can also obtain energy from oxidative phosphorylation (Diaz-Ruiz et al., 2009). This suggestion is supported by the fact that cancer cells can grow in the presence of L-carnosine when pyruvate is added to the growth medium (Holliday and McFarland, 1996). Pyruvate is thought to aid in cancer cell growth, in the presence of L-carnosine, as it is central to metabolism and connects glycolysis to the TCA cycle. Thus, it is thought that pyruvate is used by the cancer cells to derive energy from oxidative phosphorylation, rather than relying on ATP generation from glycolysis. Moreover, α-ketogluterate and oxaloacetate, which are part of the TCA cycle, also enable cancer cell growth in the presence of L-carnosine (Holliday and McFarland, 1996). The mechanistic reasons behind, L-carnosine's effects toward yeast grown on a fermentable carbon source and a non-fermentable carbon source, are analogous to L-carnosine's differing affects towards cancer and normal cells, as cancer and yeast cells have similar metabolisms (Diaz-Ruiz et al., 2009).

Holliday and McFarland in 1996 suggested the reason L-carnosine has a negative effect towards cancer cells and not normal cells, is because L-carnosine may be reacting with the reactive triose sugars, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate, which enter into the energy yielding part of glycolysis. Thus, if these triose sugars are removed by L-carnosine, it is suggested that there would be a decrease in ATP production and there would also be a decrease in the levels of pyruvate and other carbon skeletons, used in the rapid growth of cancer cells. A reduction in ATP synthesis specifically from glycolysis has been recorded in cancer cells, in the presence of L-carnosine, but not normal cells (Iovine et al., 2012; Renner et al., 2010, 2008). However, even though the reduction in triose sugars has been suggested, this has never been tested, thus the cause for the reduction in ATP remains unknown. Nevertheless, this decrease in ATP production has been suggested to be the cause of a reduction in growth rate in cancer cells, *in vivo* and *in vitro* (Renner et al., 2008). The decrease in ATP could be detrimental to cancer cells as they rely on glycolysis for energy production (Diaz-Ruiz et al., 2009).

The anti-glycation property of L-carnosine as a mechanism for the inhibition in cancer cell growth is supported by the drug tenilsetam, which prevents glycation and has been shown to kill cancer cells, dependent upon the absence of pyruvate (Holliday and McFarland, 1996). On the other hand, the CRC, homocarnosine, has been shown to have anti-glycation abilities and does not cause the inhibition of cancer cell growth (Alhamdani et al., 2007; Holliday and McFarland, 1996). However, itis known that L-carnosine is the most potent in preventing protein glycation (Alhamdani et al., 2007).

Interestingly, unlike the increase in lifespan of normal cells, which is only caused by L-carnosine, anserine has been shown to cause a reduction in the growth of cancer cells, which is dependent upon the absence of pyruvate from the growth medium (Holliday and McFarland, 1996). As such, anserine's effects towards *S. cerevisiae* were analysed and appeared to cause a small reduction in yeast viability when cells were grown on glucose as a carbon source, but not glycerol, however this result needs to be further investigated. The reason anserine may be less potent than L-carnosine is because it has differences in its properties, compared to L-carnosine, for ROS scavenging, metal chelation and anti-glycation (Alhamdani et al., 2007; Fontana et al., 2002; Kohen et al., 1988).

L-carnosine's, affect towards cancer cells and yeast cells could also be attributed to its metal chelation properties. For instance the drug curcumin, has metal chelation properties, and has been shown to inhibit the growth of cancer cells, whilst protecting against neurodegeneration. Studies showed that curcumin decreased the growth rate of yeast by chelating the metal ion, iron. However, in contrast to the L-carnosine results presented in Chapter 4, it did not cause a decrease in viability (Minear et al., 2011). The metal chelation properties of L-carnosine could be affecting the activities of glycolytic enzymes, as they rely on metal cofactors, such as Mg^{2+}, and L-carnosine could be chelating these ions, which could contribute to the decrease in ATP production observed in cancer cells, (Hipkiss et al., 2013; Rose and Harrison, 1971). However, L-histidine, which is a metal chelator (Ikeda et al., 1980), similar to L-carnosine, did not reduce the viability of yeast. Moreover L-carnosine's effect towards cancer and yeast cells is unlikely to be solely attributed to its ROS scavenging abilities, as L-histidine is also able to scavenge ROS (Kohen et al., 1988), and does not decrease the viability of yeast cells. Also, the antioxidants, ascorbic acid and quercetin have been shown to have protective effects towards yeast cells (Belinha et al., 2007; Branduardi et al., 2007).

Overall, any one (or a combination of several) of L-carnosine's properties could result in a decrease in yeast viability, similar to the decrease in cancer cell growth; the literature points towards L-carnosine's anti-glycation properties (Holliday and McFarland, 1996; Preston et al., 1998).

5.3. Future work

5.3.1. Understanding translational processes during the high-yielding production of membrane proteins

Overall this project has determined why there is an increase in Fps1 production in the high yielding strains; the presence of uORFs in the *FPS1* transcript limits translation of *FPS1* in wild-type cells, while the high yielding strains, *spt3Δ* and yTHC*BMS1,* increase Fps1 production by circumventing the uORFs. Future work for this project can be divided into three objectives:

5.3.1.1. Further analysis of the mechanisms of translation initiation in the high yielding strains

The strains *srb5Δ, spt3Δ* and *gcn5Δ* have not been reported to have an effect on initiation of translation, whereas Bms1 has been reported to decrease the levels of 40S ribosomal subunit when mutated. The

data from the high yielding strains points towards a leaky scanning mechanism, however further analysis could be done to clarify this hypothesis. The extension of uORF1 into the ORF of *FPS1* would aid in the determination of whether a leaky scanning mechanism rather than a re-initiation mechanism is occurring, if there is a leaky scanning mechanism occurring the results would be similar for when uORF1 is not extended into the ORF. If there is not a leaky scanning mechanism and it is a reinitiation mechanism, the levels of Fps1 produced would be decreased, as translation of the uORF would end after the start of the ORF of *FPS1*, preventing reinitiation. This could also be done for uORF2, to determine the extent of leaky scanning at this uORF.

Only *spt3Δ* and yTHC*BMS1* (with 0.5µg/mL doxycycline) were analysed with the new vectors, pYX222-5´Δ1-43-*FPS1*-HA_3 and pYX222-5´Δ1-215-*FPS1*-HA_3. The analysis could therefore be extended to the other high yielding strains, such as the ribosomal mutants, which would further aid in the determination of whether a leaky scanning mechanism is occurring.

Finally the effect of the length of uORF1 could be analysed, as a uORF of its size is thought to decrease re-initiation at the ORF to almost zero (Vilela and McCarthy, 2003).

5.3.1.2. Understanding the mechanism behind an increase in functional protein yields

As some of the high yielding strains appear to increase functional protein yields, and this is not dependent upon the presence of uORFs, it would be interesting to determine the corresponding mechanisms. One experiment would be to compare western blot data for the proteins, which have been shown to have an increase in functional expression. This would provide information on yield and function. Furthermore, very little analysis has been done on the characterisation of the strain yTHC*BMS1* (with 10µg/mL doxycycline); it would be interesting to obtain polysome profile data, growth rate information and Gcn4 production levels in this strain, to determine if any are a factor in increasing functional protein production.

5.3.1.3. The role of the uORF in the **FPS1** *transcript*

It would be interesting to determine whether the uORF in *FPS1* serves any other function, except for lowering *FPS1* translation. It is clear that the levels of Fps1 can be influenced by the presence of this uORF. If Fps1 levels are influenced by changes in the environment, it is likely to occur during hypo-osmotic shock or during anaerobic growth, as the Fps1 channel is needed to remove glycerol from the cell. Thus the levels of Fps1 protein under these conditions could be analysed using the pYX222-5 ´Δ1-43-*FPS1*-HA_3.

5.3.2. Manipulation of yeast metabolism to study the molecule, L-carnosine

Overall, this project has demonstrated that L-carnosine has a metabolism-dependent effect towards *S. cerevisiae*. When yeast have a respiro-fermentative metabolism, L-carnosine causes in a reduction in viability, however when yeast have a respiratory metabolism, L-carnosine does not cause a reduction in viability. To aid in the understanding of L-carnosine's effects on yeast, metabolic flux experiments

could be performed, where the levels of glycolytic components and the activities of enzymes are determined. This would aid in determining whether L-carnosine does cause a reduction in triose sugars. In addition, it would also provide information on whether L-carnosine influences glycolysis in any way. It is also clear that L-carnosine affects aromatic compounds in the yeast cell cultures, as they smell differently. It would be interesting to obtain gas chromarography data to determine whether any of the aromatic compounds have changed concentrations.

As ATP levels were increased when L-carnosine was added to yeast in a dose-dependent manner, this suggests that there could be an increase in respiration rates. Moreover, the TAM data for the 20mM and 30mM concentrations indicated that either the secondary peak was broad and flattened or may have disappeared. This suggests that respiration may be occurring in these cells. Yeast cells grown on glucose have a respiro-fermentative metabolism, so it would be interesting to see the effect of a fully anaerobic metabolism upon yeast when L-carnosine is added. Anaerobic growth can also be achieved with the use of petite yeast, which do not have a respiratory metabolism due to mutations in mitochondrial genes (Van Dijken et al., 1993). In addition it was shown (Holliday and McFarland, 1996) that the addition of sodium pyruvate to cancer cells enabled growth in the presence of L-carnosine; it would be interesting to see if the addition of pyruvate to yeast cultures prevents the decrease in viability.

Currently, 6 undergraduate project students have been screening the *S. cerevisiae* deletion set for yeast that are resistant or more sensitive to L-carnosine, to try and understand the mechanism of L-carnosine action when yeast are grown on glucose as a carbon source. So far, around 750 deletion strains have been screened, in which a third have been shown to be resistant or more sensitive to L-carnosine. For instance, one feature of the data is the finding that five mitochondrial ribosomal proteins of the large ribosomal subunit, when deleted, increase the viability of yeast when grown in the presence of L-carnosine. However, all that can be suggested is that those deletion strains that are more resistant to L-carnosine have an increase in respiration rates, and those that are more sensitive have a decrease in respiration rates.

Finally, it would be interesting to confirm the effects of the drug tenilsetam on yeast, as it is an anti-glycation drug that has been shown to reduce the growth of cancer cells (Holliday and McFarland, 1996), and is dependent upon the absence of pyruvate. If it does have similar affects to L-carnosine, this could indicate that it is L-carnosine's anti-glycation properties that result in a reduction in viability.

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Appendices

A.1. Mechanisms of translation

A.1.1. Translation in prokaryotes

Initiation of translation is different in prokaryotes compared to eukaryotes: the large ribosomal subunit is a 50S species whilst the small ribosomal subunit is a 30S species, when joined this makes the 70S ribosome. The initial stage of translation is the dissociation of the free 70S into 30S and 50S, aided by the initiation factors IF1 and IF3 (Kozak, 1999). IF2·GTP is bound by the initiator tRNA, Met-tRNA, which is recruited to the 30S subunit (Kozak, 1999). This 30S subunit with the initiation factors is then recruited to the Shine-Dalgarno (SD) sequence. The SD sequence binds to a complimentary sequence on the 16S rRNA in the small ribosomal subunit. The SD sequence in mRNA is typically 5-8 nucleotides upstream of the start codon, enabling binding of the 16S rRNA and the positioning of the start codon in the polypeptide site (P-site) (Kozak, 2005). The SD is purine (adenine and guanine) rich and can have between 3-8 nucleotides in its sequence to aid in binding to the 16S rRNA. The start codon of prokaryotes is often AUG, the same as eukaryotes. However 14% and 3% of the genome have GUG and UUG respectively as their start codons, this is because the anti-codon of Met-tRNA only needs to base pair with two nucleotides in prokaryotes (Kozak, 2005). There are some sequences without the SD sequence, but it is thought these are translated by threading free 70S at the 5´end and scanning for the start codon; these sequences are rare (Kozak, 2005). Another key difference in translation is many bacterial mRNAs are polycistronic, meaning they code for more than one polypeptide, eukaryotic mRNA are mostly monocistronic mRNA (Kozak, 2005, 1999).

A.1.2. Cap-independent mechanism of translation: IRES

Initiation of translation can also occur independently of the 5´cap, by internal initiation, with the use of internal ribosome entry sites (IRESs). IRESs are rare and were first discovered in the RNA of picornavirus (Meijer and Thomas, 2002). IRESs have secondary structures and act as landing pads for 40S ribosomal subunits. Often they still require some eIFs; for example, Hepatitis C virus requires the TC and eIF3, Poliovirus on the other hand requires some eIF and IRES trans-acting factors (ITAFs). These ITAFs are thought to either stabilise the secondary structure of the IRES or aid in the recruitment of the 40S. Some IRESs do not require any eIFs such as the Cricket Paralysis virus, which does not even require the TC as its IRES mimics the starting tRNA and can drive translation from a non-AUG (Meijer and Thomas, 2002; Filbin and Kieft, 2009). When a cell is infected by a virus translation initiation is down regulated by the phosphorylation of eIF2α; as a result some viruses have IRESs to enable translation to occur (Kozak, 1992). Cellular IRESs also occur, but are rare, they are thought to play a role in driving translation of important genes when translation has been down regulated, which could be caused by stress, mitosis or cellular proliferation (Filbin and Kieft, 2009). IRESs also occur in *S. cerevisiae*, such as in the transcript of *YAP1* (Zhou et al., 2001).

A.1.3. Leaky scanning and factors that affect translation

Leaky scanning of a start codon which matches the consensus sequence can also occur if the start codon is within 15 nucleotides or less of the 5´end of the mRNA, as the PIC needs to be clear of the 5´cap and be completely associated with the mRNA for efficient start site recognition (Kozak, 1989). Leaky scanning can aid in gene regulation, as it provides a level of translational control (Kozak, 1999). In higher eukaryotes, it has been shown that leaky scanning aids in the formation of different isoforms of proteins, when the start codons are inframe, this results in proteins that are of different lengths, due to the truncation of the N-terminal.

Translation of an ORF can be affected by stable and non-stable secondary structures. Low stability secondary structures in the 5´UTR do not affect initiation, as the scanning ribosome possesses RNA helicase activity. However stable secondary structures, such as hairpins, can halt the progression of the scanning PIC and can decrease translation by 85-95% (Kozak, 1986). At the same time, non-stable secondary structures at an optimal position of 14 nucleotides downstream of the start codon can increase translation at unfavourable start codon sequences and non-AUG start codons. The reason translation is thought to be increased is the secondary structure slows the progression of the scanning PIC, thus enabling more time for start site recognition and GTP hydrolysis. The optimal distance of 14 nucelotides down stream is due to the distance between the leading edge of the scanning PIC and the position of the start codon in the P-site, (Kozak, 1990). Translation of a mRNA can also be decreased by a GC-rich 5´UTR, as a high GC content can lead to structured mRNA of greater stability. mRNAs with high GC rich 5´UTR are often associated with the genes of growth factors and transcription factors, where low levels of the proteins are often required (Kozak, 1992).

Start site recognition is also affected by initiation factors, for instance, mutations which weaken eIF1 binding to the 40S, increases recognition of non-AUGs, as eIF1 stabilises the open conformation of the PIC and prevents the closed formation until AUG has been recognised. Upon AUG recognition the Met-tRNA$_i$ pushes the eIF1 away from the P-site enabling the Met-tRNA$_i$ to fully enter the P-site upon AUG recognition. Once eIF1 has been released this enables the release of P_i from eIF2·GTP (Hinnebusch and Lorsch, 2012). Moreover mutations in eIF2 and eIF5 also affect start codon recognition (Kozak, 1992).

In summary, the 5´UTR aids in the regulation of protein synthesis by variations in sequences, the presence of structures and deviations from the Kozak sequence.

A.1.4. Yeast uORF

uORFs occur in yeast, it has been estimated in yeast that 13% of *S. cerevisiae* transcripts may contain uORF (Lawless et al., 2009), another estimate puts it at 6% (Hood et al., 2009) (Table A.1.1). The difficulty is that only recently have the transcriptional start sites of ~70% of the yeast genome been elucidated (Lawless et al., 2009).

Translation at the ORF can also be decreased by proteins produced from the uORF, by preventing ribosomes from reaching the ORF. This is done by stalling the ribosome at the stop codon or during elongation of the uORF, which then prevents ribosomes reaching the ORF as the stalled ribosome acts as a block. For instance the transcript of *CPA1,* which encodes the small subunit of arginine specific carbamoyl phosphate synthetase in yeast, contains an uORF, however leaky scanning occurs so that both uORF and the ORF are translated. When arginine levels are high ribosomes that translate the uORF stall due to the production of arginine attenuator peptide (AAP) from the uORF. The protein promotes stalling during high arginine concentration at the stop codon. This stalling also triggers nonsense mediated decay (NMD), which degrades the mRNA. When there are low levels of arginine there is no stalling, thus, the ORF can be translated, moreover NMD does not take place. NMD is triggered by early stop codons; it is known that a higher proportion of mRNA with uORF are targeted by NMD, however this aids in gene regulation. Not all mRNA with uORF are targeted for NMD (Hood et al., 2009).

Thus, uORFs are present in higher eukaryotes and yeast and aid in the regulation of protein synthesis by decreasing the levels of translation at ORFs. Furthermore the sequences surrounding the start and stop codon, the length of the uORF and the distance between the uORF and ORF can aid in the regulation of translation at the ORFs.

Table A.1.1: Yeast genes that contain uORF. *Below is a list of selected transcripts from yeast with known uORF. Many of the uORFs have a small codon size, such as* GCN4*, but others have longer ones, moreover some transcripts have more than one uORF, adapted from (Vilela and McCarthy, 2003).*

Gene	Length of 5´UTR	uORF (Codon size)	Function
CBS1	101	uORF (4)	PET gene involved in 5´end processing of cytochrome b
CLN3	864	uORF (4)	G1 cycline
CPA1	244	uORF (26)	Carbamoyl phosphate synthetase
GCN4	591	uORF1 (4), uORF2 (2), uORF3 (4), uORF4 (4)	Transcription factor in amino acid biosynthesis
HAP4	280	uORF1 (10), uORF2 (4)	Transcription factor
INO2	105	uORF (20)	Transcription factor of phospholipid biosynthesis
SCHO9	600	uORF (55)	Protein kinase, regulates progression through G1
STA1/2/3	~100	uORF (5)	All three genes involved in glucoamylase
PPR1	50	uORF (6)	Regulatory protein controlling transcription of two genes in pyrimidine biosynthesis
YAP1	164	uORF (7)	Stress induced transcription factor
YAP2	157	uORF1 (6), uORF2 (2)	Stress induced transcription factor

A.1.5. MutantS which affect GCN4 *translation*

A range of mutations in the initiation factors of translation can affect Gcn4 levels and as a result are known as either a Gcn$^-$ or Gcd$^-$ phenotypes (Table A.1.2). For instance, four of the five subunits of eIF2B are Gcd$^-$ mutants as they cause an increase in Gcn4 levels, under non-amino acid starved conditions, thus the subunit names begin with Gcd. Mutations in proteins beginning with Gcn prevent Gcn4 translation, even during amino acid starvation (Hinnebusch, 2005). Interestingly if two mutants are put together, where one results in a Gcd$^-$ phenotype and the other in a Gcn$^-$ phenotype, the ordering in the translation initiation pathway will determine the phenotypes. For instance, mutations in *GCN2* prevent *GCN4* translation and derepression, however, if all the uORF are removed from the *GCN4* transcript, then Gcn4 protein is produced constitutively in the *GCN2* mutant (Hinnebusch, 1984). Furthermore, some mutations in the same gene can result in a Gcd$^-$ phenotype whilst others result in a Gcn$^-$ phenotype. For instance, some mutations in eIF2α can result in a Gcd$^-$ phenotype, as it decreases TC formation thus causing derepression under normal conditions, however if serine-51 is replaced by an alanine this prevents phosphorylation, resulting in a Gcn$^-$ phenotype, as translation of *GCN4* cannot take place under amino acid starvation. (Hinnebusch, 2005). Mutation and deletions of ribosomal proteins and ribosomal biogenesis proteins have also been shown to cause an increase in Gcn4 levels, due to decreased levels of 60S (Foiani et al., 1991; Steffen et al., 2008).

Table A.1.2: Mutations that affect* GCN4 *translation. *A range of translation initiation factors affect Gcn4 levels when mutated, adapted from (Hinnebusch, 2005).*

Subunit name in yeast	Mammalian name	Function
Gcn2	mGcn2	Protein kinase, activated by uncharged tRNAs causingeIF2α-P
Gcn1	-	Aids in the activation of Gcn2 by uncharged tRNAs
Gcn20	-	
Gcd1	γ subunit of eIF2B	GEF for eIF2
Gcd2	δ subunit of eIF2B	
Gcd6	ε subunit of eIF2B	
Gcd7	β subunit of eIF2B	
Gcn3	α subunit of eIF2B	
Gcd 11	γ subunit of eIF2	Binds GTP
Sui2	α subunit of eIF2	Part of the PIC and phosphorylated at serine-51 under amino acid starvation
Sui3	β subunit of eIF2	Part of the PIC
Gcd10	-	subunit of tRNA methyltransferase, mutations decrease methylation of adenine 58 especially in the starting tRNA
Gcd14	-	

A.1.5.1. Mammalian cells and GCN4 *regulation*

In mammalian cells, eIF2α can be phophorylated under a range of stressful conditions such as a decrease in hemin, viral infections, ER stress and decrease in amino acids. These stressful conditions activate a rage of protein kinases such as heme-regulated inhibitor (HRI), dsRNA activated protein kinase (PKR), pancreatic protein kinase activated by ER stress (PERK) and Gcn2. In yeast there is only one protein kinase known to phophorylate eIF2α, Gcn2. Interestingly the addition of HRI and PKR to

yeast can result in the phosphorylation of eIF2α. The *GCN4* homologue in mammals is *ATF4*, which also has uORF which regulates its expression (Hinnebusch, 2005).

A.1.5.2. Other stresses which activate GCN4 *in yeast*

As yeast only has one protein kinase which phosphorylates eIF2α, unlike mammalian cells, it is interesting to note that many other stresses, besides amino acid starvation, have been shown to activate the translation of *GCN4*, for instance purine starvation, fusel alcohols, glucose starvation, hydrogen peroxide, osmotic stress and rapamycin (Hinnebusch, 2005).

Fusel alcohols: During nitrogen starvation, amino acids are metabolised to fusel alcohols, such as butanol. The addition of butanol at 1% inhibits the GEF activity of eIF2B, independently of eIF2α phosphorylation. This inhibition decreases the TC and thus causes an initiation block resulting in an increase in Gcn4 levels (Smirnova et al., 2005).

Hydrogen peroxide: A block in the initiation of translation can also be caused by the addition of hydrogen peroxide to yeast cultures and cause increased Gcn4, which is dependent on eIF2α phosphorylation and Gcn2 and its HisRS domain, Gcn1 and Gcn20 (Shenton et al., 2006).

Purine starvation: This causes an increase in Gcn4 levels and is dependent on the HisRS domain of Gcn2 and results in the phosphorylation of eIF2α (Hinnebusch, 2005).

Osmotic shock: The addition of high levels of NaCl to yeast cultures causes initiation blocks, which are dependent on Gcn2, Gcn20 and Gcn1 causing the phosphorylation of eIF2α and an increase in Gcn4 (Shenton et al., 2006). It is also thought an initiation block is brought about by eIF1A being targeted and thus the PIC doesn't associate with the 5´cap efficiently (Simpson and Ashe, 2012).

Glucose starvation: Glucose is the preferred carbon source for yeast. Growth on medium with no glucose causes an initiation block in a few minutes. This initiation block is independent of eIF2α phosphorylation and Gcn2 nor is it dependent on Tap42 of the TOR pathway. It appears instead the removal of glucose causes the dissociation of eIF4A from the 5´cap, thus preventing the association of the PIC with the 5´cap. This initiation block is reversible when glucose is added back into the cultures. Some mutations in the glucose repression pathway, are resistant to the removal of glucose, which causes an inhibition in translation, however it is likely these mutants, such as *hxk2Δ* and *reg1Δ* act as if they are grown on very little glucose in the first place. It is known that moving yeast from a non-fermentable carbon source to media containing no carbon source, causes no change in polysome profiles (Ashe et al., 2000). Interestingly glucose limitation on the other hand by moving cultures from 2% glucose to 0.05% glucose causes a transient increase in eIF2α phosphorylation after 4h and an increase in Gcn4 by 10 fold after 6h which began to decline after 8h. eIF2α phosphorylation was shown to be dependent on the HisRS domain of Gcn2, which appears to be activated by uncharged tRNAs as there is a decrease in the amino acid pool size in the vacuole under glucose limitation (Yang et al., 2000).

Rapamycin addition: TOR is a phosohatidylinositol kinase. In yeast, there are two types of TOR, TOR1 and TOR2. TOR2 is mostly involved in the organisation of the actin cytoskeleton. TOR1 is thought to aid in the regulation between cellular growth and nutrient availability, as it can influence ribosomal biogenesis and the levels of protein translation. It is important to coordinate nutrient availability to protein production as it is a very energy consuming process: over 50% of all transcription in exponentially growing yeast are involved in ribosomal biogenesis (Jorgensen et al., 2004). Not only does the down regulation of TOR1 by rapamycin or nutrient availability affect ribosomes, but the addition of rapamycin to yeast cells causes them to spend a greater amount of time in G1 of the cell cycle and enter into stationary phase (Powers and Walter, 1999). The addition of rapamycin to yeast cells causes a down regulation of the TOR proteins. This down regulation in TOR has then been shown to result in dephosphorylation of Gcn2 at serine-577. This phosphorylation is present during amino acid starvation, however when removed this causes an increase in Gcn2 affinity for uncharged tRNAs, resulting in its activation and thus the phosphorylation of eIF2α, causing an increase in Gcn4 levels. It is thought the dephosphorylation occurs by the actions of Sit4, which is a phosphatase, but under normal conditions where TOR1 levels are high Sit4 activity is inhibited by the binding of Tap42 which is activated by TOR1 (Hinnebusch, 2005). Moreover, TOR1 can also regulate initiation of translation by the phosphorylation of the eIF4E-binding protein, Eap1. When TOR1 activity has been decreased this causes dephosphorylation of Eap1 which binds eIF4E decreasing the interaction between the PIC and the 5´cap (Cherkasova and Hinnebusch, 2003). TOR1 also regulates ribosomal biogenesis according to nutrient availability: when nutrients are low or upon the addition of rapamycin, there is a down regulation of transcription of rRNA (Powers and Walter, 1999).

In conclusion, the stress resistance transcription factor Gcn4 regulates over 500 genes during many different stresses. Moreover, the regulation of *GCN4* is controlled at the level of translation by the presence of uORFs, which prevent the translation of *GCN4* during non-stress conditions, but enable translation during initiation blocks due to the mechanism of reinitiation of translation.

To conclude eukaryotic translation initiation is a complex mechanism that enables regulation of protein synthesis during certain conditions, such as different environmental stresses. The complexity in translation initiation is aided by the structure and sequence of the 5´UTR and the sequence surrounding the start codon. Moreover, uORFs often decrease translation at the ORF, however uORFs enable further regulation of protein expression. In the case of *GCN4* uORFs they aid in the expression of Gcn4 only during times of stress and enable a change in gene expression, such as the production of genes involved in amino acid biosynthesis (Hinnebusch and Fink, 1983) and the production of chaperone proteins during the unfolded protein response (UPR) (Patil et al., 2004).

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A.2. The properties of L-carnosine

A.2.1. L-carnosine is a physiological buffer in muscles

L-carnosine is found at high concentrations in skeletal muscle. In humans the average L-carnosine concentration is 20mmol kg^{-1} dry muscle mass (Mannion et al., 1992). In other mammals however, concentrations can be much higher at 100 mmol kg^{-1} dry muscle mass (Dunnett and Harris, 1997). The concentration of L-carnosine is higher in type 2 muscle fibres than type 1 fibres (Culbertson et al. 2010), this is also the case for L-carnosine concentrations in humans (Abe, 2000). The reason that L-carnosine is at greater concentrations in type 2, fast twitch muscle is anaerobic metabolism is greater in type 2 muscle fibres than in type 1, slow twitch muscle (Abe, 2000). During intensive exercise, this results in hypoxia, causing anaerobic metabolism to take place, resulting in an increase of lactic acid and protons (H^+). This increase in hydrogen ions causes a reduction in physiological pH (Mannion et al. 1992), which is detrimental for survival of the organism as low pH inhibits glycolytic enzymes, such as phosphofructokinase, decreasing glycolytic flux and preventing muscle contraction (Abe, 2000). Consequently, animals require buffers within their muscles of which L-carnosine and other imidazole containing compounds, such as histidine, anserine and ophidine (balenine) are examples (Abe 2000; Mannion et al., 1992). Buffers such as L-carnosine are able to neutralise the acids which accumulate from anaerobic exercise; the buffering capacity of L-carnosine in humans is thought to be higher in type 2 muscle than type 1 (Abe, 2000). As L-carnosine and other CRC such as anserine are buffers in muscle there is a strong correlation in animals for the concentration of CRC and high levels of sprinting activity (Abe, 2000). This also seems to be the case for humans since sprinters and body builders have some of the highest recorded concentrations of L-carnosine, moreover L-carnosine levels in muscle can be increased by sprinting (Culbertson et al., 2010). CRC concentrations also correlate with an increase in hypoxia, as deep-sea diving marine mammals contain large amounts of CRC in their muscle (Culbertson et al. 2010).Thus L-carnosine and CRC are important for anaerobic exercise and buffers against the acidic conditions in vertebrates.

A.2.2. L-Carnosine decreases muscle fatigue

A muscle is made from parallel muscle fibres which are multinucleated cells and each muscle fibre is divided into sarcomeres, which contain myosin and actin filaments. During contractions the actin filaments slide along the myosin filaments resulting in the shortening of the sarcomere and a muscle contraction. During a muscle contraction the muscle fibre is depolarised by the binding of acetylcholine to receptors which has been released from the motorneurone axons. This depolarisation causes the sacroplasmic reticulum (SR) to release calcium ions (Ca^{2+}), which binds to troponin C, moving tropomyosin, and enabling myosin heads to bind to the actin filament. Myosin tightly binds to actin when bound by ADP, at which point a conformational change occurs in myosin and ADP is released, and the myosin head moves, so that the myosin filament pulls on the actin filament. ATP then binds myosin, which causes it to dissociate from actin, this ATP is hydrolysed to ADP and P_i enabling the myosin head to bind to the actin filament, but further down. During relaxation the calcium ions are

pumped back into the SR by calcium ATPase and thus tropomyosin blocks the binding site of actin (Becker et al., 2006).

L-Carnosine and CRC have been shown to enable muscle contraction when fatigued and also allow a muscle to contract longer before the onset of fatigue. This is known as Severin's effect. Muscle fatigue can be caused by deregulation of calcium release form the SR. Acidification of muscles can inhibit calcium release from SR (Rubtsov 2001). As discussed above L-carnosine acts as a buffer and can decrease acidification of muscles. Moreover the increase in hydrogen ions can react with superoxide to form a peroxyl radical, which is more reactive than superoxide; as L-carnosine is a buffer, this prevents the formation of peroxyl radicals (Boldyrev, 2012). ROS levels can increase greatly during exercise as there is estimated to be a 20 fold increase in oxygen consumption and about 2-4% of oxygen consumed is thought to result in ROS production; ROS also inhibit calcium channels. L-Carnosine and CRC can scavenge ROS, which can enable a prolonged period of time before fatigue sets in (Rubtsov, 2001).

Imidazole containing compounds such as L-carnosine, CRC and caffeine are known to increase calcium release from SR (Rubtsov, 2001). (Lamont and Miller, 1992) showed that L-carnosine and to a lesser extent anserine and homocarnosine can increase muscle tension of rat and frog muscle fibres at physiological concentrations in the presence of calcium ions. Interestingly invertebrates that do not contain L-carnosine, such as crab muscle, did not show an increase in muscle tension. They also showed that L-carnosine increases calcium sensitivity, this may be caused by improving troponin C sensitivity to calcium ions, but it is unknown why. L-carnosine and to a greater extent anserine have been shown to activate myosin ATPase at physiological concentrations (Avena and Bowen, 1969). These multiple effects of L-carnosine could all contribute to the Severin's effect.

It has been shown in rowers that the greater the concentration of muscle L-carnosine the higher the performance (Baguet et al., 2010). β-alanine supplementation causes an increase in muscle L-carnosine by 60-80%, results in greater performance levels than placebo and can also delay fatigue in trained and untrained athletes (Culbertson et al., 2010; Del Favero et al., 2012). The reason β-alanine supplementation increases L-carnosine levels in muscle is that β-alanine is found at lower concentrations than L–histidine inside cells. Thus, β-alanine concentrations could be the rate limiting step in L-carnosine synthesis as β -alanine can either be taken up from the diet or can be synthesised in the liver form the catabolism of uracil and thymine (Caruso et al., 2012). Conversely a diet deficient in L–histidine results in a decrease in muscle L-carnosine in mice (Parker et al., 1985). After β-alanine supplementation, L-carnosine begin to fall over several weeks, as L-carnosine may be being released from muscles or reacting with ROS or reactive sugars (Harris et al., 2012). Overall L-carnosine and CRC decrease fatigue and in turn increase performance as they buffer against the build up of hydrogen ions during anaerobic exercise and also prevent the formation of more potent ROS and scavenge ROS that also contribute to muscle fatigue.

A.2.3. L-Carnosine and CRC have a protective role in nerve cells

L-carnosine is found throughout the CNS at ~2mM (Renner et al., 2008; Bauer, 2005). Although it is found at high concentrations in the olfactory bulb (2-5mM), it is not synthesised in olfactory neurones, but instead in glial cells (Bauer, 2005). Homocarnosine is found at higher concentrations in the CNS, due to the availability of GABA in the CNS (Bellia et al., 2011). Interestingly chick muscle fibres supplemented with GABA can synthesis homocarnosine due to the broad specificity of carnosine synthetase (Bauer, 2005).

As L-carnosine and other CRC are found throughout the brain it is speculated that they may play a role in neuromodulation (Trombley et al., 2000). L-carnosine is speculated to be involved in neuromodulation as zinc and copper ions participate in neuronal excitability. For example, it has been shown *in vitro* that L-carnosine decreases neuronal excitability as it chelates metal ions, but this may not happen *in vivo* (Trombley et al., 2000). L-carnosine has also been speculated to be a neurotransmitter (Burd et al., 1982) in the olfactory bulb, however there is no direct evidence to support this hypothesis (Trombley et al., 2000). Instead it is thought L-carnosine may play a protective role as the olfactory nerves come into direct contact with the external environment (Bellia et al., 2011) and may protect against ROS, metal ions and reactive sugars (De Marchis et al., 2000).

A.2.4. L-Carnosine is an antioxidant

An "oxidant" is an oxidising agent, which is a compound that can accept electrons, and as a result is reduced; an "antioxidant" is a reducing agent that donates electrons and is itself oxidised. When these reactions occur together the process is known as a redox reaction. In biology, a redox reaction typically comprises the donation of electrons accompanied by the donation of hydrogen or the removal of oxygen. In an organism, there is a balance between antioxidants and oxidants to enable normal cellular function. When there are more oxidants than antioxidants, oxidative stress occurs, conversely when there are greater levels of antioxidants than oxidants this causes reductive stress. ROS and reactive nitrogen species (RNS) are examples of oxidants and cause damage to macromolecules such as DNA, lipids and proteins.

There are two forms of oxidants: radical and non-radical. Radicals have an unpaired electron, such as superoxide $O_2^{\cdot -}$, hydroxyl $OH^{\cdot}$, nitric oxide $NO^{\cdot}$ and peroxyl $ROO^{\cdot}$ and as a result they are very reactive. Non-radicals include hydrogen peroxide H_2O_2, organic peroxides, aldehydes, hypochlorous acid, oxygen and singlet oxygen. Different types of ROS oxidants can be generated during metabolism, specifically from the mitochondria, where oxygen is reduced to water by the donation of 4 electrons, and thus 4 hydrogen ions, during the production of ATP. This process is enhanced during ageing and more ROS are formed. ROS can also be generated by pathogens and the immune system (Kohen and Nyska, 2002). The RNS nitric oxide is produced to control vasodilation and is involved in neurotransmission, neuromodulation and synaptic plasticity (Calabrese et al., 2005). ROS are used as

second messengers inside cells. ROS can also come from the external environment form air pollution, food, UV-light and γ-irradiation.

Transition metals contain unpaired electrons and thus are radicals. These radicals can readily react with ROS, such as iron with hydrogen peroxide in the Fenton reaction which results in the production of hydroxyl radicals, which are more reactive than hydrogen peroxide.

$Fe^{2+} + H_2O_2 \rightarrow OH^{\cdot} + OH^{-} + Fe^{3+}$

The metal ions which participate in this reaction are loosely bound to macromolecules, as a result greater levels of damage are done as ROS often have short half-lives of a few seconds, as ROS are produced in the vicinity of the macromolecules.

As ROS and RNS result in the damage of macromolecules, they play a role in the ageing process which was first suggested by Herman Denham in 1956. As ROS can be detrimental to the cell, the cell has enzymatic and non-enzymatic antioxidants. For instance SOD is an enzymatic antioxidant present in most aerobic organisms; two superoxide radicals can spontaneously react to form hydrogen peroxide and oxygen, however this reaction is slow and SOD enzymes speed up this reaction. Other antioxidants donate electrons and as a result scavenge radicals (Figure A.2.1), which prevents the radical from causing macromolecule damage. The scavenger is converted to a non-reactive radical, which can be regenerated by the donation of an electron, and thus a hydrogen. For instance oxidised glutathione is reduced by NADH. The antioxidant ascorbic acid also donates electrons. It is interesting to note that many of the non-enzymatic antioxidants have secondary functions. For example, ascorbic acid is a co-factor for enzymes, glutathione functions in metabolism ,and tocopherol stabilises membranes (Kohen and Nyska, 2002). L-Carnosine also appears to have multiple roles within the cell and is an antioxidant. Thus, the oxidants ROS and RNS are needed for normal cellular functions, however the levels must be controlled to prevent cellular damage and this role is performed by cellular antioxidants.

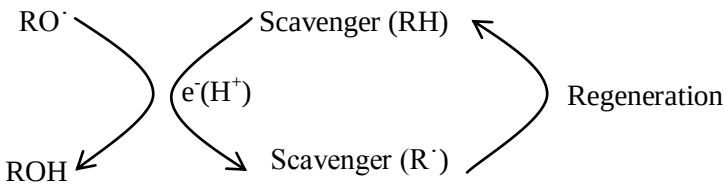


Figure A.2.1: Diagrammatical representation of antioxidant scavenging mechanism. *Scavengers donate electrons and thus hydrogen ions to ROS, to quench them, adapted from (Kohen and Nyska, 2002).*

Much of the earlier works on L-carnosine were done in Russia and focussed on L-carnosine antioxidant properties. L-carnosine was found to scavenge ROS, such as hydroxyl radicals and superoxide and was found to chelate copper, zinc, colbalt and to a lesser extent iron (Boldyrev et al., 1999).

A.2.5. L-carnosine scavenges ROS and RNS

Many CRC, even L-histidine, scavenge ROS and RNS to differing extents (Kohen et al., 1988). L-carnosine gives electrons to free radicals and thus prevents their damaging effects (Alpsoy et al., 2011). It is thought that L-carnosine donates electrons from its imidazole ring (Hipkiss et al., 1995). Also L-carnosine has been shown to form a transfer charge complex with superoxide, thus effectively buffering against ROS activity (Boldyrev et al., 1999).

The addition of L-carnosine *in vivo* and *in vitro* has been shown to reduce DNA damage caused by ROS. Oxidative damage to the nucleoside deoxyguanisine results in the formation of 8-hydroxydeoxyguanisine. L-carnosine has been shown to prevent hydroxyl radicals causing oxidative damage to the nucleoside deoxyguanisine when hydroxyl radicals are formed in the reaction between Cu^{2+} and ascorbic acid *in vitro*. Also the addition of L-carnosine to cultured human fibroblasts, subjected to nutritional insult caused by the medium not being changed for 5 weeks, was shown to decrease the levels of 8-hydroxydeoxyguanisine in a dose-dependent manner as it is thought during this time L-carnosine is scavenging ROS which have formed in the media. The decrease in oxidative damage to deoxyguanisine is attributed to L-carnosine's ability to chelate Cu^{2+} and scavenge ROS (Kantha et al., 1996). Conversely (Datta et al., 1993) showed that L-carnosine increased further the oxidative damage done by hydroxyl radicals to free deoxyguanisine nucleosides when nickel ions are used in the Fenton reaction. It is also known that L-histidine can form complexes with nickel to generate ROS from H_2O_2, this maybe the case for L-carnosine.

DNA damage can also be caused by hydrogen peroxide when in the presence of iron ions from ferritin, as this results in the Fenton reaction and the formation of hydroxyl radicals. The addition of L-carnosine and homocarnosine reduces DNA fragmentation levels as hydroxyl radicals are scavenged. Unlike Cu^{2+} ions, iron ions are not chelated as well by L-carnosine thus the prevention of DNA fragmentation is attributed to its ROS scavenging properties (J. H. Kang 2010; (Decker et al., 2000).

Anserine, and to a lesser extent L-carnosine and L-histidine, have been shown, in a dose-dependent manner, to prevent nitration of the amino acid tyrosine, resulting in 3-nitrotyrosine caused by the RNS peroxynitrile ($ONOO^-$). L-carnosine is thought to either scavenge peroxynitrile, or scavenge ROS, such as hydroxyl free radicals, which are the result of the peroxynitrile degradation (Fontana et al., 2002)

It is interesting to note that many antioxidant therapies have failed because ROS and RNS are short lived. For success, the antioxidant needs to be present at high concentrations near the site affected. Moreover ROS and RNS are important in cellular function such as gene activation, development, regulation of the immune system, vasodilation and cellular growth; consequently the large doses of antioxidants to treat diseases may be detrimental (Kohen and Nyska, 2002). In conclusion, it is clear that L-carnosine is a ROS scavenger and may scavenge RNS, however L-carnosine could be preventing the damaged caused by the degradation products of peroxynitrile instead. Moreover, L-carnosine could be scavenging metal ions when these are used in the reaction mixtures to produce

ROS. At the same time though it is clear that antioxidant therapies have had no success, thus it remains to be seen if one of L-carnosine physiological roles is as a ROS scavenger *in vivo.*

A.2.6. L-Carnosine affects the second messenger function of ROS

ROS are known to participate in gene expression; since L-carnosine decreases ROS levels, L-carnosine may also have an effect on signalling cascades and gene expression.

Homocysteine (HC) is a structural analogue of glutamate and as such can activate glutamate N-methyl-D-aspartate (NMDA) receptors. HC is a naturally occurring intermediate of methionine and cysteine metabolism. At high levels, HC can cause hyperhomocysteinemia, which results in cardiovascular and neurodegenerative disease. When HC activates glutamate N-methyl-D-aspartate (NMDA) receptors, this causes an increase in cellular ROS and activates signalling cascades that control cellular survival. Activation by glutamate causes short term activation of ERK1/2 and inhibition of JNK which enhances cellular survival (Boldyrev, 2012), as they regulate gene expression. Long-term activation of NMDA, which occurs with HC, causes apoptosis and necrosis, due to the damage by ROS. L-carnosine pre-treatment of cerebellum granule cells, treated with HC, prevented free-radical formation and as a result decreased the number of apoptotic and necrotic cells. The treatment with L-carnosine also prevented the long-term activation of ERK1/2 and prevented JNK activation (Boldyrev, 2009; Kulebyakin et al., 2012). *In vivo* L-carnosine can protect rats from HC toxicity and is shown by increasing memory (Boldyrev, 2012).

Intestinal epithelial cells (IECs) secrete cytokines and chemokines, which are both anti- and pro-inflammatory, as IECs act as a barrier against bacteria. IECs such as Caco-2 cells secrete IL-8 upon treatment with H_2O_2. When pre-treated with L-carnosine, Caco-2 cells did not secrete IL-8. Glutathione, anserine and L-histidine also decreased IL-8 secretion as they caused a decrease in IL-8 mRNA levels. L-carnosine on the other hand did not decrease IL-8 mRNA levels upon treatment with H_2O_2, but instead decreased protein synthesis of IL-8. H_2O_2 treatment of Caco-2 cells causes the phosphorylation of eIF4E via the phosphorylation of ERK1/2 and AKT. L-carnosine treatment was shown to inhibit this phosphorylation, possibly through ROS scavenging of H_2O_2 products, and thus decrease IL-8 expression (Son et al., 2008).

Also as mentioned in the text, during oxidative stress of cells, this causes an increase in HSP and SOD levels, upon the treatment with L-carnosine this causes a decrease in ROS and as a result a decrease in HSP and SOD levels (Calabrese et al., 2005). Thus, as L-carnosine can scavenge ROS this results in altered cellular signalling and can affect gene expression.

A.2.7. L-Carnosine protects cellular anti-oxidants

Cu,Zn-Superoxide dismutase (SOD) is an antioxidant that converts superoxide to hydrogen peroxide and oxygen. However Cu,Zn-SOD can be glycated by reactive sugars, resulting in fragmentation. Furthermore when Cu,Zn-SOD is damaged by fructose, this can result in the release of Cu^{2+}, which,

via the Fenton reaction, can produce ROS (Ukeda et al., 2002). This production of ROS can inactivate other Cu,Zn-SOD (Stvolinskii et al., 2003). L-carnosine can prevent glycation of Cu,Zn-SOD and can scavenge ROS. As a result L-carnosine has been shown to increase the activity of Cu,Zn-SOD; moreover L-histidine and to a lesser extent anserine, homocarnosine and imidazole can also protect against Cu,Zn-SOD inactivation *in vitro* in the presence of reactive sugars (Ukeda et al., 2002).

Carbon tetrachloride is carcinogenic and can cause the formation of ROS, and as such can cause DNA damage. Carbon tetrachloride can also decrease the activities of Cu,Zn-SOD and glutathione-peroxidase. It also decreases the levels of glutathione, in cultured human lymphocytes. The addition of L-carnosine at physiological concentrations in the presence of CCL4, restores the activity of Cu,Zn-SOD, glutathione-peroxidase and increases the levels of glutathione. Interestingly ascorbic acid also increases Cu,Zn-SOD activity and restores glutathione levels. However ascorbic acid had no significant effect on glutathione-peroxidase (Alpsoy et al., 2011).

L-carnosine as a supplement has been shown to protect cerebral cytosolic Cu,Zn-SOD in senescence accelerated mice (SAM) which have an increase in ROS formation (Stvolinskii et al., 2003). L-carnosine has also been shown to protect other proteins, such as the copper transporting protein: ceruloplasmin, against oxidative stress (Stvolinskii et al., 2003). Thus, it is clear that not only does L-carnosine prevent damage from ROS by scavenging but can also aid in the protection of other cellular antioxidants, which will also decrease ROS formation.

A.2.8. L-Carnosine chelates metal ions

L-carnosine can chelate metal ions since it has several binding sites for cations: two imidazole nitrogens, a carboxylate group, an amino group and the peptide bond. The β-alanine component of L-carnosine can also fold toward the imidazole ring, aiding chelation. L-carnosine can form complexes with copper, cobalt, nickel, zinc and weaker complexes with iron (Baran, 2000) while anserine also chelates copper (Kohen et al., 1988). The complex, Zn-carnosine (Figure A.2.2), also referred to as polaprezinc or L-CAZ, has a polymeric structure between L-carnosine and zinc ions. L-CAZ is an approved anti-ulcer drug, which is thought to adhere to the mucosal linings *via* L-carnosine allowing zinc to be released slowly from the complex into gastric ulcers (Matsukura and Tanaka, 2000). The zinc then aids in wound healing. Zinc also increase HSP72 in mucosal cells protecting the mucosal lining from damage (Mikami et al., 2006). L-CAZ also inhibits the growth of *Helicobacter pylori*. Interestingly D-CAZ, which is made from the optical isomer D-carnosine, does not have a significant effect on the treatment of ulcers (Matsukura and Tanaka, 2000).

The metal chelation abilities of L-carnosine and to a greater extent L-histidine, have been shown to increase the activity of the gluconeogenic enzyme fructose-1,6-bisphosphotase, as this enzyme is inhibited by Zn^{2+} ions (Ikeda et al., 1980). The metal complex of zinc and L-carnosine, have been shown to scavenge superoxide whilst Cu-carnosine complex has been found to have SOD activity

against superoxide (Stuerenburg, 2000). As discussed L-carnosine's ability to chelate metal ions also aids in the reduction in ROS formation from the Fenton reaction and as such protects cells.

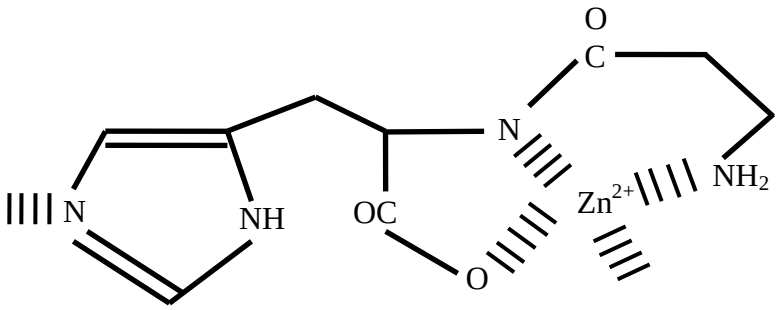
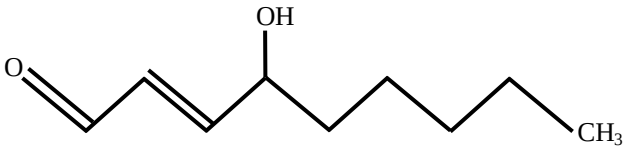


Figure A.2.2: Representation of how L-carnosine chelates the metal ion zinc. *Each zinc is bound by two L-carnosines, resulting in a polymeric structure enabling the slow release of zinc ions to treat stomach ulcers, adapted from (Matsukura and Tanaka, 2000).*

A.2.9. L-Carnosine protects against lipid oxidation

When ROS react with polyunsaturated fatty acids they undergo lipid peroxidation, this results in many different aldehyde products such as 4-hydroxyl-trans-3-nonenal (4-HNE) and malondialdehyde (MDA) (Figure A.2.3). 4-HNE is mostly generated from the polyunsaturated fatty acids linoleic acid and arachidonic acid whereas MDA is mostly generated from arachidonic acid. MDA and 4-HNE are reactive themselves and can create further damage to macromolecules such as proteins by reacting with amino-groups and amino acid side chains (Sasa Frank and Kostner, 2012). Lipid peroxidation has been linked to atherosclerosis, diabetes, cancer and neurodegeneration (Kim et al., 2011), as 4-HNE and MDA can react with the amino acid side chains of lysine, L-histidine and cysteine, to form advanced lipid peroxidation end products. The antioxidants SOD, glutathione-peroxidase and glutathione can protect against LPO (Kim et al., 2011; Aldini et al., 2002b). However 4-HNE does have biological functions at low concentrations, for instance in cellular proliferation and gene expression (Aldini et al., 2002b).

4-Hydroxyl-trans-3-nonenal (4-HNE)



Malondialdehyde (MDA)

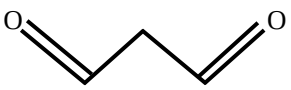


Figure A.2.3: Diagram of 4-hydroxyl-trans-3-nonenal (4-HNE) and Malondialdehyde (MDA). *Both lipid peroxidation products are very reactive and can cause cellular damage adapted from (Sasa Frank and Kostner, 2012).*

L-carnosine and CRC have been shown to protect membranes against LPO, as they can chelate metal ions and scavenge ROS which cause LPO (Stuerenburg, 2000). For instance L-carnosine and anserine, and to a lesser extent homocarnosine, L-histidine, imidazole and histamine, have been shown to decrease LPO of linoleic acid in the presence of the peroxy-radical initiator 2 2'-azobis(2.4-dimethyl valeronitrile) (AMVN). In contrast, β-alanine alone has no affect against LPO, however, it plays an important role in the antioxidant activity of L-carnosine as it can protect against LPO better than L-histidine on its own (Kohen et al., 1988).

L-carnosine and anserine, and to a lesser extent L-histidine and N-acetyl-carnosine, have been shown to quench 4-HNE to form carnosine-HNE adducts (Figure A.2.4) (Aldini et al., 2002a). Moreover, anserine-HNE adducts have been identified in rat muscle (Aldini et al., 2002b). The addition of L-carnosine prevents the cross-linking of the protein α-crystallin, caused by the lipid-peroxidation product MDA; L-carnosine also decreases the levels of carbonyl-groups on proteins, caused by MDA, which can result in protein intra and inter-crosslinking (Hipkiss et al., 1998). *In vivo* studies of L-carnosine have shown that the supplementation of L-carnosine at 5% to a rat's diet for 6 weeks, decreased the levels of MDA in serum, skin and liver, compared to control (Kim et al., 2011)

L-carnosine prevents the damage caused by carbon tetrachloride CCl_4, which is carcinogenic and can cause DNA mutations. The reason for this is CCl_4 is metabolised by cytochrome P_{450} resulting in the trichloromethyl radical $^{\cdot}CCl_3$, which in turn can form the trichloromethyl peroxy radical, $^{\cdot}OOCCl_3$. These radicals can remove hydrogen atoms from polyunsaturated fatty acids in membranes, in turn producing fatty acid free radicals such as MDA. The addition of CCl_4 to human lymphocytes causes DNA damage, noted by an increase in sister chromatid exchange (SCE). It can also cause an increase in MDA levels. Treatment with L-carnosine at physiological concentrations restores SCE levels to control; ascorbic acid has the same effect. L-carnosine also reduced MDA levels to control, ascorbic acid had no effect (Alpsoy et al., 2011). Consequently, L-carnosine decreases DNA mutations because of its ROS scavenging properties as seen for ascorbic acid. However L-carnosine, unlike ascorbic acid, decreased MDA levels, which can be attributed to its ability to quench reactive aldehydes. Overall L-carnosine prevents LPO initially by decreasing the levels of ROS and secondly by reacting with the reactive products formed from the reaction of ROS with polyunsaturated fatty acids, such as MDA and 4-HNE.

4-Hydroxyl-trans-3-nonenal (4-HNE)

Figure A.2.4: Structure of 4-hydroxyl-trans-3-nonenal (4-HNE) and L-carnosine-HNE adduct formed during reaction between L-carnosine and HNE*. L-carnosine binds 4-HNE and this prevents 4-HNE from reacting with other cellular proteins, which would have detrimental effects to the cell, taken from (Aldini et al., 2002a).*

A.2.10. L-Carnosine protects against glycation

The reaction of sugars with proteins is referred to as glycation and is a result of the Maillard reaction or browning reaction, as a solution containing sugar and protein turns slowly brown (Price et al., 2001). The Maillard reaction is a non-enzymatic reaction, which is caused by condensation between an amine group of a protein side chain and a carboxyl group of a sugar. Intra- and inter-molecular cross-linking results in the formation of advanced glycation end-products (AGEs) (Miller et al., 2003).

AGEs are thought to contribute to the ageing process, to be detrimental to the cell, and to cause age-related diseases, such as diabetes, neurodegenerative and cardiovascular diseases (Price et al., 2001). Damaged proteins are usually degraded by the proteasome via proteolysis, but AGEs inhibit proteolysis (Hipkiss et al., 2002). AGE formation may also be the result of an increase in ROS and a decrease in protein turnover during ageing (Hipkiss and Brownson, 2000). Many different sugars, which have aldehyde or ketone groups, will react with proteins to from AGEs, which result in an increase in carbonyl groups in proteins; ROS and UV-light can also result in the formation of carbonyl groups on amino acid side chains, which in turn can aid in cross-linking (Brownson and Hipkiss, 2000).

Methyl glyoxal is very toxic to the cell as it is a dicarbonyl compound and can promote cross-linking of proteins (Figure A.2.5) (Miller et al., 2003). MG is formed spontaneously from the triose sugars glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate (De Arriba et al., 2007). MG like many other sugars reacts typically with the side chain amino group of lysine when next to a L-histidine (Hipkiss et al., 1995).

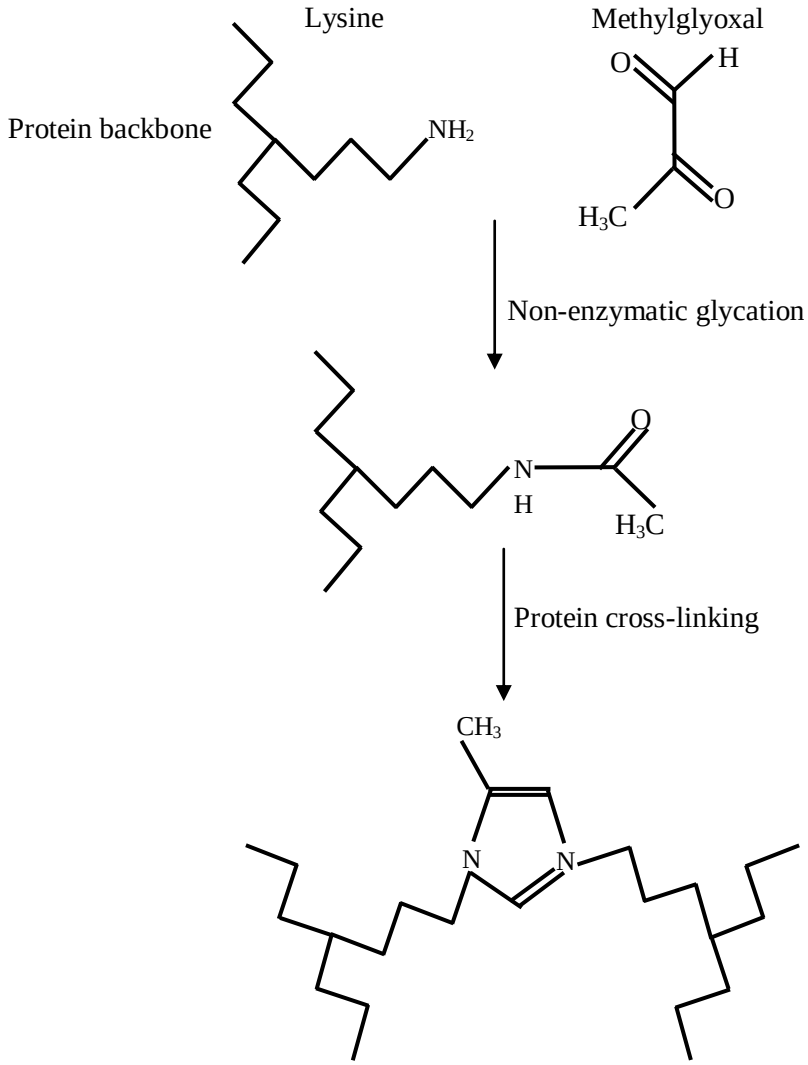


Figure A.2.5: Representation of methylglyoxal glycation with lysine and potential cross-linking structure. *Methylglyoxal spontaneously forms from the breakdown of dihydroxyacetone phosphate, it is very reactive and plays a role in AGE formation adapted from (Miller et al., 2003).*

(Hipkiss et al., 1995) showed that L-carnosine readily reacts with sugars to form a brown solution, indicatative of the Maillard reaction and the glycation of L-carnosine. They also showed that in a mixture containing the peptide α-acetyl-lysine-histidine amide, lysine is readily glycated by deoxyribose, but in the presence of L-carnosine, the glycation to lysine is decreased. This is thought to be due to L-carnosine acting as a sacrificial peptide, as L-carnosine resembles the favoured glycation site on peptide chains, which is a lysine adjacent to a L-histidine (Hipkiss et al., 1995). The addition of

L-carnosine to the reaction between the sugar deoxyribose and α-crystallin, causes a decrease in the cross-linking of α-crystallin (Hipkiss et al., 1995). L-carnosine has also been shown to react with AGEs to form carnosinylated proteins, this in turn decreases the number of free carbonyl groups and may prevent the AGE from reacting further (Brownson and Hipkiss, 2000), as a result this may aid in degradation as heavily oxidised proteins inhibit proteolysis (Hipkiss, 1998).

The fate of carnosinylated proteins is unknown, however, unlike glycated lysine, which is mutagenic, glycated L-carnosine has been shown not to be (Hipkiss et al., 1995), furthermore carnosinylated proteins may form the age-pigment lipofuscin, which has low reactivity, which is common in muscle and brain (Brownson and Hipkiss, 2000). L-carnosine has also been shown to increase proteolysis of slow-turn over proteins in fibroblasts, thus L-carnosine could actively increase proteolysis (Hipkiss and Brownson, 2000). Evidence for an increase in proteolysis activities in the presence of L-carnosine is that cells exposed to amyloid-β, have an increase in glucose consumption, as proteolysis is an energy demanding mechanism (Hipkiss et al., 1998). L-carnosine has also been shown to reverse protein-aldehyde cross linking, thus enabling rejuvenation (Boldyrev et al., 1999).

The addition of L-carnosine to rat brain endothelial cells has been shown to protect against the toxic effects of albumin-AGE, this has also been shown to be the case in CHO, where L-carnosine protects the cells from lysine-deoxyribose toxicity (Hipkiss, 1998). To conclude reactive sugars result in the formation of AGE products which results in damaged proteins and protein aggregation. L-carnosine has been shown to act as a sacrificial peptide and thus decrease protein glycation this in turn protects cells from the toxic effects of AGEs.

A.2.11. L-Carnosine has potential in the treatment of age related diseases

Ageing is a multifactorial process of which one of the causes is an increase in oxidants, such as ROS and an increase in protein mis-folding; this can cause age-related diseases such as neurodegeneration, cardiovascular disease, cancer, sarcopenia, type 2 diabetes, cataracts and many others (Kohen et al., 1988).

Neurodegenerative diseases are typically associated with an increase in mis-folded proteins and a dysfunction in proteolysis. This leads to amyloidβ plaques (Aβ) in Alzheimer's disease (AD), Lewy bodies in Parkinson's and inclusion bodies in Huntington's disease. In addition, neurodegeneration is often associated with mitochondrial dysfunction, which is caused by ROS and RNS but can also result in further production of ROS and RNS which can increase protein mis-folding further (Bellia et al., 2011; Calabrese et al., 2005). The body has natural defences against oxidative stress, such as the non-enzymatic antioxidants, vitamin C, vitamin E and glutathione, and enzymatic antioxidants, such as Cu,Zn-SOD, glutathione peroxidise and catalase. Nonetheless, the production of antioxidants exceeds the body's natural defences, resulting in cellular damage (Alpsoy et al., 2011).

AD is associated with increased levels of free zinc ions, which have been shown to promote Aβ aggregation and mitochondrial dysfunction (Corona et al., 2011). Even though zinc ions are

detrimental for the development of dementia as they are free radicals and will react with ROS to form more potent ROS, zinc and other transition metals, such as copper, are also required for normal brain development and function. For example, zinc is involved in gene expression, neurotransmission in memory, enzyme complexes and neuronal development (Matsukura and Tanaka, 2000; Mikami et al., 2006; Trombley et al., 2000). As a result zinc deficiencies result in Alzheimer-like cognitive problems (Corona et al., 2011).

L-carnosine concentrations are lower in AD patients in the CNS (Bellia et al., 2011). L-carnosine, homocarnosine and β-alanine, have been shown to protect cultured rat brain endothelial cells against the toxic Aβ(25-35) fragment, which was attributed to their ROS scavenging and metal chelation ability, but also their anti-glycation properties; SOD, which protects against ROS, protected cells but not to the same extent (Preston et al., 1998). Furthermore, *in vivo* studies using 3XTg-AD mice, which express mutant Aβ and develop an AD phenotype, demonstrated that L-carnosine supplementation decreased Aβ plaque formation and increased the long term memory of AD mice. 3XTg-AD mice were also shown to have a decrease in the activity of Complex 1, 2 and 4 of the mitochondria. Upon L-carnosine treatment, this restored Complex 1 activity to control levels and interestingly increased Complex 2 and 4 activities above that of control levels. This ability was attributed to the metal chelation properties and ROS scavenging properties of L-carnosine. (Corona et al., 2011). Furthermore it has been suggested that L-carnosine can directly prevent the formation of Aβ plaques (Figure A.2.6) (Hipkiss et al., 1998).

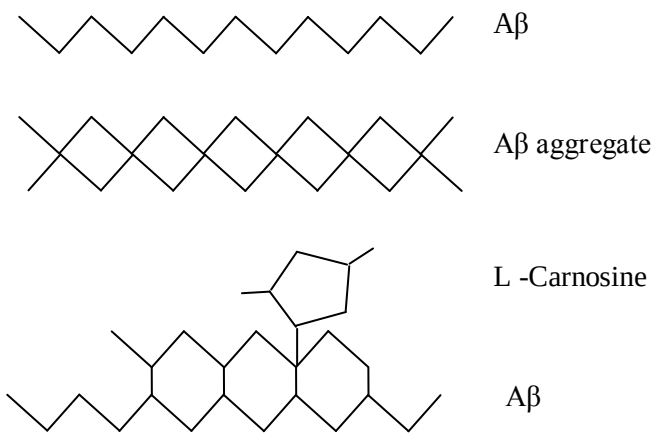


Figure A.2.6: Representing potential mechanism of action of how L-carnosine prevents Aβ aggregation*. L-carnosine could react with Aβ, this would prevent it from further reacting with its self and with other proteins, which would result in protein aggregation which is detrimental to the cell, adapted from (Hipkiss et al., 1998).*

Type 2 diabetes is an age-related disease also associated with obesity. Uncontrolled type 2 diabetics have hyperglycaemia, hyperlipidaemia and insulin insensitivity. Diabetic patients also have an increase in AGEs, which contribute to the development of secondary diseases, such as cataracts, hypertension

and cardiovascular disease (Aldini et al., 2011; Hipkiss et al., 1998). Increased L-carnosine levels, caused by an allele variation, which causes a decrease in carnosinase, has been associated with a decrease in diabetes incidence (Sauerhöfer et al., 2007).

Mice have low levels of serum carnosinase compared to humans, thus transgenic mice expressing human carnosinase have higher levels of carnosinase in the serum and thus lower levels in of L-carnosine. (Sauerhöfer et al., 2007) showed that diabetic mice developed hyperglycaemia at 8-10 weeks, mice expressing human carnosinase developed hyperglycaemia at 6-7 weeks, and mice supplemented with L-carnosine developed hyperglycaemia at 17-20 weeks. These data also correlated with insulin levels, where transgenic mice expressing human carnosinase had the highest levels.

Zucker rats, which have a defect in leptin receptors have a diabetic phenotype (hyperlipidaemia, hypertension and renal disease). The supplementation of D and L-carnosine decreased the levels of triglycerides, cholesterol and insulin compared to control Zucker rats, they also had a decrease in blood pressure and kidney damage. Moreover, supplementation with D and L-carnosine in Zucker rats decreased cross-linking of collagen. These beneficial effects of D and L-carnosine can be attributed to an increase in carnosine-HNE adducts in the urine, indicating that D and L-carnosine remove toxic products from the body. It is interesting to note that the antioxidants, vitamins C and E, have no effect on Zucker rats suggesting that it is the anti-glycation properties and the ability to remove of LPO products that may be the relevant mechanisms. L-carnosine has therefore been suggested as a treatment of diabetes (Aldini et al., 2011).

L-carnosine has been shown to prevent the protein aggregation of α-crystallin, which is a protein chaperone in the lens of the eye that prevents cataracts (Yan and Harding, 2006); L-carnosine is found in eye lenses at 25 μM, but this level decreases with age. L-carnosine in the form of N-acetylcarnosine has been shown to treat cataracts by decreasing opaqueness of the lens and improving the eye sight of older adults (Babizhayev et al., 2009; Wang et al., 2000), which can be attributed to its anti-glycation and ROS scavenging properties. Currently L-carnosine eye drops are used in the treatment of cataracts (Gariballa and Sinclair, 2000).

Parkinson's patients, who have been treated with L-carnosine in addition to their normal medication, have been shown to have improved neurological symptoms, decreased oxidation of proteins and lipids in the blood serum and also increased Cu,Zn-SOD activity in eyrthrocytes compared to control patients. Moreover, L-carnosine supplementation has been shown to be beneficial to patients who have suffered ischemic brain stoke (Boldyrev, 2012). Overall these findings suggest that L-carnosine supplementation could be used in the treatment of age-related diseases, primarily on account of its ROS scavenging properties, along with its ability to prevent macromolecule damage from reactive sugars and LPO products. In conclusion, L-carnosine has been shown to treat a range of age-related disease in model organisms and humans, such as dementia, diabetes and cataracts. There appears to be multiple mechanisms of action such as ROS scavenging, metal chelation, however as other CRC do not have similar affects the main mechanism seems to be attributed to L-carnosine ability to act as a

sacrificial peptide for glycation and is able to form adducts with LPO products, such as 4-HNE, to make L-carnosine-HNE.

If L-carnosine were to be used routinely in the treatment of age related diseases, serum carnosinase, which rapidly breaks down L-carnosine, would be a potential problem. Thus CRC which are more resistant to serum carnosinease than L-carnosine could offer useful alternatives (Bellia et al., 2011). D-carnosine, which has a different spatial orientation than L-carnosine is also resistant to serum carnosinase; D-carnosine was shown to be taken up from the intestine of rats and decrease LPO (Aldini et al., 2011). Alternatively, β-alanine supplementation has been shown to increase L-carnosine levels in muscle (Culbertson et al., 2010).

(Fontana et al., 2002) showed that sulphonamide pseudodipeptide of L-carnosine, which has a sulphonamide junction rather than a peptide bond (Figure A.2.7), referred to as tauryl-histidine, protected tyrosine from peroxynitrite nitration. This pseudodipeptide is resistant to carnosinase and could potentially be used in the treatment of age related disease.

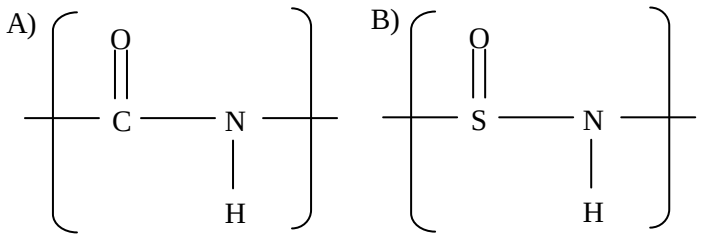


Figure A.2.7: Diagram of a peptide bond (A) and a sulphonamide junction (B). *The sulphonamide pseudodipeptide of L-carnosine has a sulphonamide junction rather than a peptide bond, making it resistant to carnosinase, adapted from (Fontana et al., 2002).*

At the same time though, metal ions, ROS and LPO products do play a role in normal cellular function, the long term treatment with L-carnosine or CRC may have detrimental effects. For instance the absence of serum carnosinase, results in carnosinemia causing mental retardation (Hipkiss, 2000) .

A.2.12 References

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A.3. Vector maps and sequences

Where restriction sites in red are single cutters and those in blue are double cutters.

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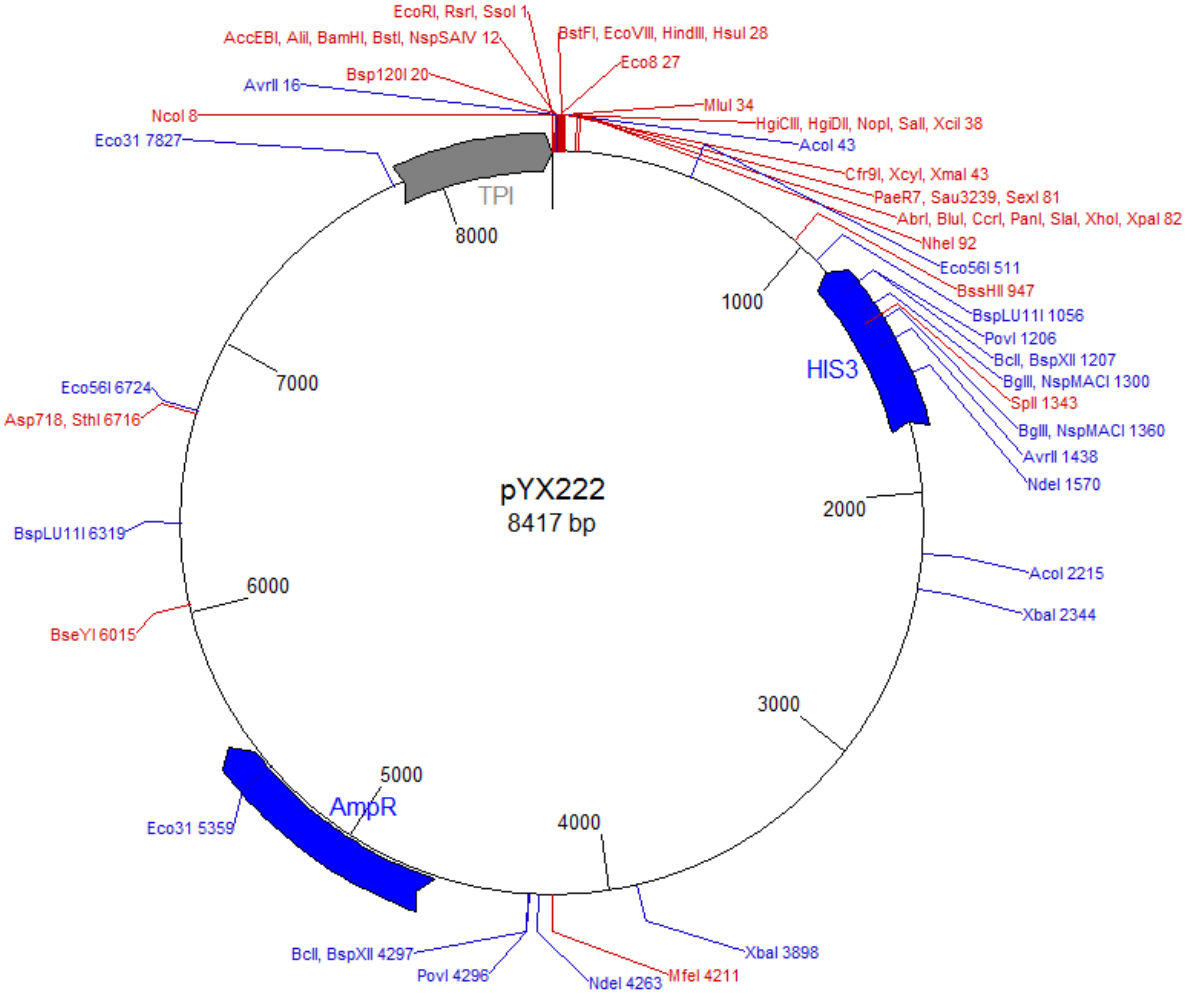

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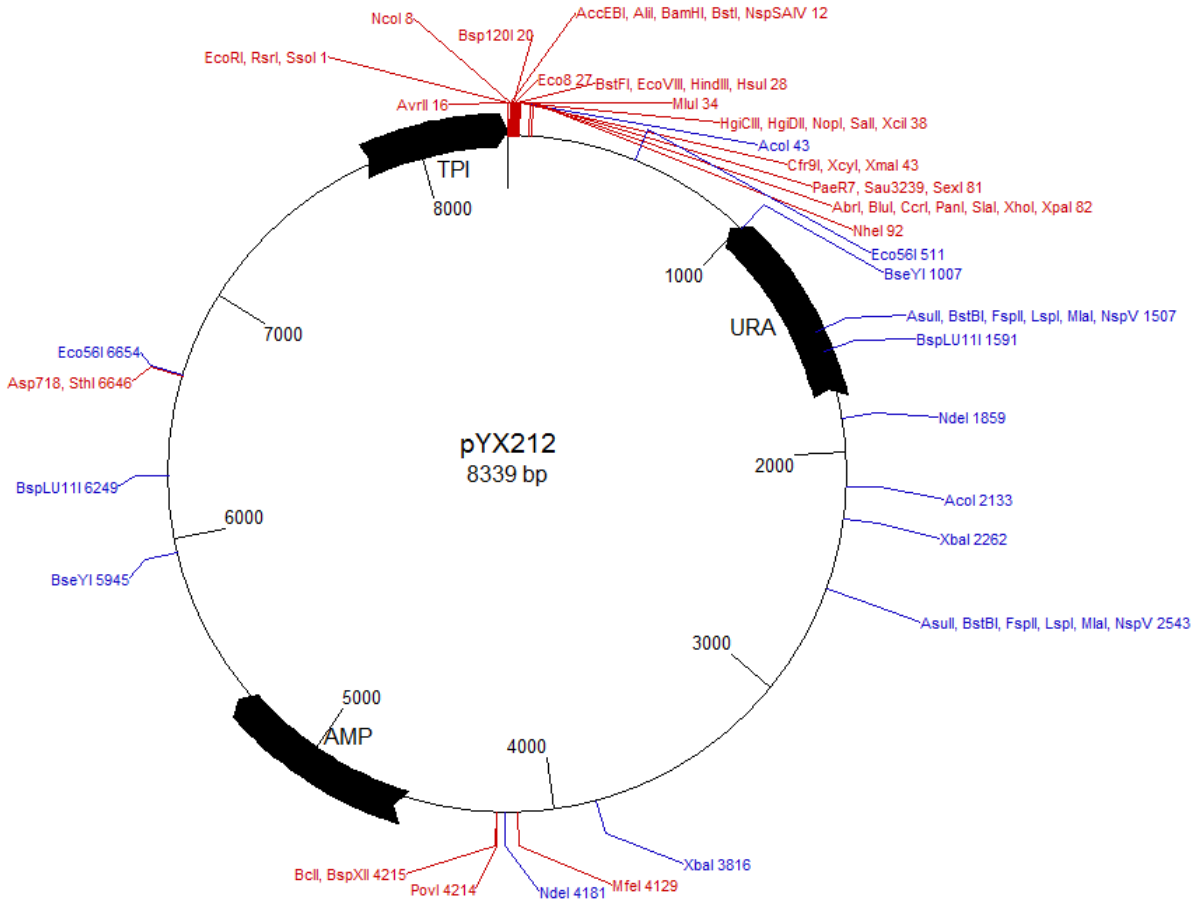

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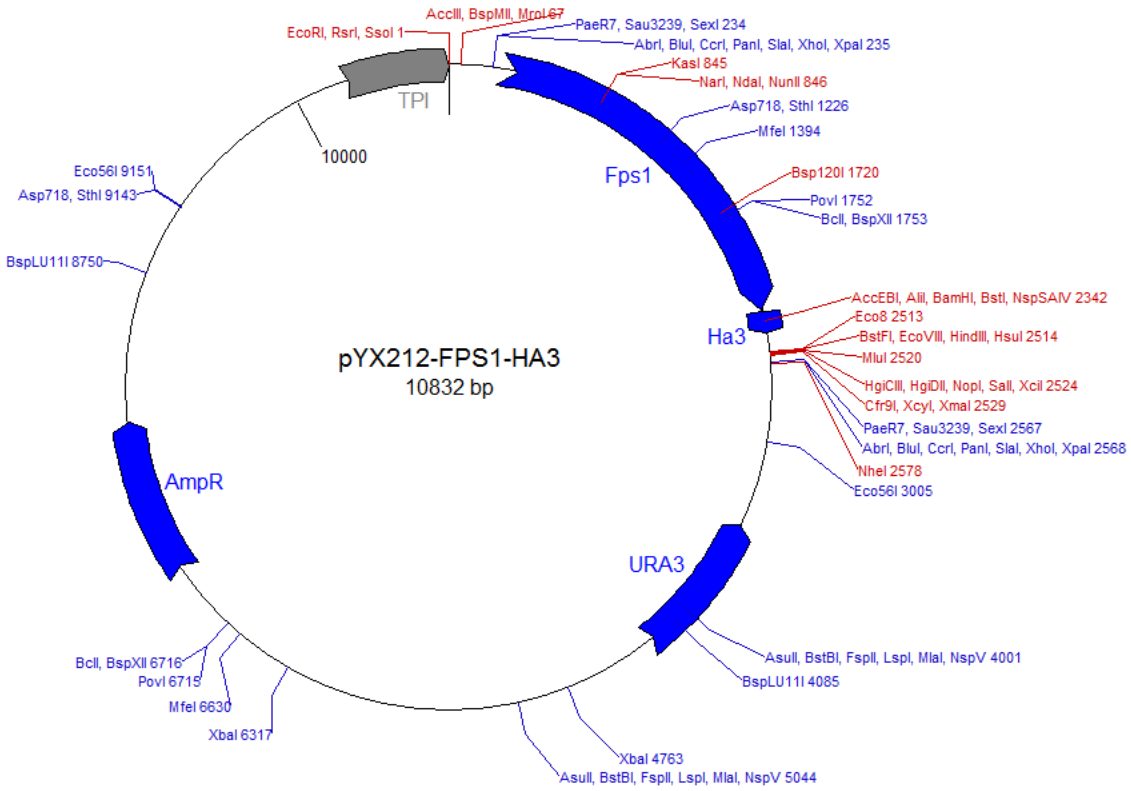

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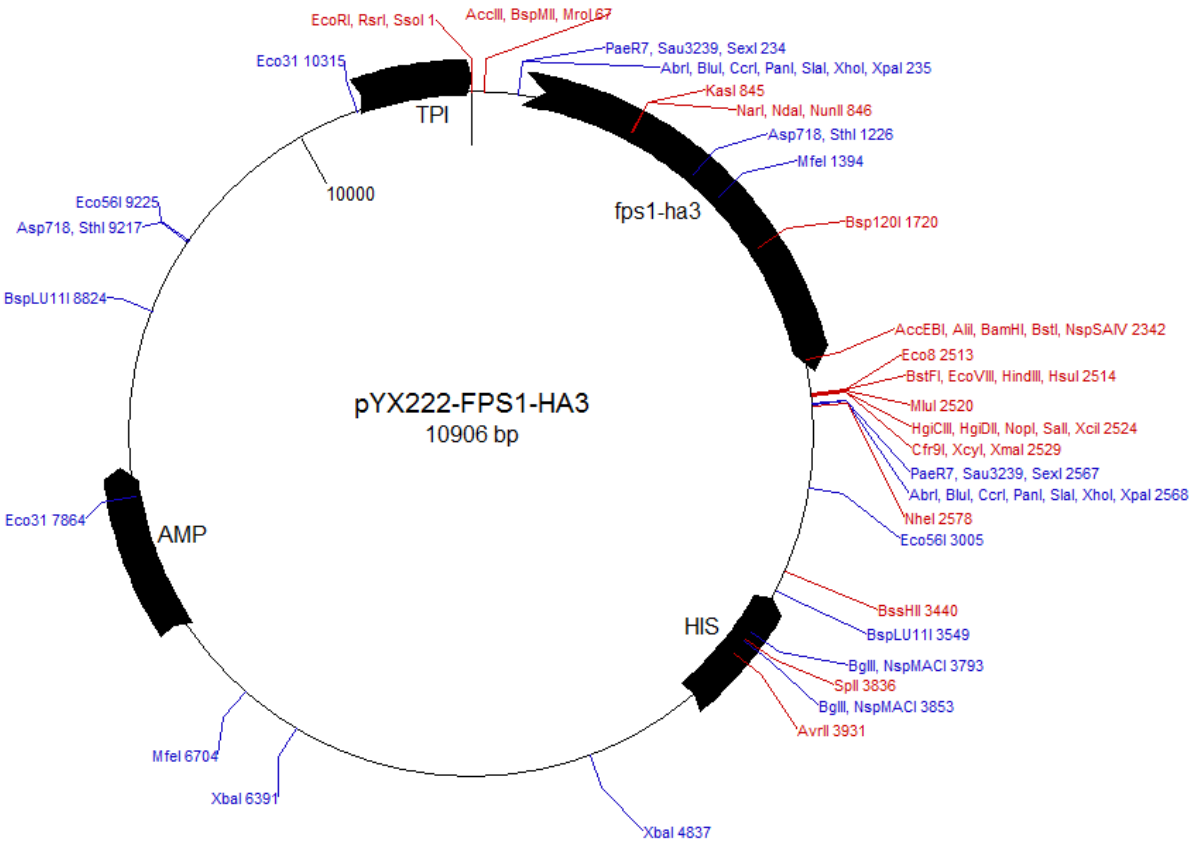

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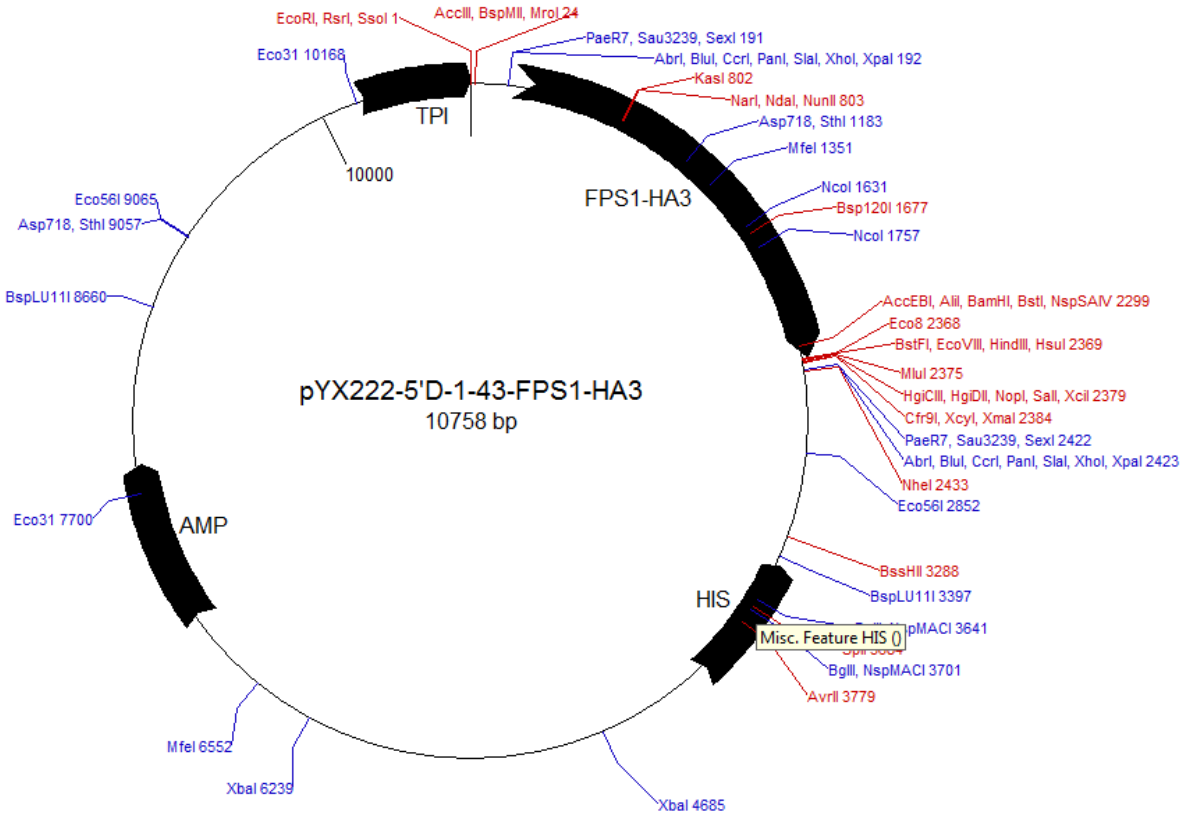

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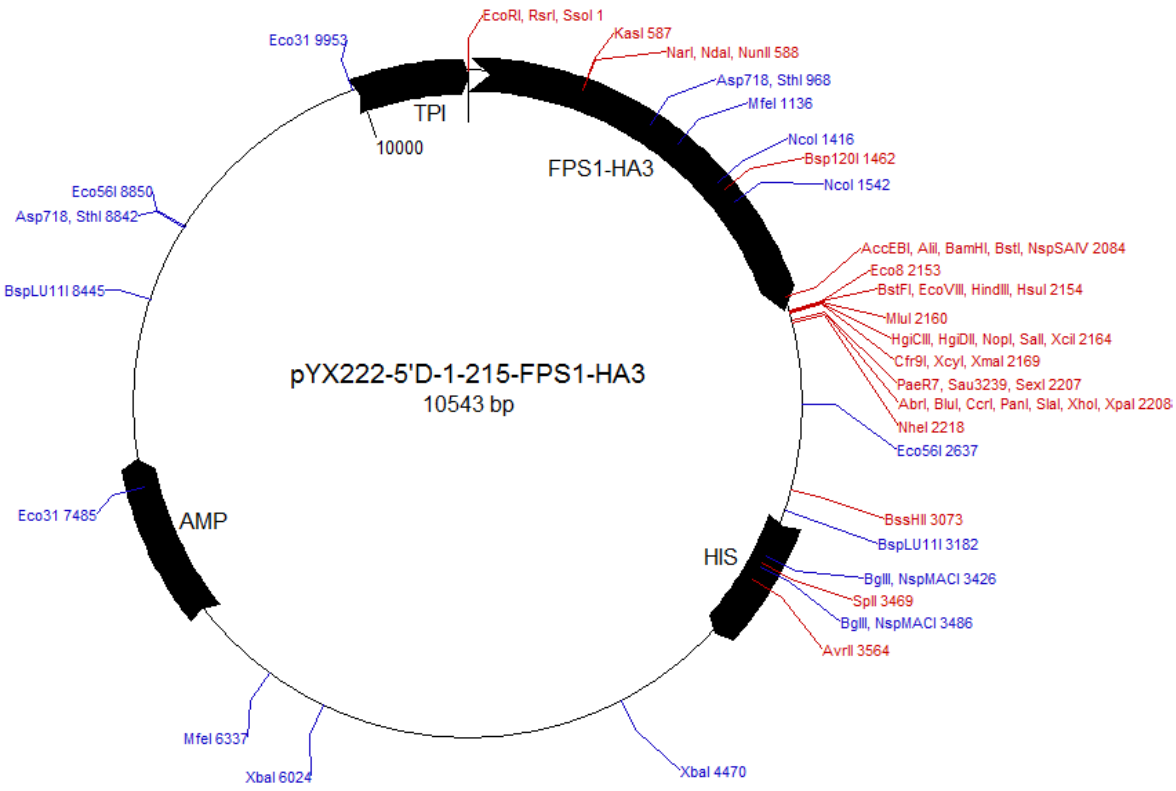
A.3.6: pYX222-5´Δ-1-215-FPS1-*HA*$_3$



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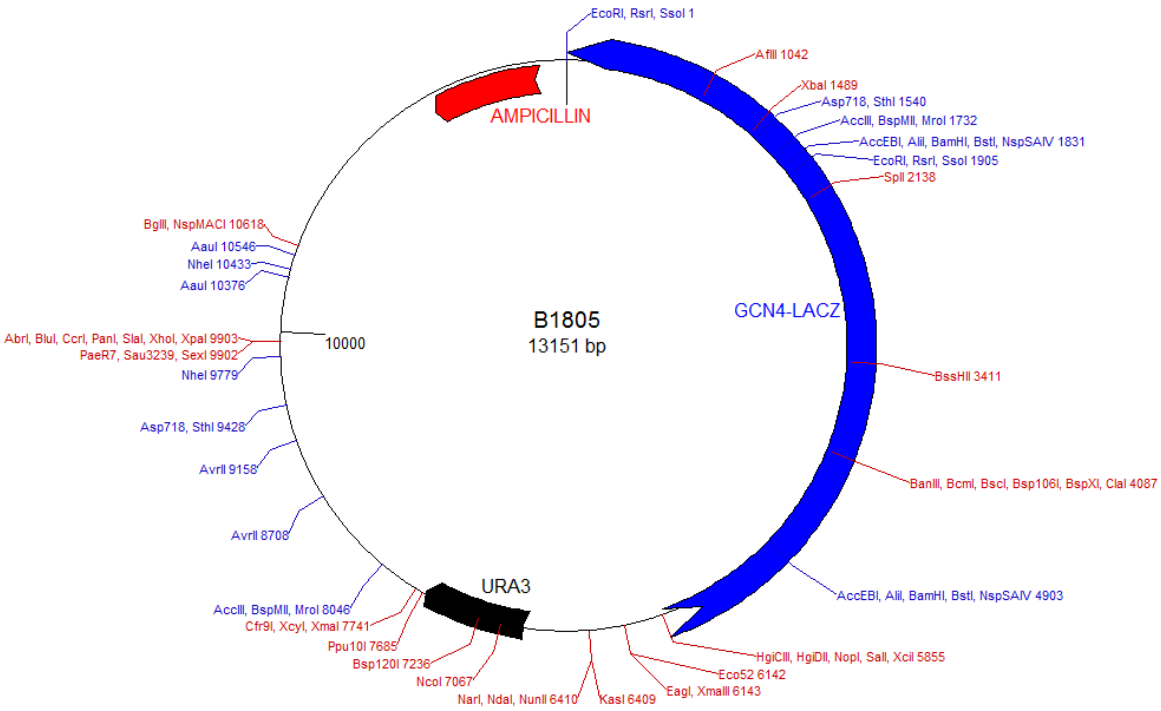

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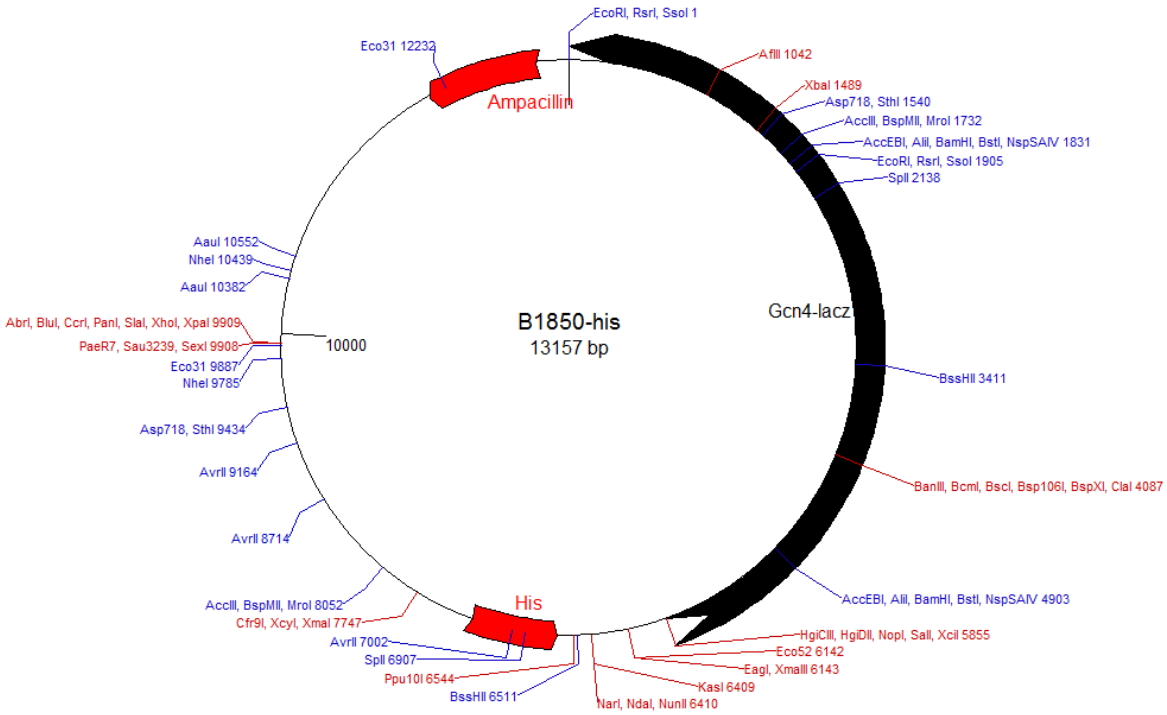

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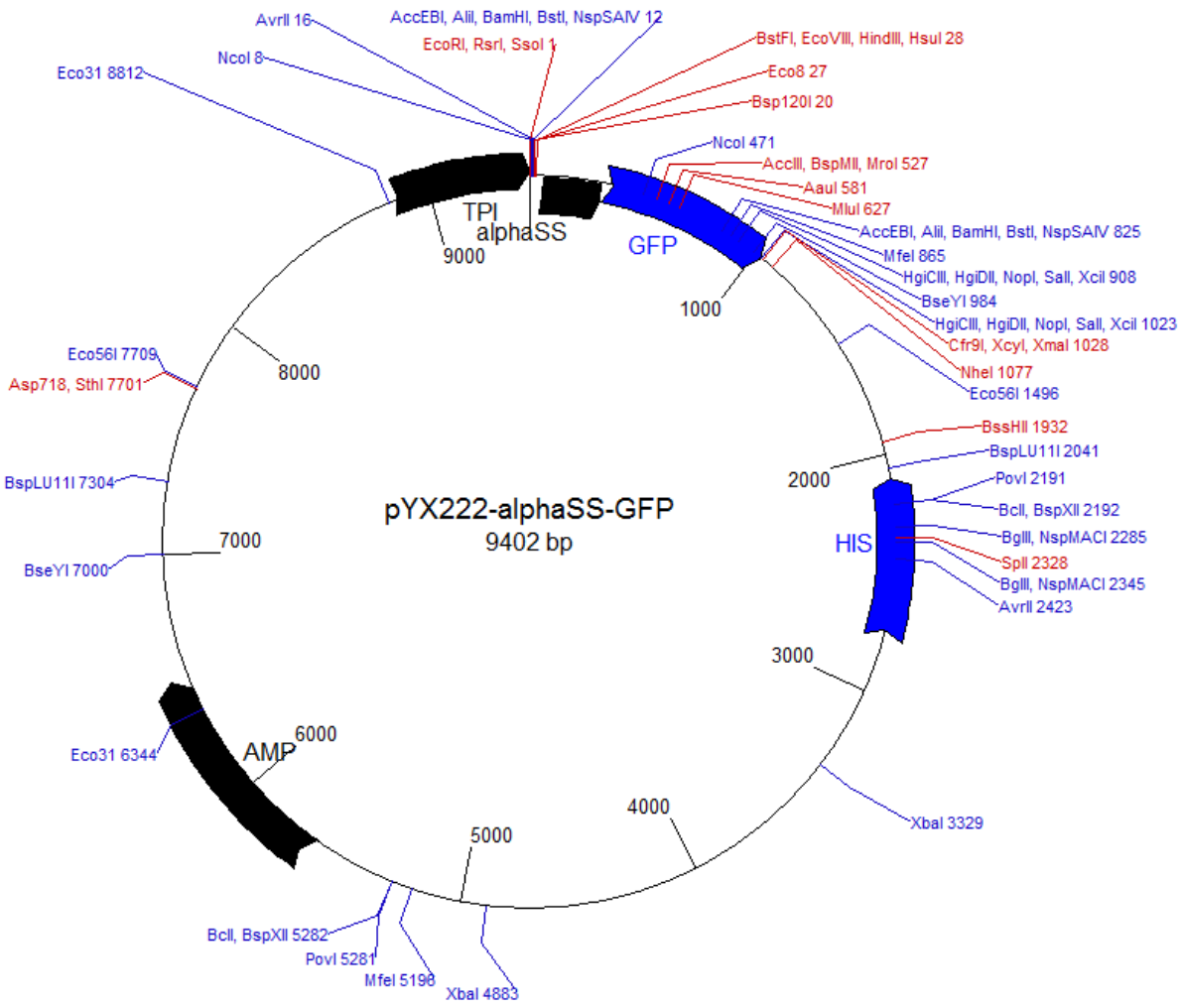

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A.4. Publications and presentations resulting from this research

Publications:

Hipkiss, A.R., Cartwright, S.P., Bromley, C., Gross, S.R., Bill, R.M., 2013. Carnosine: can understanding its actions on energy metabolism and protein homeostasis inform its therapeutic potential? Chem Cent J 7, 38.

Cartwright, S.P., Bill, R.M., Hipkiss, A.R., 2012. L-carnosine affects the growth of Saccharomyces cerevisiae in a metabolism-dependent manner. PLoS One.;7(9):e45006.

Darby, R.A.J., Cartwright, S.P., Dilworth, M.V., Bill, R.M., 2012. Which yeast species shall I choose? Saccharomyces cerevisiae versus Pichia pastoris (review). Methods Mol. Biol. 866, 11–23.

Bawa, Z., Bland, C.E., Bonander, N., Bora, N., Cartwright, S.P., Clare, M., Conner, M.T., Darby, R.A.J., Dilworth, M.V., Holmes, W.J., Jamshad, M., Routledge, S.J., Gross, S.R., Bill, R.M., 2011. Understanding the yeast host cell response to recombinant membrane protein production. Biochem. Soc. Trans. 39, 719–723.

Presentations:

Translation UK, 2013, The University of Kent, Canterbury, UK: Understanding the mechanisms underpinning high-yielding membrane protein production in *S. cerevisiae.*

The 5th Midlands Biophysics Network Symposium, 2012, Aston University, Birmingham, UK: Understanding the mechanisms underpinning high-yielding membrane protein production in *S. cerevisiae.*

The 6th Recombinant Protein Production Conference, 2011, University of Natural Resources and Life Sciences, *Vienna*, Austria: Understanding the mechanisms underpinning high-yielding membrane protein production in *S. cerevisiae.*