Assessing the role of Hsp70 in prion propagation in *Saccharomyces cerevisiae*

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Declaration of Authorship

This thesis has not previously been submitted in whole or in part to this or any other University for any other degree. This thesis is the sole work of the author, with the exception of the generation of the Ssa1 mutant library, which was carried out by Dr. Harriet Loovers.

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List of abbreviations

ABC: ATP binding cassette

ADP: Adenosine triphosphate

AMP: Ampicillin

AOX: Alcohol oxidase

APS: Ammonium persulfate

ATP: Adenosine triphosphate

BSE: Bovine spongiform encephalopathy

CD: Circular dichroism

CHL: Chloramphenicol

CJD: Creutzfeldt–Jakob disease

CNS: Central nervous system

CTD: C-terminal domain

CWI: Cell wall integrity signaling

DNA: Deoxyribonucleic acid

DNTPs: Deoxynucleotide triphosphates

DTT: Dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

ER: Endoplasmic reticulum

5-FOA: 5-Fluoro-orotic Acid

GdnHCL: Guanidine Hydrochloride

GPI: Glycosyl phosphatidylinositol

HSE: Heat shock element

HSF: Heat shock factor

HSP: Heat shock protein

ISCMs: Inactivating stop codon mutations

KCL: Potassium chloride

LB: Luria- bertani

LDH: Lactate dehydrogenase

LiAC: Lithium acetate

MAP: Mitogen activated protein

MCAC: Metal chelate affinity chromatography

MGT: Mean generation time

NEF: Nucleotide exchange factor

NTD: Amino-terminal domain

OD: Optical density

ONPG: Ortho-Nitrophenyl-β-galactoside

OR: Oligopeptide repeat

PBD: Peptide binding domain

PCR: Polymerase chain reaction

PDR: Pleiotropic drug resistance

PEG: Polyethylene glycol

PEP: Phosphoenolpyruvate

PIC: Protease inhibitor cocktail

PK: Pyruvate kinase

PMSF: Phenylmethanesulfonylfluoride

PrD: Prion domain

PVDF: Polyvinylidene Fluoride

RF: Release factors

RNA: Ribonucleic acid

RPM: Rotations per minute

RT-PCR: Reverse transcript- Polymerase chain reaction

SBF: Swi4/6 cell cycle box-binding factor

SC: Synthetic complex

SDM: Site-directed mutagenesis

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SGD: Saccharomyces genome database

SNPS: Single-nucleotide polymorphisms

SSA: Stress-seventy superfamily

stH_20: Sterile water

T.S: Thermosensitive

TAE: Tris base, acetic acid and EDTA

TBS: Tris buffered saline

TeMED: Tetramethylethylenediamine

TF: Transcription factor

TPR: Tetratricopeptide domain

TSE: Transmissible spongioform encephalopathy

UPR: Unfolded protein response

UPRE: Unfolded protein response element

UPS: Ubiquitin proteosome system

YPD: Yeast dextrose medium

YPG: Yeast peptone glycerol

YPGAL: Yeast peptone galactose

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Summary

The term Prion (Proteinaceous infectious) was first described by Stanley Prusiner in 1982. Prions are infectious proteins and are responsible for many neurodegenerative diseases, collectively termed as transmissible spongioform encephalopathies, including; BSE, vBSE, scrapie and CJD. Prions are also present in fungi. There have been a number of prion proteins discovered in the yeast *Saccharomyces cerevisiae*. Probably the most studied of these is the [*PSI*$^{+}$], which is the prion form of the protein Sup35, which is required for the release of nascent polypeptide chains from the ribosome during translation termination. Studies indicate that the *de novo* formation and propagation of yeast prions require the function of protein chaperones and co-chaperones. The heat shock proteins (HSP) such as Hsp40, Hsp70 and Hsp104, are essential for prion propagation in yeast. Hsp70 is a highly conserved protein composed of an N-terminal ATPase domain, a peptide-binding domain (PBD) and a C-terminal domain. In this study we describe a genetic screen that identifies an array of mutants within different domains of the major cytosolic Hsp70 chaperones of yeast, Ssa1-4 which impair the propagation of [*PSI*$^{+}$]. A majority of isolated mutants are located within the ATPase domain and have no significant affect on Hsp70 function. The PBD mutant Ssa1^{F475S}, which impairs [*PSI*$^{+}$] propagation also appears to make Ssa1 non-functional and may implicate Ssa1 in an array of cellular functions. We also highlight differences between the Hsp70 cytosolic Ssa family members with respect to prion propagation and Hsp70 function.

1.0 Mammalian prions

The term Prion (Proteinaceous infectious) was first coined by Stanley Prusiner in 1982 (Prusiner 1982a)**.** Prions are responsible for a class of neurodegenerative diseases termed as transmissible spongiform encephalopathies (TSE) (Prusiner 1982a). Bovine spongiform encephalopathy (BSE), Scrapie and Creutzfeldt-Jakob disease (CJD) are prion diseases that manifest in cows, sheep and humans, respectively (Prusiner 1998)**.** The most prominent symptom of CJD is progressive dementia whereas Scrapie and BSE generally display ataxic symptoms. Strong evidence lead researchers to the conclusion that prions are proteinaceous infectious particles that are devoid of nucleic acid.

1.1 PrP Protein

The *PRNP* gene encodes the PrP^{c} protein in mammals, which is expressed in the central nervous system (CNS). PrP is a cell membrane protein that is anchored by glycosyl phosphatidyl inositol (GPI) (Prusiner 1998). The normal function of this protein is not yet clear. Evidence suggests it may be involved in a range of functions such as sleep physiology, oxidative burst compensation, apoptosis, signal transduction and interactions with extracellular matrix (Mouillet *et al*., 2000). The PrP^{c} protein can be converted to form the prion form of the protein; PrP^{sc} (Prusiner 1998). PrP^{c} is typically composed of α-helical structure, soluble and highly sensitive to protease digestion, when converted to the prion form (PrP^{sc}) a portion of the α-helical structure is refolded into ß-sheet form and the protein becomes insoluble, is amyloid rich and becomes resistant to protease digestion (Prusiner 1998). These structural changes are also accompanied by physiochemical properties of PrP being altered. The amino acid sequence of PrP^{sc} corresponds to that encoded by the *PRNP* gene.

1.1.1 Prion Transmission

Prions can be established in three different ways. They can arise spontaneously, be inherited by way of a predisposing mutation or may be contracted by infection (Prusiner 1998). Sporadic conversion of human CJD occurs rarely, affecting one in a million annually. There are more than twenty mutations of the PrP gene known to cause inherited human prion diseases. A common polymorphism at codon 129 of the human PrP gene (*PRNP*), where either methionine (M) or valine (V) can be encoded, is a key determinant of susceptibility to sporadic and acquired prion diseases (Asante *et al*., 2002). These mutations can lead to aberrant behavior of the PrP protein. A common way for prions to appear is by contraction through infection. This is when the host is infected by TSE agents; this is how vCJD is established.

1.2 Fungal Prions

Prions are not exclusive to mammals; they are also present in other eukaryotes such as fungi. Studies revealed that the yeast *Saccharomyces cerevisiae* contains proteins that can spontaneously convert to prions. This was first observed by Brian Cox when he discovered a non-mendelian factor which he termed [PSI^+] (Cox 1965). Another non-mendelian element, [*URE3*], was also discovered during a genetic screen to isolate nitrogen metabolism mutants (Lacroute 1971, Aigle and Lacroute, 1975). For years researchers postulated over the molecular mechanisms of these non-mendelian elements until Reed Wickner proposed that [PSI^+] is the prion form of the Sup35 (translation termination factor) and [*URE3*] is the prion form of the Ure2 (regulator in nitrogen catabolism) (Wickner 1994). Mammalian prions are known to cause neurodegenerative diseases, yet it was unclear whether the presence of fungal prions were detrimental, beneficial or neutral. The [HET-s] prion is present in the

filamentous fungus *Podospora anserina,* and is involved in controlling a fungal somatic allorecognition process called heterokaryon incompatibility. Filamentous fungi grow as an interconnected network of filaments forming a syncytial structure. Fusion of somatic cells occurs between strains, which then leads to the formation of vegetative heterokaryons (Glass and Kaneko, 2003). Organisms that form vegetative heterokaryons display genetic systems allowing them to recognize self from non-self. *P. anserina* possesses a set of genes termed *het* genes (Saupe 2000). The het-s gene is a member of this family and exists in two polymorphic variants; het-S and het-s and only heterokaryons in which both nuclear components have compatible *het* gene constitution are viable. The Het-s protein can convert into the prion [Het-s] and this incompatibility reaction only occurs when the protein is in its prion state. This suggests that the presence of fungal prions may be beneficial for carrying out natural cellular roles.

It is debatable whether the presence of yeast prions are advantageous or a disease of yeast. Studies have proposed that the presence of [*PSI*$^+$] is an advantage to cells when surviving stress (Eaglestone *et al*., 1999) and for evolvability (True *et al*., 2004, True and Lindquist, 2000). However these studies were all preformed by measuring growth, but yeast spends most of its time in stationary phase. Therefore, studies were undertaken to examine the distribution of the prions [*PSI*$^+$ and [*URE3*] in seventy wild-yeast strains (Nakayashiki *et al*., 2005). It was postulated that even if prions were of a mild disadvantage they should be found in the wild. No wild-type strains were found to contain [*PSI*$^+$] or [*URE3*], however the prion [*PIN*$^+$] was isolated in eleven strains. Similarly previous studies found [*PSI*$^+$] to be absent from nine clinical isolates (Resende *et al*., 2003) and two industrial strains of *S. cerevisiae* (Chernoff *et*

al.,, 2000). This may indicate that prions are detrimental to there host as if they were advantageous distribution in wild yeast would be expected.

1.2.1 Properties of yeast prions

Prions are infectious proteins and horizontal transmission in yeast and filamentous fungi occurs by cytoplasmic mixing, which occurs by mating parental strains even if nuclei do not fuse mating of filamentous fungi occurs when two colonies of filamentous fungi grow towards each other and their hyphae fuse to allow cytoplasmic mixing. Along with transmission of prions during cytoplasmic mixing, viruses may also be transferred. In order to distinguish prions from viruses, three genetic properties were assigned to self-inactivating prions that are not found in nucleic acid replicons (Wickner 1994). The first genetic property that a prion must possess is reversible curing. If a prion can be cured from a yeast strain, it should be able to arise again *de novo* as the protein capable of undergoing the prion change is still present and could spontaneously covert into the prion form. In contrast, if a virus is cured, it should not reappear again, unless reintroduced from the outside. The second genetic property prions must display is that the overproduction of the protein must increase the frequency with which the prion arises. One would expect that by simply increasing the concentration of the protein capable of undergoing the prion change, the frequency of which the prion forms *de novo* would also increase. In contrast there are no chromosomally encoded proteins whose overproduction will increase the frequency in which nucleic acid replicons arise *de novo*, in fact they will not arise *de novo* at all. The third genetic criteria that a prion protein must possess is that the gene for the protein required for the propagation of the prion, and the phenotype when the prion is present is similar to that of a mutation of the gene for the protein. For prions

that simply inactivate the protein function, similarities in phenotypes between presence of the prion and mutations of that protein would be expected, for example, a null mutant for the protein should have the same phenotype as a strain that contains the prion form of protein (if the prion is an inactive form of the protein) (Wickner 1994**).** Both **[***PSI*$^{+}$] and [*URE3*] both fit the genetic criteria needed to classify them as prions.

1.2.2 The [*PSI*$^{+}$] prion

As mentioned above [*PSI*$^{+}$] is the prion form of the translation termination factor Sup35.Termination of protein synthesis occurs when the stop codons (TAA, TGA or TAG) of the mRNA enters the ribosomal A site. Stop codons are recognized by the class I release factors (RF), which also promote the hydrolysis of ester bonds in peptidyl t-RNA in the peptidyl transfer center of the large ribosomal subunit (Nakamura and Ito, 2003). In eukaryotes the eRF1, encoded by the *SUP45* gene, recognizes all three stop codons (Kisselev *et al.* 2003). In eukaryotes translation termination also requires the eRF3 (Stansfield *et al*., 1995), which is encoded by the *SUP35* gene. The GTPase activity of eRF3 couples codon recognition and a peptidyl RNA hydrolysis mediated by eRF1 to ensure rapid and efficient peptide release (Salas-Marco and Bedwell, 2004). The structure of Sup35 can be divided into three regions; the N-terminal, which is 130 amino acids long, which contains a prion domain (PrD), this domain is required for the formation and propagation of prions and is typically high in Glutamine and Aspargine residues, a highly charged middle domain and the C-terminus, which carries out the translation termination function. The PrD contains five imperfect regions of an octapeptide sequence YQQYNPQGG similar to the octapeptide PHGGGWGQ repeat present in the PrP protein. Deletion of

these repeats interferes with [*PSI*$^+$] propagation (Ter-Avanesyan *et al*., 1994) and addition of repeats leads to an increase in frequency of [*PSI*$^+$] appearance (Liu and Lindquist ,1999). Similarly, adding repeats in PrP can cause inherited CJD (Owen *et al*., 1989) but is not necessary for scrapie propagation (Flechsig *et al*., 2000). Aggregation of Sup35 in [*PSI*$^+$] cells causes nonsense suppression due to the reduced pool of functional Sup35 (inefficient translation termination) (Figure 1.1). Strains containing the *ade2-1* nonsense allele cannot grow without adenine and are red when grown on limiting amounts; this red color is due to the accumulation of a pigmented substrate of Ade2. Partial suppression of the *ade2-1* nonsense allele by [*PSI*$^+$], which also requires the weakly UAA suppressing tRNA *SUQ5*, allows growth on media lacking adenine and eliminates pigmentation (Cox 1965**).** In other words, this system allows the detection of the presence of [*PSI*$^+$], if the prion is not present, the strain will grow as red colonies and if the prion is present cells will grow as white colonies on limiting adenine.

1.2.3 [*URE3*]

When yeast has good nitrogen source e.g. ammonia or glutamine, it turns off synthesis of enzymes and transporters needed for assimilating poor nitrogen sources such as proline and allonate. The Ure2 protein mediates this process, known as nitrogen catabolite repression. It represses genes that express enzymes and transporters needed by yeast cells in the utilization of poor nitrogen sources in the presence of a good nitrogen source by sequestering the transcription factor Gln3 and Gat3/Nil1 in the

cytoplasm (Beck and Hall, 1999, Cox *et al*., 2000), which prevent their entry into the

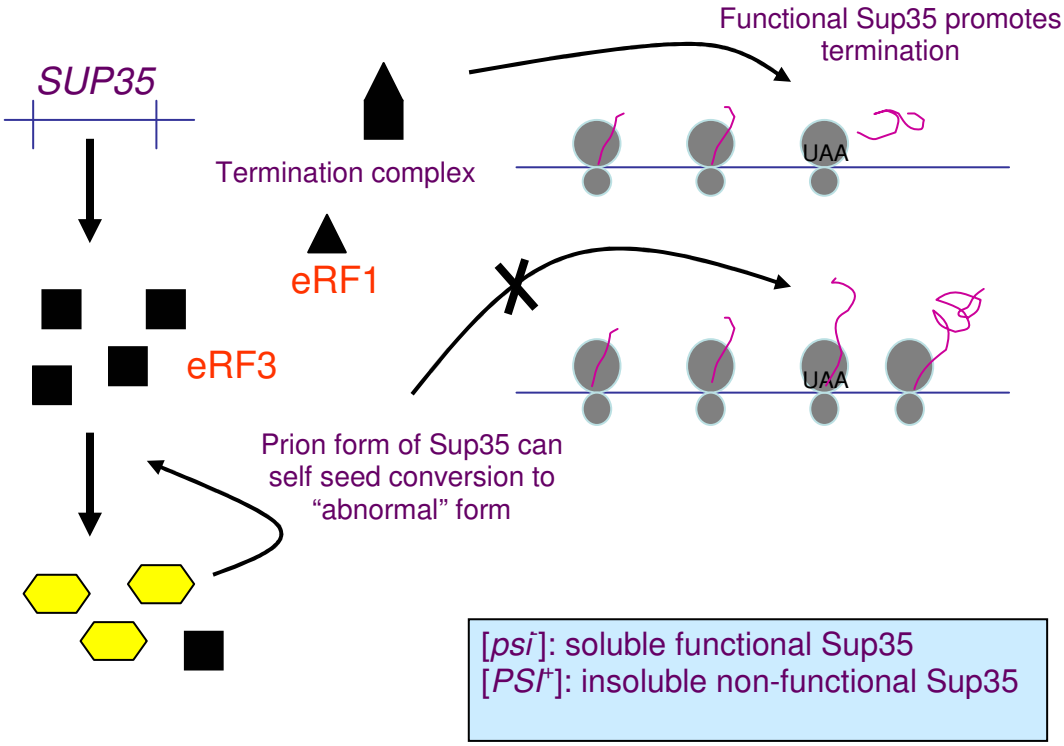


Figure 1.1 Spontaneous formation of [*PSI*$^+$]. In *S.cerevisiae,* the *SUP35* gene encodes the protein Sup35, also known as eRF3 (eukaryotic release factor) (black squares). Under normal circumstances, eRF3 binds to another release factor (black triangle); eRF1 to form the termination complex. The termination complex is responsible for the correct release of nascent polypeptides from the ribosome post translation. However, the Sup35 protein can undergo spontaneous conversion to a prion form of the protein (yellow hexagon). Once the prion form is established, it can then recruit and convert native Sup35 into the prion form. The termination complex is unable to form, as the prion form of Sup35 is unable to find with eRF1, which results in insoluble non-functional Sup35 unable to assist in the release of nascent polypeptide chains from ribosome.

nucleus. [*URE3*] is a nonchromosomal mutation that was isolated in a genetic screen, to identify nitrogen metabolite genes, that gave rise to Ure2 mutants (Lacrout 1971).The [*URE3*]element displays the three genetic criteria in order for it to be classified as a prion (Wickner 1994). Firstly it is cured by millimolar concentrations of guanidine hydrochloride, but [*URE3*] can arise post curing, overproducing the Ure2 protein increases the frequency of [*URE3*] *de novo* generation and the [*URE3*]

phenotype is similar to that of loss of function mutations in Ure2 (Wickner 1994). Ure2 is a two-domain protein, containing an N-terminal (1-65 amino acids) and a C terminal (66-354 amino acids). Overproduction of the N-terminal alone is sufficient to induce [*URE3*] appearance at a rate of approximately 100-fold higher than overexpression of the full length Ure2 protein (Masison and Wickner, 1995). In contrast, the C-terminal domain is sufficient for carrying out nitrogen regulation (Masison *et al.,* 1997). In addition, the prion domain (PrD) is sufficient to propagate [*URE3*] in complete absence of the C-terminal domain (Masison *et al*., 1997). Similarly to Sup35, the Ure2 PrD is composed of 40% aspargine residues and deletion of a run of these residues diminishes the ability of overproduced Ure2 to induce prion formation (Maddelein and Wickner, 1999).

1.2.4 [*PIN*$^+$]

Although Sup35 induces the *de novo* formation of the [*PSI*$^+$] (Chernoff *et al*., 1993), it was noted that this was a strain specific phenomenon (Derkatch *et al*., 1997). In fact the ability to overexpress Sup35 to induce [*PSI*$^+$] appearance was proved to be dependent on a second nonchromosomal agent termed [*PIN*$^+$] (*PSI* inducibility) (Derkatch *et al*., 1997). Subsequently, an examination to isolate potential prions by analyzing proteins containing regions rich in N/Q residues, resulted in the discovery of the Rnq1 protein, which can be found in an aggregated state in some strains and a soluble state in others (Sondheimer and Lindquist, 2000)

The function of Rnq1 is unknown. [*PIN*$^+$] proved to be the prion form of the Rnq1 protein; overexpression of Rnq1 increases the frequency with which [*PIN*$^+$] arises and the propagation of [*PIN*$^+$] is dependent on the presence of Rnq1 (Derkatch *et al.,*

2001). The [*PIN*$^+$] prion is so far the only prion to be isolated in natural isolates of *S.cerevisiae.* Data so far suggests that [*PIN*$^+$] prion aggregates act as an imperfect template on which Sup35 molecules misfold and assemble into infectious prion aggregates. This procedure, termed "cross-seeding", occurs only at the initial step of *de novo* formation of [*PSI*$^+$] and subsequently the two proteins stably co-aggregate (Bagriantsev and Liebman, 2004). There is also evidence for co-localization during initial cross-seeding (Derkatch *et al.,*2004, Salnikova *et al.,* 2005). Furthermore, yeast-two hybrid studies and co-immunoprecipitation analysis have detected interactions between soluble forms of Rnq1 and Sup35 *in vivo* (Tuite *et al.,* 2008).

1.2.4 Discovery of new yeast prions

Since the discovery of [*PSI*$^+$], [*URE3*] and [*PIN*$^+$] many genetic screens have gone underway to search for novel prions in yeast. Recently an unbiased screen identified a possible ten new prions whose overexpression facilitated the *de novo* appearance of [*PSI*$^+$] (Derkatch *et al*., 2001). As Ure2 was isolated in this screen as facilitating the *de novo* appearance of [*PSI*$^+$], it was postulated that some of these proteins might in fact be prions themselves, prions include Cyc8, New1 and Swi1 (Table 1.1). Further screens have identified twenty-four potential new yeast prions (Alberti *et al*., 2009). [*OCT*$^+$] is the prion form of the protein Cyc8 (Patel *et al*., 2009).The cyc8-Tup1 global transcriptional repressor complex (Smith and Johnson, 2000) forms one of the largest gene regulatory systems controlling over seven percent of yeast genes (Green and Johnson, 2004)**.** Analogous to other yeast prions, overexpression of the glutamine-rich region induces a heritable dominant [OCT$^+$] phenotype that is transmitted cytoplasmically and is dependent on the presence of the Cyc8 protein (Patel *et al*., 2009). The SWI/SNF evolutionary conserved ATP chromatin remodeling

complex plays an important role in transcriptional regulation (Martens and Winston, 2003). This complex regulates the expression of approximately 6% of total genes, either through gene activation or repression (Sudarsanam *et al.*2000).Similar to other prion proteins, Swi1 also contains an N-terminal domain rich in glutamine and aspargine residues. Swi1 aggregates in [*SWI*$^+$] cells but not in non prion cells and cells containing [*SWI*$^+$] show a partial loss of function phenotype (Du *et al*., 2008). The [*SWI*$^+$] prion is eliminated by the presence of guanidine or loss of the Swi1 protein and is dominantly and cytoplasmically transmitted (Du *et al*., 2008). The findings that the proteins Cyc8 and Snf1 can propagate as prions shows that prionization may lead to the mass activation or repression of yeast genes. The protein Mca1 is a metacaspase that regulates apoptosis in *S.cerevisiae*. Mca1 was isolated as a potential prion as it harbors a Q/N rich region in its N-terminal (Nemecek *et al*., 2009). Additionally, the prion forming domain of Mca1 contains several imperfect repeats of QQYG residues which is similar to repeats in the Sup35 protein (Nemecek *et al*., 2009).

1.3 Protein chaperones

As polypeptide chains are released from the ribosome, it may change its conformation more than once, before reaching its functioning biologically active structure. Protein folding is a structured process and evolution has created a simplified pathway to achieve this. Protein chaperones catalyze protein folding and prevent protein misfolding; normally many chaperones will be involved in this process. Another function of protein chaperones is to recover proteins that have not correctly folded or have become aggregated. Misfolding of proteins may be a consequence of the cell being exposed to environmental stresses such as heat shock, osmolarity and acidity.

Native protein	Cellular function	Prion form	
Sup35	Efficient translation termination	*[PSI⁺]*	**Genetically and biochemically confirmed**
Ure2	Utilisation of poor Nitrogen sources	*[URE3]*	
Rnq1	Unknown	*[PIN⁺]*	
Cyc8	Transcriptional co-repressor/co-activator	*[OCT⁺]*	**Strong genetic proof**
Mca1	Regulation of apoptosis	*[MCA]*	
Swi1	Regulation of transcription	*[SWI⁺]*	
New1	Unknown	*[NU⁺]*	

Table 1.1 Prions present in *S.cerevisiae*. There is strong genetic and biochemical data that the proteins Sup35, Ure2 and Rnq1 are capable of spontaneously converting into prion forms. According to strong genetic evidence, the more recently discovered Cyc8, Mca1, Swi1 and New1 are possible prion proteins, yet more biochemical analysis must be carried out.

Genetic screens have uncovered protein chaperones and cochaperones such as Hsp70, Hsp104, Hsp40, Sse1 and Sti1 as having an essential role in the propagation of yeast prions.

1.3.1 Hsp70

The Hsp70 family of chaperones is highly conserved forming part of the ubiquitous protein folding system (Wegele *et al.*, 2004). Hsp70 assists in protein folding and prevents aggregation of misfolded proteins due to heat shock. Further functions of Hsp70 include translocation of polypeptides across membranes and it also plays a role

in macromolecule assembly e.g. clathrin coats (Schlossman *et al.,* 1984). Hsp70 is a highly conserved protein, it is found in all organisms from archaebacteria to plants to humans. The bacterial Hsp70 (Dnak) shares fifty percent identity with eukaryotic Hsp70 proteins (Daugaard *et al*., 2007). Hsp70 also shows functional conservation e.g. *Drosophila* Hsp70 expressed in mammalian cells efficiently protects them against heat stress (Pelham 1984). *S.cerevisiae* encodes five organelle specific and nine cytosolic Hsp70's (Table1.2). Kar2 is a lumenal Hsp70, which functions in conjunction with the transmembrane signal inducer Ire1 and the transcription factor Hac1 as part of the unfolded protein response in the endoplasmic reticulum. Yeast cytosolic Hsp70s are composed of the; Ssa (stress seventy subfamily a) (Ssa1, Ssa2, Ssa3, and Ssa4) and Ssb (Ssb1 and Ssb2), Sse1, Sse2 and Ssz1.

1.3.1.1 Structure of Hsp70

Hsp70 contains a conserved N-terminal ATPase (44-kDa), a peptide-binding domain (18-kDa) and a C-terminal domain lid domain (10-kDa). Functioning of Hsp70 depends on the binding and hydrolysis of ATP; in an ATP bound state there is rapid substrate exchange between the chaperone and surrounding environment. When ATP is hydrolyzed to ADP, the chaperone is in a closed state, the substrate remains tightly bound to the peptide binding domain (Mayer *et al*., 2001) (Fig. 1.2). Optimal Hsp70 function is achieved by communication between the peptide-binding domain and ATPase domain; binding of the substrate stimulates ATP hydrolysis in proportion to the proteins binding affinity (Mayer *et al*., 2000). The ATPase domain of Hsp70 is composed of two lobes, which consists of four subdomains (IA, IB, IIA and IIB), lobe one consists of IA and IB and the other lobe is comprised of IIA and IIB. The lobes

Gene name	Location	Function
SSA1	Cytosol	Constitutively expressed molecular chaperone, that binds newly-translated proteins to assist in proper folding and prevent aggregation/misfolding
SSA2	Cytosol	Constitutively expressed molecular chaperone, that binds newly-translated proteins to assist in proper folding and prevent aggregation/misfolding
SSA3	Cytosol	Heat induced molecular chaperone, that binds newly-translated proteins to assist in proper folding and prevent aggregation/misfolding
SSA4	Cytosol	Heat induced molecular chaperone, that binds newly-translated proteins to assist in proper folding and prevent aggregation/misfolding
SSE1	Cytosol	Nucleotide exchange factors (NEF) for Hsp70 chaperones during protein refolding.
SSE2	Cytosol	Nucleotide exchange factors (NEF) for Hsp70 chaperones during protein refolding.
SSB1	Cytosol	The chaperone activity of Ssb1 is localized to the ribosome as part of the ribosome-associated complex RAC.
SSB2	Cytosol	The chaperone activity of Ssb2 is localized to the ribosome as part of the ribosome-associated complex RAC.
SSZ1	Cytosol	The activity of Ssz1p is localized to the ribosome where it functions as part of the ribosome-associated complex (RAC),
KAR2	Lumen of the ER	Binds to secretory and transmembrane precursor proteins to prevent their misfolding
LHS1	Lumen of the ER	Lhs1 localizes to the ER and shares 24% amino acid identity with the other ER HSP70 protein, Kar2. These two proteins reciprocally regulate each other. Lhs1 only participates in post-translational import.
SSC1	Mitochondria	*SSC1* encodes an essential chaperone that is the key component of the import motor sub-complex that mediates the transit of precursor proteins through the TIM23 complex.
SSQ1	Mitochondria	Required for assembly of iron/sulfur clusters into proteins at a step after cluster synthesis, and for maturation of Yfh1p, which is a homolog of human frataxin implicated in Friedreich's ataxia,
ECM10	Mitochondria	Plays a role in protein translocation.

Table 1.2 *S.cerevisiae* Hsp70 family: *S.cerevisiae* encodes five-organelle specific and nine cytosolic Hsp70's

are connected at their bases through subdomains IA and IIA. ATP binds to the ATPase domain in a cleft between IB and IIB (O'Brien *et al*., 1996, Jiang *et al*., 2005). The DnaK Hsp70 peptide-binding domain is composed of two anti-parallel ß-sheets that are comprised of four ß-strands each, which forms a ß-sandwich structure. The first ß-sheet is comprised of ß3, ß6, ß7 and ß8 and the second ß-sheet is comprised of ß5, ß4, ß1 and ß2 with four upward protruding loops (Zhu *et al*., 1996). The peptide-binding cavity is formed by ß-sheets one and two and loops L_{12} and L_{34}. The peptide-binding cavity is primarily hydrophobic, but the surface potential around the cavity is mainly negative, therefore charges in the substrate also contribute to binding affinity. Hsp70 favours peptides that contain an interior hydrophobic core flanked with basic residues, with an optimal substrate length of eight residues for ATPase stimulation (Jordan and McMacken, 1995). The C-terminal domain (CTD) lid is linked to the ß-sandwich PBD through a network of Hydrogen bonds and ionic interactions between residues in the lid and residues present in loops L_{45} and L_{12}. The CTD is not necessary for inter-domain communication but is necessary for peptide binding.

1.3.1.2 Hsp70 mechanism of action

The functioning of Hsp70 depends on the binding and hydrolysis of ATP. Information regarding the mechanism of action of Hsp70 is mostly acquired from studies utilizing *E.coli* Hsp70, DnaK, which has 50% identity to mammalian Hsp70 and thought to function in a similar manner. The function of Hsp70 is dependent on coordinated action from all three domains (ATPase, PBD and CTD). Substrate binding occurs in peptide binding cavity, which is formed by ß-sheets one and two and loops L_{12} and L_{34}

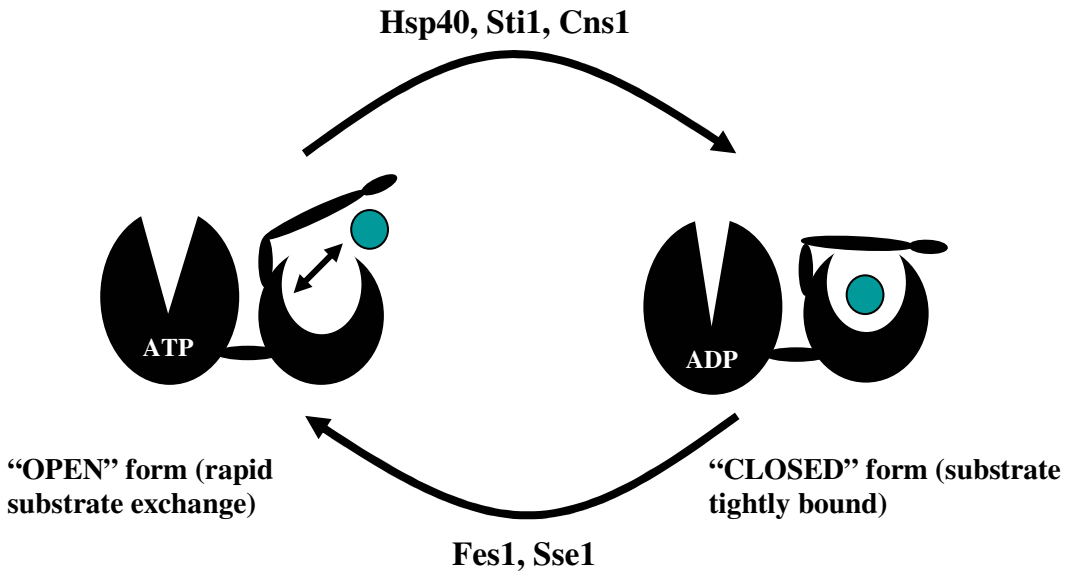


Figure 1.2 Schematic diagram showing the Hsp70 binding cycle. In an ATP bound state Hsp70 is in an open conformation allowing rapid substrate exchange. ATP is hydrolyzed to ADP causing Hsp70 to become into a closed state in which the substrate is tightly bound. The ATPase cycle is regulated by the presence of co-chaperones such as Hsp40, that stimulates ATP hydrolysis. The nucleotide exchange factors Fes1 and Sse1 assist in returning the Hsp70 into the "open" conformation.

in the PBD, the binding affinity and kinetics is dependent on the presence or absence of ATP in the ATPase domain. In an ATP bound state there is rapid substrate exchange in the PBD, conversely when in an ADP bound state the PBD has a higher affinity for its substrates and displays slower exchange rates of peptides (Figure 1.2). The major structural difference between the ATP and ADP bound states of Hsp70 is the position of the CTD. In an ADP bound state the CTD lid is positioned over the peptide binding cavity, therefore reduces the release of substrates (Han and Christen, 2003). Upon ATP hydrolysis, structural changes in the ATPase domain induce conformational changes in the PBD and CTD, which result in substrate trapping.

Correspondingly, substrate binding to the PBD alters its structure, which results in a signal being transmitted to the ATPase domain that stimulates ATP hydrolysis, therefore increasing substrate-binding affinity. Conversely, Hsp70 Nucleotide exchange factors (NEFs), promote the release of ADP, thereby facilitating the binding of ATP and reinstating the PBD to a low affinity state allowing rapid substrate exchange. The Hsp70 mechanism of action strongly suggests that inter-domain communication between the Hsp70 domains is critical for the correct functioning of Hsp70.

1.3.1.3 Hsp70 cytosolic members of *S.cerevisiae*

S.cerevisiae encodes nine cytosolic members of the Hsp70 family that are subdivided into classes Ssa (Ssa1, Ssa2, Ssa3, and Ssa4), Ssb (Ssb1 and Ssb2), Sse (Sse1 and Sse2) and Ssz1. The highly homologous Ssa proteins differ in their expression patterns, Ssa2 is constitutively expressed at high levels, *SSA1* is also constitutively expressed but at lower levels than *SSA2*. However, *SSA3* and *SSA4* are not expressed under vegetative conditions but expression is dramatically increased when the cell encounters stress (Werner-Washburne *et al.,* 1987, Boorstein and Craig 1990b, Boorstein and Craig, 1990a). The Ssa family consists of four proteins (Ssa1-Ssa4), and at least one of these is required for growth (Werner-Washburne *et al*., 1987). Ssa1 and Ssa2 have 97% homology, the heat-inducible Ssa3/Ssa4 are 88% identical to one another and are 80% identical to Ssa1/2 (Table 1.3) (Sharma *et al*., 2009). Ssa1 negatively regulates its own expression (Stone and Craig, 1990).

	Ssa1	Ssa2	Ssa3
Ssa2	97%		
Ssa3	79%	79%	
Ssa4	81%	81%	87%

Table 1.3 Percent amino acid identity of Ssa proteins. Table adapted from (Sharma *et al*. 2009).

The activity of cytosolic Hsp70 chaperones Ssb1, Ssb2 and Ssz1 are localized to the ribosome. They form part of a complex, ribosome-associated complex (RAC), which includes either Ssb1/2, Ssz1 and the DnaJ homologue Zuo1 (Pfund *et al*., 1998, Gautschi *et al*., 2001). This complex binds both the active ribosome and the associated nascent polypeptide chain to assist with translational fidelity and ensure correct protein folding (Gautschi *et al.,* 2002, Rakwalska and Rospert, 2004). Both Ssb1/2 and Ssz1 are structurally similar to other Hsp70's, containing an ATPase domain and a peptide-binding domain (Pfund *et al*., 1998). Unlike other heat shock genes in which expression is induced by heat, the expression of *SSB1/2* is repressed (Craig and Jacobsen, 1985), instead the expression of *SSB1/2* is coordinated with expression of ribosomal protein genes (Lopez *et al*., 1999). Ssz1 differs from other Hsp70's as its ATPase domain and PBD activities do not appear to be necessary to carry out its functions (Hundley *et al*., 2002, Huang *et al*., 2005). It has been postulated that the function of Ssz1 is not to bind unfolded protein, but to assist in the ATPase stimulation of Ssb1/2 by the DnaJ protein Zuo1 (Huang *et al*., 2005). The Sse family of cytosolic Hsp70's act as nucleotide exchange factors for Ssa1 (section 1.3.2.3).

1.3.2 Regulatory roles of Hsp70 co-factors

Hsp70 is involved in many cellular functions and requires the activity of co-chaperones and NEFs to carry out essential cellular functions and to regulate its own functions. Hsp70 functions in concert with the co-chaperones such as Hsp40 and Hsp90 and the ATPase cycle of Hsp70 is regulated by components that stimulate ATP hydrolysis and NEFs such as Sse1 and Fes1.

1.3.2.1 Hsp40

One important co-chaperone that plays a major role in regulating Hsp70 interaction with substrates is Hsp40. The Hsp40 is ubiquitous and is represented by multiple isoforms, with twenty-two being expressed in *S.cerevisiae.* The Hsp40 co-chaperone family (also known as J proteins) are defined by a conserved stretch of approximately seventy residues, which is known as the J domain (Craig *et al*., 2006, Walsh *et al*., 2004). Hsp40 is the most abundant and is an obligatory regulator of Hsp70 in *S.cerevisiae*. The co-chaperone Hsp40 functions in two ways to assist the function of Hsp70; it can bind to unfolded substrates and present them to Hsp70 (Laufen *et al*., 1999) and it can physically interact with Hsp70 and stimulate ATP hydrolysis (Cyr *et al*., 1992) (Figure1.2). Hsp40 can be defined as a chaperone itself as it binds specific hydrophobic peptide, which are also bound by Hsp70 and contributes to the prevention of protein aggregation, (Lian *et al*., 2007, Moriyama *et al*., 2000). Hsp40 also binds directly to Hsp70, stimulating ATP hydrolysis by up to 1000-fold and functions to coordinate ATP hydrolysis in the PBD (Laufen *et al*., 1999, Liberek *et al*., 1991). Particular Hsp70s display specificity for a single J protein (Walsh *et al.,*

2004, Sahi and Craig, 2007), e.g. in *S.cerevisiae* the cytosolic Hsp70 members Ssa1/2 specifically interact with the hsp40 members; Sis1, Ydj1, Swa2, Hij1 and Djp1 (Hettema *et al*., 1998, Horton *et al*., 2001, Kryndushkin *et al*., 2002). The specificity of the interaction between members of the Hsp70 family and the Hsp40 family, allows Hsp70 to perform various functions, e.g. a main function of Ssa1/2 is general protein folding in the cytosol. However, the Hsp40 member Sis1 assists Ssa1/2 on ribosomes (Horton *et al*., 2001) in translation and Ydj1 is involved in the recruitment of Ssa1/2 to protein folding on ER membranes (Brodsky *et al*.,1998). The ability of Hsp40 to interact with various substrates and its ability to stimulate the ATPase cycle of Hsp70 results in Hsp70 binding a wider range of substrates than it would independently (Misselwitz *et al*., 1998).

1.3.2.2 Sti1 and Cns1

Sti1 and Cns1 are both strong regulators of ATPase activity of the Hsp70 member Ssa1. Sti1 was originally discovered as a Hsp90 chaperone that inhibits the ATPase activity of Hsp90 by acting as a non-competitive inhibitor (Prodromou *et al*. 1999) (Richter *et al*. 2003). Sti1 has also been identified as a potent ATPase activator of the Hsp70 Ssa members (Wegele *et al*. 2003b). Hop1 is the mammalian homologue of Sti1 (Honoré *et al*. 1992, Smith *et al*. 1993) and binds to the C -termini of both Hsp70 and Hsp90, providing a physical link between both chaperones (Brinker *et al*. 2002). Binding of Sti1 to Hsp70 and Hsp90 involves tetratricopeptide (TPR) domains, which consist of TPR motifs which are degenerate sequences that are thirty-four amino acid in length (Richter *et al*., 2003, Lamb *et al*., 1995), that form a structurally conserved tandem array of two anti- parallel α-helices. Multiple TPR motifs adopt a right-handed super helix structure, which creates a groove with a large surface area. Both

Hsp70/Ssa1 and Hsp90 contain the conserved C-terminus residues EEVD that mediates their interactions with the TPR domains of Sti1 (Scheufler *et al*., 2000). The Cns1 protein also contains a TPR domain and can activate basal Ssa1 ATPase activity by thirty-fold (Hainzl *et al*., 2004).

1.3.2.3 Hsp70 NEFs Sse1 and Fes1

When in an ADP bound state, Hsp70 has a weak interaction with its substrate, conversely, in an ADP bound state the Hsp70 substrate becomes tightly bound, therefore, the ADP-ATP exchange is critical for regulating the Hsp70 cycle. NEFs trigger substrate release by shifting the equilibrium towards an ATP bound state. Fes1 and Sse1 are well known NEFs in *S.cerevisiae* (Kabani, *et al*., 2002a). Sse1 and Fes1 exhibit functional redundancy as overexpression of *FES1* recovers strains harboring a lethal Δ*sse1* Δ*sse2* double deletion (Raviol *et al*., 2006), although recovery seems to be strain specific (C. Moran, personal communication).

Sse1 is also a member of the cytosolic Hsp70 family, comprised of an ATPase domain, a PBD containing an extended linker region separating the ß and α subdomains of the domain and an extended C-terminal region (Liu and Hendrickson, 2007; Easton *et al*., 2000). Sse1 requires ATP for its activation and upon binding of ATP Sse1 adopts a conformation required for association with Ssa1. It is thought that the ATPase domains of Ssa1 and Sse1 associate assymetrically so that lobe I of Ssa1 contacts lobe II of Sse1 and the C-terminal α-helical domain of Sse1 plays a role in this interaction (Andréasson *et al.,* 2008). Fes1 is also a NEF present in yeast (Kabani *et al*., 2002a), it is homologous to the mammalian HspBP1 which causes nucleotide dissociation in both Ssa1 and mammalian Hsc70 (Kabani *et al*., 2002b). Fes1

facilitates translation, a process in which Ssa1 and Ydj1 have been implicated in (Brodsky *et al*., 1998, Horton *et al*., 2001).

1.3.3 Hsp90

Hsp90 was originally identified as one of the several conserved heat shock proteins as it exhibits general protective properties, preventing aggregation of non-native proteins (Wiech *et al*., 1992). However, Hsp90 appears to be more selective than other heat shock proteins, reacting with only a specific subset of proteins (Picard 2002). Hsp90 is a highly conserved protein and is extremely abundant in nearly all organisms and cell types prior to cellular stress. Budding yeast contain two Hsp90 genes which encode very similar isoforms of Hsp90; Hsc82 and Hsp82 which have 97% sequence identity . Hsc82 is constitutively expressed at high levels and is only slightly induced by heat shock, whereas Hsp82 is strongly induced by both heat and stress (Borkovich *et al*., 1989).

1.3.3.1 Hsp90 structure

Hsp90 is a flexible dimer, with each monomer consisting of three domains, each consisting of an N domain, connected by a long linker sequence to an M –domain that is followed by a C-terminal dimerization domain. The function of Hsp90 is dependent on ATP hydrolysis (Panaretou *et al*., 1998, Obermann *et al*., 1998); the N-terminal possesses a deep ATP binding pocket (Prodromou *et al.,* 1997). The rate of ATP hydrolysis is slow (in yeast one molecule of ATP is hydrolyzed every one/two minutes) (Panaretou *et al*., 1998, Scheibel *et al*., 1998). The slow rate of ATP hydrolysis suggests that Hsp90 undergoes a complex conformational rearrangement due to ATP binding. Upon ATP binding a short segment on the N-domain known as

the ATP lid, changes its position and flaps over the binding pocket. This releases a short N-terminal sequence from its original position (Richter *et al*. 2002). Subsequently this segment binds to the respective N-domain of other subunit in dimer producing a strand-swapped, transiently dimerized N-terminal conformation (Ali *et al*., 2006, Richter *et al*., 2006). This N-terminal rearrangement results in further conformational changes throughout the dimer leading to a twisted and compact structure.

1.3.3.2 Hsp90 function and cofactors

The Hsp90 chaperone differs from Hsp70 as its client proteins typically compose of kinase or transcription factors that rely on Hsp90 for correct signaling. Hsp90 binds clients that are already considerably folded and induces structural changes and protein associations in order for clients to obtain their full activity. Hsp90 client folding pathway involves Hsp70 and co-chaperones involved in regulating Hsp90. Initially, client proteins interact with Hsp40 (Hernández *et al*., 2002ab), which targets them to Hsp70; proteins are transferred from Hsp70 to Hsp90 in order for folding to be completed. The co-chaperone Sti1 is thought to facilitate transfer by binding both Hsp70 and Hsp90 via TPR domains, forming a bridge on which proteins can be transferred (Smith *et al*., 1993, Chen *et al*., 1996, Chen and Smith, 1998). Sti1 stimulates the ATPase activity of Hsp70 but inhibits the ATPase activity of Hsp90 (Wegele *et al*., 2003b, Richter *et al*., 2003). It is thought that in addition to forming a physical link between Hsp70 and Hsp90, Sti1 may affect client folding through regulation of conformation and ATPase cycles of the two chaperones (Johnson *et al*., 1998, Smith and Johnson, 2000, Hernández *et al*., 2002b).

An example of an Hsp90 client is the stress activated mitogen activated protein (MAP) kinase protein Slt2. MAPK modules consist of protein kinases that stimulate each other in series resulting in activation of a terminal MAPK. The Slt2 protein is part of the cell wall integrity (CWI) signaling pathway. The CWI signaling pathway is induced by cell wall damage caused by heat and chemical agents. Cell wall damage is transmitted by protein kinase C to a MAP kinase module terminating in the MAPK Slt2 (Paravicini *et al*., 1992, Torres *et al*., 1991, Lee and Levin, 1992). Slt2 phosphorylates and activates two transcription factors, Rlm1 and the Swi4/6 cell-cycle box-binding factor (SBF). Rlm1 and SBF activate expression of distinct sets of genes with predicted roles in cell wall synthesis and repair (Lagorce *et al.,* 2003). A Yeast-two hybrid screen that was undertaken to isolate Hsp90 binding partners isolated Slt2 as a Hsp90 binding partner (Millson *et al*., 2005) and further studies revealed that Hsp90 binds specifically to the dually Thr/Tyr-phosphorylated, stress activated form of Slt2p ((Y-P,T-P) Slt2) and also to the MAP kinase domain within this (Y-P,T-P) Slt2 (Millson *et al*., 2005). Mutant analysis of Hsp90 revealed that the chaperones function for (Y-P,T-P) Slt2 to activate its downstream target; the transcription Rlm1 (Millson *et al*., 2005), implicating Hsp90 as a component of the CWI signaling pathway. More recently the Hsp70 NEF Sse1 has also been implicated in the CWI signaling pathway, through a partnership with Hsp90 and Slt2 (Shaner *et al*., 2008).

1.3.4 Hsp104

Hsp104 is a member of the class1 Clp/Hsp100 family of hexameric AAA^+ ATPases (ATPases associated with various cellular activities) protein superfamily (Neuwald *et al*., 1999). The clp/Hsp100 family is composed of Hsp100 proteins from bacteria,

plants and fungi. Hsp104 is a protein disaggregase that recovers proteins that have becomes aggregated due to stresses such as heat shock, hydrogen peroxide (Godon *et al*., 1998), ethanol and near freezing cold shock (Kandror *et al*., 2004). Hsp104 substrates include unfolded polypeptides and amorphous aggregates from various proteins. It is not required for cellular viability, as a Δ*hsp104* strain shows no defect in normal cellular growth but exhibits severe defects at elevated temperatures exhibiting the importance of the chaperone in cellular thermotolerance.

1.3.4.1 Structure and function of Hsp104

Members of the class Clp/Hsp100 family, members of this family contain variable C and N-terminus domains and two highly conserved nucleotide binding domains (NBD1 and NBD2), that are separated by a variable middle domain. The highly conserved NBD domains are equipped with a multitude of accessory motifs and domains that grant different substrate specificities and functions (Hanson and Whiteheart, 2005), but are not identical to one another in structure or function. The active state of yeast Hsp104 and its bacterial homologue ClpB is typically a hexameric ring (Figure 1.3). Studies utilizing electron microscopy observing negatively stained Hsp104 revealed a barrel shaped hexamer of approximately 155 Å diameter (Parsell *et al*., 1994). The hexameric state of Hsp104 exhibits a higher ATPase rate than the monomer and is stabilized by high protein concentration, ATP or ADP and low ionic strength (Schirmer *et al.*1998, Schirmer *et al.* 2004). NBD1 drives the major constitutive Hsp104 ATPase activity (Schirmer *et al*., 1998, Hattendorf and Lindquist, 2002), whereas oligomerization is dependent on NBD2 (Parsell *et al*., 1994, Schirmer *et al*., 2004). ATPase activities of both domains is necessary for full protein remodeling activity and is dependent on allosteric

communication between and within domains of Hsp104 (Schirmer *et al*., 2001, Hattendorf and Lindquist, 2002, Cashikar *et al*., 2002, Doyle *et al*., 2007). In Hsp104 communication between NBDs, is thought to be mediated by Arginine fingers (R334 and R765) that extend into the ATPase binding site of adjacent subunits and participate in hydrolysis of ATP (Karata *et al*., 1999). Studies on the Hsp104 homologue, ClpB of *T.thermophilis,* reveals the middle region is formed by two antiparallel coiled-coil motifs connected near the interface of NBD1 and NBD2 *(*Lee *et al*., 2003). The role of the coiled-coil region in Hsp104 is unclear, deletion of this region in ClpB results in inhibition of protein remodeling activities but hexamerization still occurs (Mogk *et al*., 2003). Single point mutations in a conserved eleven amino acid region (helix L3) of the coiled-coil domain of Hsp104 results in extremely diverse functional defects (Schirmer *et al*., 2004). A loss of function mutation in the equivalent region of the *Arabidopsis* Hsp101 is restored by suppressor mutations in both NBD1 and the axial channel loops of the hexamer, this result is indicative of a functional link between the coiled-coil and these regions (Lee *et al*., 2005). The coiled-coil region of Hsp104 drives inter-domain communication between NBD1 and NBD2 in Hsp104 (Cashikar *et al*., 2002) and acts as a regulatory device in ClpB by coupling translocation activity to DnaK chaperone activity (Haslberger *et al*., 2007). A mechanistic model of Hsp104 is that it operates by extracting polypeptide chains from aggregates and processing them through the central channel of Hsp100/ ClpB hexamer, a process which is dependent on ATP hydrolysis (Grimminger *et al*., 2004). This process in conjunction with the Hsp70/Hsp40 chaperone system carries out the disaggregase function of Hsp104 (Fig 1.3). Recent studies indicate that the M domain is essential for protein dissaggregation but dispensable for ATPase and

substrate translocation activities and controls the Hsp104 remodeling activities in a Hsp70/Hsp40 dependent manner (Sielaff and Tsai, 2010).

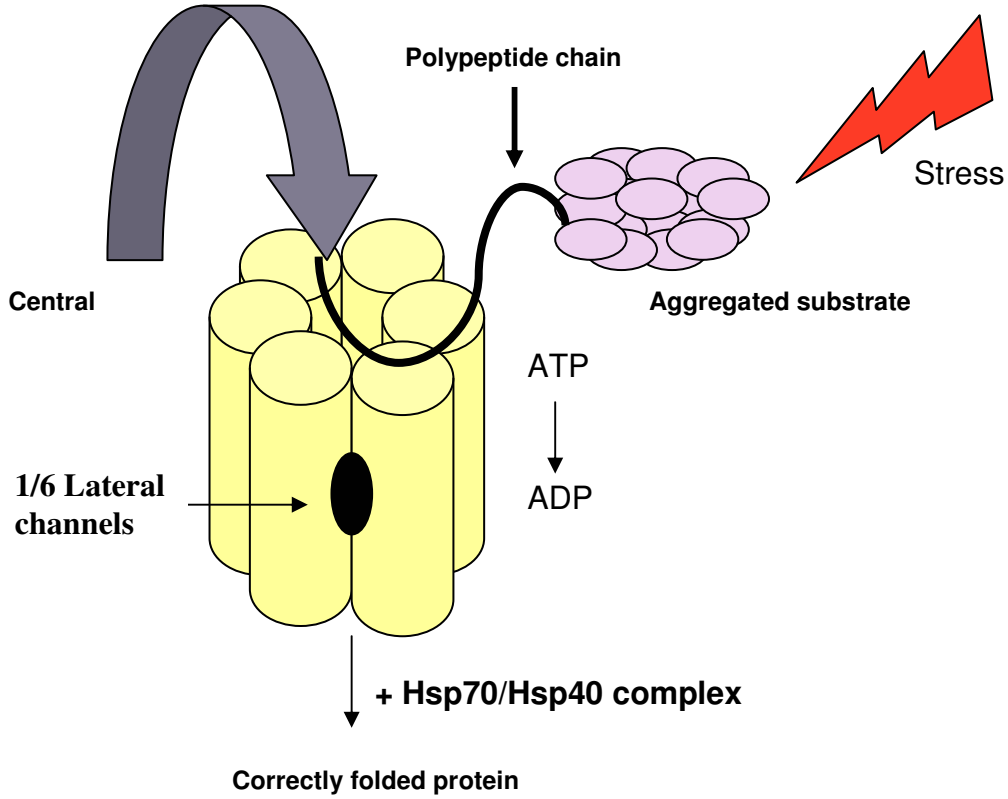


Figure 1.3 Mechanistic model of Hsp104. When the cell encounters stress, proteins become misfolded and aggregated. Hsp104 operates by extracting polypeptide chains from aggregates and processing them through the central channel of Hsp100/ Clpb hexamer, a process that is dependent on ATP hydrolysis. Hsp104 operates in concert with Hsp70 and Hsp40 to ensure correct folding of proteins.

1.4 Factors affecting prion propagation in *S.cerevisiae*

Propagation of yeast prions requires normal abundance and activity of many protein chaperones including Hsp70, Hsp40 and Hsp104. Hsp70 is regulated by many co-chaperones whose affect on prion propagation is probably mediated through Hsp70

(Figure 1.4). The Hsp70 NEFs, Sse1 and Fes1 have also been implicated as playing a role in prion propagation. Chaperones, co-chaperones and Hsp70 NEFs have been implicated in the maintenance and propagation of yeast prions.

1.4.1 Affects of Hsp70 and co-chaperones on prion propagation

Co-purification studies of the prion proteins Sup35 and Rnq1 showed that the cytosolic Hsp70 members; Ssa1 and Ssb1and their co-chaperones Ydj1, Sis1 and Sse1 bind to the prion form of the Sup35 protein in yeast (Allen *et al*., 2005, Bagriantsev *et al*., 2008, Sondheimer *et al*., 2001). Ssa1/2 were the most abundant proteins to co-purify with Sup35 from [*PSI*$^+$] cells, which is indicative that Hsp70 directly interacts with the prion form of Sup35 *in vivo* and its co-chaperones may affect prion propagation in a Hsp70 mediated manner. Many groups have monitored the affects of chaperones on amyloid and the kinetics of Sup35 and Ure2 formation, or their prion determining regions into amyloid (Krzewska and Melki, 2006, Shorter and Lindquist, 2008, Savistchenko *et al*., 2008). These studies concluded that fragmentation of amyloid or inhibition of amyloid assembly are consistent with binding interactions of the different chaperones with *ex vivo* soluble and prion forms of proteins.

In addition to biochemical studies on the involvement of Hsp70 in prion propagation, numerous groups have carried out genetic studies. Overexpression of Ssa1 in yeast cells causes loss of [*URE3*] but not doe not cause loss of the [*PSI*$^+$] prion (Schwimmer and Masison, 2002). Mutational analysis of Ssa1 and Ssa2 has further implicated Hsp70 as playing a role in prion propagation (Jung *et al*., 2000, Jones and Masison, 2003, Loovers *et al*., 2007). Isolation of mutations in the PBD and ATPase domain highlight the importance of the ATPase cycle and inter-domain communication of Hsp70 in prion

propagation. The Ssa1 dominant mutation L483W, designated the name *SSA1-21* (Jung *et al*., 2000) and the G481D (Loovers *et al*., 2007) mutants are the only isolated mutations present in the PBD of Ssa1 that impair [PSI^+] propagation in yeast. These mutations have no detectable effects on stress tolerance or cell growth suggesting the

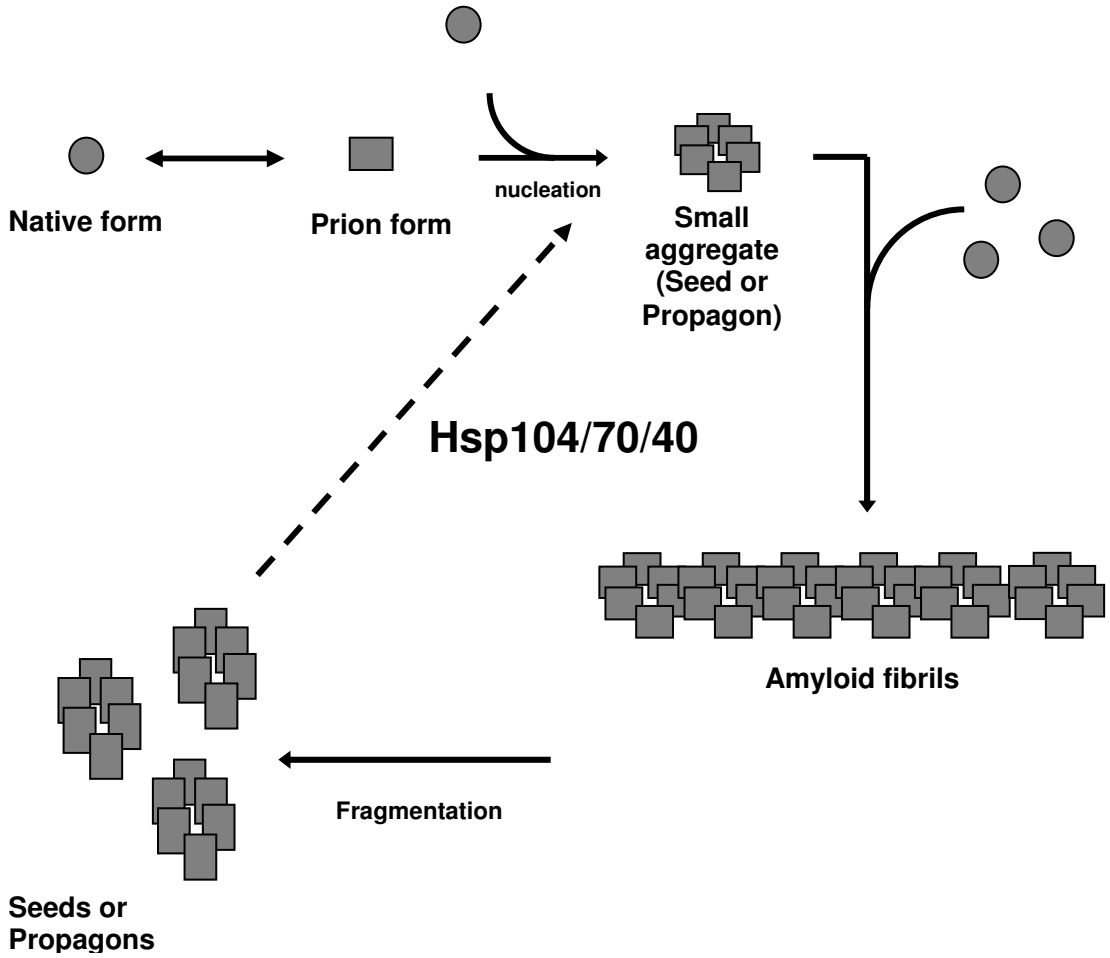


Figure 1.4 Involvement of protein chaperones in prion propagation. In yeast, specific native proteins such as Sup35 can spontaneously convert into the prion form. Once the prion form has been established, it can then recruit and convert native protein into the prion form, forming aggregates which enlarge and form amyloid fibrils. In order for prions to propagate, they must be passed on to daughter cells. This is achieved by the fragmentation of amyloid fibrils into prion seeds, which are then passed on to daughter cells during cell division. Protein chaperones such as Hsp40, Hsp70 and Hsp104 play a crucial role in this process.

affects are specific and do not affect functions of Hsp70 (Jung *et al*., 2000, Loovers *et al*., 2007). To gain further insight into how Ssa1 affects prion propagation, further mutagenesis screens were carried out, a majority of mutations that impaired prion propagation were located in the ATPase domain of Ssa1 and Ssa2 (Jones and Masison, 2003, Loovers *et al*., 2007). This is indicative that regulation of chaperone activity was more important than direct substrate interactions for the inhibitory effect. A further genetic screen to isolate second-site suppressors of the *SSA1-21,* isolated mutations that suppress anti prion effects of *SSA1-21* and a number of ATPase mutants (Jones and Masison, 2003, Loovers *et al*., 2007). The location of these second -site mutants suggests that they suppress anti-prion affects of mutants by reducing ATP hydrolysis through interaction with Hsp40 or TPR co-chaperones or through weakening substrate trapping.

Subsequent analysis confirmed that altering the ATPase cycle has an affect on prion propagation (Jones *et al*., 2004), e.g. for example, either increasing ATPase activity by overexpressing Sti1 or decreasing ADP release by deleting the NEF Fes1 weakens the [*PSI*$^+$] phenotype (Jones *et al*., 2004). This is indicative that shifting the ATPase cycle of Hsp70 to favor an ADP bound state appears to impair [*PSI*$^+$] propagation. Despite their high levels of conservation, different Ssa members appear to have different affects on prion propagation. Overexpression of Ssa1 but not Ssa2 cures [*URE3*], additionally [*URE3*] is weaker in cells lacking Ssa1 but not in cells lacking in Ssa2 (Roberts *et al*., 2004; Sharma and Masison, 2008; Sharma *et al*., 2009). Other members of the cytosolic Hsp70's also have an affect on prion propagation; Ssb1 and

Ssb2 were found to antagonize the *de novo* formation of [*PSI*$^+$] (Chernoff *et al*., 1999; Allen *et al*., 2005).

Another member of the cytosolic Hsp70 family, Sse1 is also thought to play a role in prion propagation (Fan *et al*., 2007, Sadlish *et al*., 2008, Kryndushkin and Wickner, 2007). In addition to its role in prion propagation, recent studies have shown that the Ssa1 and Ssb1 NEF is also required for the *de novo* formation of [*PSI*$^+$] and also prion variant determination (Fan *et al*., 2007). Studies show that overproduction of Sse1 dramatically enhances [*PSI*$^+$] formation, whereas deletion of Sse1 severely inhibits [*PSI*$^+$] formation (Fan *et al*., 2007; Shaner *et al*., 2008; Sadlish *et al*., 2008,). The abundance of Sse1 also has an affect on the [*URE3*] prion; overproduction of Sse1 can efficiently cure [*URE3*] whereas deletion of Sse1 blocks [*URE3*] propagation (Kryndushkin and Wickner ,2007). Results from these studies are indicative that Sse1 affects propagation of prions through Ssa1 function. It has been suggested that the NEF function of Sse1 is necessary for maintaining sufficient levels of substrate free Ssa1 (Sadlish *et al*., 2008). However, Sse1 also exhibits NEF independent activity in relation to prion propagation; stimulation of *in vitro* nucleation of the Sup35NM (prion forming domain of Sup35) (Sadlish *et al*., 2008). The above information suggests that propagation of different prions relies on different Hsp70 functions**.**

1.4.2 Affects of Hsp40 on prion propagation

Studies utilizing both [*PSI*$^+$] and [*URE3*] have identified complex roles for Hsp40 co-chaperones in prion propagation and assembly of amyloid-like fibrils. The Sis1 and Ydj1 members of the Hsp40 family have been found to physically interact with large Sup35 aggregates (Bagriantsev *et al.,* 2008) and [*PSI*$^+$] propagation has been shown to be dependent on Sis1 (Higurashi *et al*., 2008, Tipton *et al*., 2008), whereas Ydj1 in

combination with cognate Hsp70 destabilizes weak variants of [*PSI*$^+$] (Kushnirov *et al.,* 2000). Apj1 is another member of the Hsp40 family in yeast, and overexpression of this co-chaperone cures certain variants of [*PSI*$^+$] *(*Kryndushkin *et al*., 2002). Apj1 shares high homology with Ydj1 but its cellular function remains unclear. Hsp40 has also been directly implicated with Sup35 fibril assembly *in vitro* (Krzewska and Melki, 2006; Shorter and Lindquist, 2008).

Hsp40s also play a role in the propagation of the [*URE3*] prion, Sis1 is required for [*URE3*] propagation (Higurashi *et al*. 2008), while overexpression of Ydj1 cures yeast of [*URE3*] (Moriyama *et al*., 2000). Ydj1 binds to soluble Ure2 in the presence or absence of its prion domain and when added to purified Ure2 it inhibits formation of amyloid in a concentration dependent manner, indicating Ydj1 binds to Ure2 and prevents fibril formation (Savistchenko *et al.,* 2008, Lian *et al.,* 2007). However, the addition of Ydj1 to Ure2 after polymerization begins, does not affect amyloid formation and additionally, cells over producing Ydj1 have longer prion polymers (Kryndushkin and Wickner, 2007), indicating that Ydj1 may interfere with polymer fragmentation rather than polymer assembly. Studies have also revealed that the J domain of Ydj1 is sufficient for the elimination of [*URE3*], the J domain of Ydj1 mediates interaction with Hsp70 and is responsible for stimulating ATPase activity, suggesting that the regulation of Ssa1 by Ydj1 is crucial for the [*URE3*] curing process (Sharma *et al*., 2009; Masison *et al*., 2009).

The Hsp40 cytosolic member, Sis1, was discovered to be essential for prion propagation through its association with the Rnq1 protein in [*PIN*$^+$] cells (Sondheimer *et al*., 2001). Sis1 is the only member of the Hsp40 family that is required for the propagation of [*PSI*$^+$], [*URE3*] and [*PIN*$^+$] (Aron *et al.,* 2007, Higurashi *et al.,* 2008).

The Sis1 protein binds specifically to Rnq1, to a region outside of the prion determining domain, and this binding is important for [*PIN*$^+$] propagation (Lopez *et al.,* 2003, Douglas *et al.* 2008). Ssa1 also interacts with Rnq1, and the Sis1pG/F region is believed to aid cooperation between Sis1 and Ssa1 in substrate interactions. The interaction of Sis1 and Ssa1 with Rnq1 occurs only in [*PIN*+] cells (Sondheimer *et al*., 2001), and inhibition of the ability of Sis1 to stimulate ATPase activity of Ssa1 also impairs [*PIN*$^+$] propagation, suggesting Sis1 acts as a Hsp70 co-chaperone with respect to [*PIN*$^+$] propagation (Higurashi *et al*., 2008, Lopez *et al.,* 2003, Aron *et al*., 2007). In cells depleted of Sis1 and Hsp104, there is an increase in Rnq1 polymers size and the number of [*PIN*$^+$] seeds decreases (Aron *et al*., 2007). Both these effects appear to be due to altered Sis1 interactions with both Hsp70 and Hsp104 and indicates that Sis1 is necessary for fragmentation of Rnq1 polymers which is required for [*PIN*$^+$] prion propagation (Aron *et al*., 2007). Sup35 polymers also increase in size in the absence of Sis1 or due to the inhibition of Hsp104 (Kryndushkin *et al*., 2003, Higurashi *et al*., 2008). These results suggest that Sis1 may work in conjunction with Hsp70 to deliver prion substrates to Hsp104 (Tipton *et al*., 2008).

1.4.3 Affects of Hsp104 on prion propagation

In yeast, prions are passed from generation to generation by transmitting aggregated prion seeds in cytoplasm from mother to daughter cells. Prion seeds can recruit and convert native proteins of identical amino acid sequence into the prion form, thus propagating the prion (Figure 1.4). Abundance of the protein chaperone Hsp104 is critical in the propagation of [*PSI*$^+$] prion (Chernoff *et al.,*1995), deletion or overexpression results in the loss of the prion and the appearance of [*psi*$^-$] cells. Intact

Hsp104 is also required for the propagation of other yeast prions although unlike [*PSI*$^+$] they are not cured by Hsp104 overproduction.

Decreased production or inactivation of Hsp104 results in an increased size and number of Sup35 aggregates (Wegrzyn *et al*., 2001). Treatment of cells by GdnHCl, a chemical known to cure yeast prions via Hsp104 inactivation (Ferreira *et al*., 2001), or down regulation of its production, also exhibit an increased size of Sup35 aggregates. The increase in polymer size and decrease in polymer number can be explained by the assumption that Hsp104 breaks down prion polymers into smaller oligomers, therefore deletion or inhibition of Hsp104 results in the generation of larger polymers and therefore a smaller number of seeds (Kushnirov and Ter-Avanesyan, 1998). The decrease in polymer levels per cell results in the decreased efficiency of prion conversion, resulting in loss of the [*PSI*$^+$] prion during cellular divisions (Cox *et al*., 2003).

The dissaggregation-based model of Hsp104 may also explain how overproduction of the chaperone cures the [*PSI*$^+$] prion. There is a high possibility that high abundance of the Hsp104 chaperone eliminates [*PSI*$^+$] by shearing Sup35 polymers into monomers, although direct evidence is lacking. Studies utilizing fluorescent microscopy analyzed the affects of Hsp104 overproduction on a Sup35-GFP aggregated structure (Wegrzyn *et al.,*2001). Diffusion of the structure was visible in [*PSI*$^+$] cells; however, it is not clear whether the diffusion reflects monomerization or a decrease in the Sup35-GFP aggregate size. Biochemical analysis confirm that [*PSI*$^+$] cells overexpressing Hsp104 accumulate a larger proportion of soluble Sup35 (Paushkin *et al.,*1996), that behaves as a monomeric protein in semi denaturing conditions (Kryndushkin *et al*., 2003). However, it is not clear whether monomeric

Sup35 present in cells overproducing Hsp104 originates from prion aggregates or is synthesized *de novo* when prion propagation is inhibited, although it is thought that monomers originating from prion aggregates are likely to be misfolded.

However, there is also an alternative hypothesis on how excess Hsp104 cures cells of the [*PSI*$^+$] prion. In cells overexpressing Hsp104 in which monomers were detected, the remaining Sup35 polymers were larger in comparison to extracts from the same strain expressing normal levels of Hsp104 (Kryndushkin *et al*., 2003). Fluorescent microscopy studies of overproduced Sup35-GFP in the presence of excess Hsp104 formed large filaments in [*PSI*$^+$] cells (Zhou *et al*., 2001), such filaments have no been observed in [*PSI*$^+$] cells expressing normal abundance of Hsp104, but have been observed in [*psi*$^-$] cultures overproducing Sup35-GFP (Zhou *et al.,,* 2001). Further studies showed that these filaments are associated with some cytoskeletal components of the endocytic/vacuolar network and are interpreted as intermediates in the prion *de novo* formation pathway (Ganusova *et al*., 2006).

Further studies on the mechanism of how overproducing Hsp104 cures the [*PSI*$^+$] prion, the Masison group proposed that the amino terminal domain (NTD), is necessary for this process (Hung and Masison, 2006). The NTD is a highly conserved domain among Hsp100 proteins and contains two structurally similar repeats R1 and R2 (Lo *et al*., 2001), the function of this domain in unknown. Mutations of the Hsp104 NTD were isolated that improved [*PSI*$^+$] propagation and removal of the NTD also supported [*PSI*$^+$], thermotolerance and protein dissaggregation, yet overexpression of this construct failed to cure [*PSI*$^+$] (Hung and Masison, 2006). This suggests that an intact NTD is required for curing by Hsp104 overexpression, despite

having both normal thermotolerance and protein dissaggregation function increasing these activities is not sufficient for curing. These results imply that the mechanism by which excess Hsp104 cures the [*PSI*$^+$] prion is not dependent on Hsp104's function of disaggregating of proteins (Hung and Masison, 2006). It also implies that the NTD of Hsp104 is not required for interacting with Hsp104 substrates such as Sup35. However, overexpression of the NTD alone is not sufficient to cure [*PSI*$^+$], suggesting that the dissaggregation function of Hsp104 may still be necessary for this process. One possibility is that the NTD directs Hsp104 function to subcellular locations required for eliminating [*PSI*$^+$]. The exact mechanism by which overexpression of Hsp104 cures [*PSI*$^+$] remains to be uncovered.

It also appears that Hsp104 does not function independently in curing of the [*PSI*$^+$] prion by its overexpression. Recent studies have indicated that the Hsp70 and Hsp90 co-chaperones Sti1 and Cpr7 may be involved in this process (Moosavi *et al*., 2010). The N-terminal of Hsp104 contains a TPR motif required for interactions with TPR containing co-chaperones. The Hsp104 TPR motif is dispensable for curing, however cells expressing Sti1 defective in Hsp70 and Hsp90 interaction cured less efficiently. Also the presence of the Hsp90 inhibitor radicicol abolishes curing, implying Sti1 acts in curing through Hsp70 and Hsp90 interactions (Reidy and Masison, 2010). Similar results were also observed by another study, deletion of the Hsp70 and Hsp90 co-chaperones lead to a significant reduction in the generation of [*psi*$^-$] cells by Hsp104 overexpression. Deletion of the Sti1 and Cpr7 genes does not modify curing of [*PSI*$^+$] by guanidine, which inhibits the ATPase activity of Hsp104, but does not block the elimination of [*PSI*$^+$] by overexpression of either a Hsp104 mutant deficient in ATPase activity or a trap mutant that binds substrate and does not release it

(Moosavi *et al*., 2010). These results support the hypothesis that Hsp104 overexpression elimination of [*PSI*$^+$] is not simply a consequence of complete dissolution of prion aggregates but rather through a consequence of a mechanism distinct from remodeling of Hsp104.

It is unclear whether Hsp104 recognizes specific regions of prion proteins. Some reports implicate the Sup35 prion domain (PrD), which is located between residues 40 and 97, which includes 5.5 imperfect copies of the oligopeptide repeat (OR) as a possible element responsible for [*PSI*$^+$] transmission to daughter cells (Chernoff 2004, Osherovich *et al*., 2004). As prion propagation is dependent on Hsp104, it is conceivable to think that the ORs may acts as a site of interaction for Hsp104. However, studies carried out using Sup35 derivatives with a shuffled PrD (maintained amino acid sequence but lacked repetitive order of), are still able to propagate as prions in a Hsp104 dependent manner (Ross *et al*., 2005). Further to this not all prions contain the OR regions, e.g. it is absent in Ure2. Conversely, some data suggest that the middle domain of Sup35, which is located outside the PrD, influence effects of Hsp104 on prion (Liu *et al*., 2002). Collectively, this information suggests that the Hsp104 chaperone may recognize a variety of sequences in amyloid conformation or the role of the ORs is to make amyloid accessible to Hsp104 rather than interacting with the chaperone directly.

Mutational analysis of the Hsp104 gene suggests that the chaperone may have different mechanisms for dissaggregation of prion polymers and dissaggregation of heat-damaged proteins (Lee *et al.,* 2003, Cashikar *et al*., 2002, Kurahashi and Nakamura 2007). Mutations were isolated in Hsp104 that affected prion propagation

but not thermotolerance, all theses mutations were situated near the lateral channel of the Hsp104 hexamer (Figure 1.3), according to an alignment with the *T.thermophilus* ClpB sequence (Kurahashi and Nakamura 2007). This channels ~10-to-30 Å wide, thus it was proposed that this channel could be able to thread amyloid fibers, pointing to the possible ability of Hsp104 to use the lateral channels for dissaggregation of prion polymers, while the central pore is used for disaggregating heat damaged proteins (Lee *et al.,* 2003, Cashikar *et al*., 2002, Kurahashi and Nakamura, 2007).

1.5.0 Other factors affecting prion propagation in *S.cerevisiae*

In addition to effects of protein chaperones on propagation in yeast, it is possible that other cellular components or processes may also have an effect.

1.5.1 Sup45 (eRF1)

As discussed in section 1.2.2, Sup45 physically and functionally interacts with Sup35 to form a release factor complex that is essential for the release of nascent peptides from the ribosome during translation termination. The Sup35 protein interacts with the C-terminal of Sup45 (Eurwilaichitr *et al.,* 1999). Presumably once Sup45 forms a complex with Sup35, the Sup35 molecule is not available to undergo conversion into [*PSI*$^+$]. Studies analyzing the affects of overexpression of Sup45 on prion propagation found that overexpression significantly reduced the frequency of the induced *de novo* formation of [*PSI*$^+$] but did not affect prion propagation (Derkatch *et al*.,1998). It is possible that this interaction between Sup35 and Sup45 stabilizes Sup35 conformation enough to prevent imperfect seeding by the [*PIN*$^+$] but not to prevent polymerization once seeds are established. Alternatively, Sup45 could interact directly with other chaperones or could interact with the Rnq1 protein directly.

1.5.2 The ubiquitin-proteosome system (UPS)

One mechanism in yeast cells to eliminate damaged, misfolded or defective proteins that may cause damage to the cell, is the ubiquitin-proteosome system (UPS) (Hershko and Ciechanover, 1998). Once a protein has become damaged, it is targeted for degradation by binding a 76-amino acid protein named ubiquiton (UB) through the sequential action of many enzymes, including UB conjugating enzymes (Ubc). This can be reversed by various deubiquitinating enzymes, that include UB specific proteases (UBP) that take apart UB conjugates and release free UB (Wilkinson 2000). It has been shown that alterations to the UPS can affect the *de novo* appearance of [*PSI*$^+$] (Allen *et al*., 2007). As previously discussed overproduction of Hsp104 eliminates the [*PSI*$^+$] prion. Studies have demonstrated that deletion of genes (*UBC4* and *UBC6*), involved in the UPS has a decreased affect on [*PSI*$^+$] elimination by Hsp104 overproduction (Allen *et al*., 2007). Furthermore, it was found that in the presence of Rnq1, ΔUbc4 facilitates the spontaneous *de novo* formation of [*PSI*$^+$] (Allen *et al*., 2007). The deletion of Ubc4 had a slight increase on the induction of Hsps but this increase was not sufficient to explain effects on [*PSI*$^+$] (Allen *et al*., 2007). It was also observed that in strains containing the Ubc4 deletion there was an increase in the abundance of Ssa bound Sup35, which may suggest that misfolded Sup35 is more abundant or more accessible in the absence of Ubc4 (Allen *et al.,* 2007). Furthermore, it was found that there was an increase in the proportion of [*PSI*$^+$] cells containing large aggregated Sup35 structures (Allen *et al.,* 2007). It was hypothesized that alterations to the UPS induce an adaptive response that results in the accumulation of large aggresome-like aggregates that promote the *de novo* formation of prions and prion recovery by chaperone treatment (Allen *et al.,* 2007). However, it

is important to note that there is no evidence that Sup35 is ubiquitinated and therefore these affects may be indirect.

1.5.3 NatA acetyltransferases

The Nat^{α} –acetyltransferases; NatA, NatB and NAtC are involved in the N-terminal acetylation of approximately 50% yeast protesome, which is critical for the normal cellular functions of many proteins (Polevoda *et al.,* 1999). It is believed that mutations to the Nat^{α} –acetyltransferases reduces the affinity of their substrates to protein binding partners. In order to explore cellular regulation of prion proteins, the interaction between the [*PSI*$^{+}$] prion and the three Nat^{α} –acetyltransferases; NatA, NatB and NAtC was analyzed. In strains lacking NatB and NatC there was no effect on [*PSI*$^{+}$] propagation, however in cells lacking NatA the [*PSI*$^{+}$] phenotype is lost. Despite the change in phenotype, NatA mutants continue to propagate heritable [*PSI*$^{+}$], suggesting that strains lacking NatA function the [*PSI*$^{+}$] complex were uncoupled from their phenotypic effects (Pezza *et al*., 2009). It was found that this change in stability was due to failure to establish the translational termination defect which is observed upon [*PSI*$^{+}$] formation (Pezza *et al*., 2009). This effect was not a result of impaired direct acetylation of Sup35 as it is not a substrate of NatA. Furthermore, it appears other factors that affect the *de novo* formation and propagation of [*PSI*$^{+}$] such as Hsp70, Hsp104 and other co-chaperones are not crucial targets of NatA (Pezza *et al*., 2009). Substrates of NatA include the ubiquitin-conjugating enzyme Ubc4 and the cytosolic Hsp70 members Ssb1 and Ssb2 are proven substrates of the NatA (Huang *et al*., 1987, Polevoda *et al*., 1999), modify the prion cycle (Chernoff *et al*., 1999, Allen *et al.,* 2007) and have demonstrated synthetic reactions with NatA (Gautschi *et al*., 2003, Pan *et al*., 2006). Despite these

connections, cells lacking Ubc4, Ssb1 and Ssb2 display the [*PSI*$^+$] nonsense suppression phenotypes, making them unlikely crucial targets of NatA. As Sup35 or none of the main factors involved in the *de novo* formation and propagation of [*PSI*$^+$] appear to be crucial targets of NatA, it was hypothesized that the affects of NatA on prion propagation may be due to the binding of a trans-factor(s) to Sup35, which facilitates the establishment of the translation termination defect in [*PSI*$^+$] (Pezza *et al.,* 2009).

1.5.4 Polarisome transport of aggregated proteins

It has previously been shown that damaged protein aggregates accumulate in mother cells but are absent in daughter cells (Aguilaniu *et al*., 2003). A new mechanistic basis has been uncovered for this process in yeast (Liu *et al*., 2010). Previous findings' involving asymmetric segregation of damaged proteins between the mother and daughter cells suggests this process is dependent on the anti-aging gene *SIR2* (Aguilaniu *et al*., 2003) . This gene encodes a NAD dependent deacetylase that retards aging in organisms ranging from yeast to mammals (Finkel *et al*., 2009). Analysis carried out, using synthetic gene arrays, looking for genes required for survival in the presence of *sir2* mutants, resulted in a large number of genes encoding components of the polarisome being observed (Liu *et al*., 2010). The polarisome is a structure located at the distal end of the yeast bud that forms a focal point for polymerization of actin monomers into cables that extend from bud tip to mother cell. The function of these actin cables is the transportation of cargo from mother to daughter cell and to support growth. In *sir2* mutant strains, there is a substantial amount of unfolded actin monomers and additionally the chaperone required for actin folding is relatively inactive in *sir2* mutants. Previous work has shown that *SIR2* functions in

asymmetrical segregation through Hsp104 (Erjavec *et al.,* 2007). Studies showed the application of heat shock caused formation of aggregates in the mother and bud, however aggregates were transferred back to mother via the actin cables were they fused to protein aggregates in mother (Liu *et al*., 2010). Although this is a novel mechanism and further studies will be necessary, this process may affect prion propagation. As previously discussed, Hsp104 acts as a disaggregase to break down prion aggregates into seeds, which are passed onto the daughter cell during cell division in order for prions to propagate. Overexpressing Hsp104 may lead to increases in shipping of aggregates from daughter cells back to mother cells, which would explain why overproduction of Hsp104 impairs prion propagation. In addition, larger Sup35 polymers were observed in strains over expressing Hsp104, this could be due to aggregates from daughter cell binding to previously formed aggregates in mother cell. However, at present this is just a theory.

1.5.5 Involvement of Sla1 in prion propagation

Similarities between cytoskeletal assembly and nucleated polymerization of prion proteins is suggestive that similar overlapping proteins may assist in both processes (Lansbury and Caughey, 1995). By carrying out yeast two-hybrid studies, it was found that the C-terminal domain of the cytoskeletal assembly protein Sla1 interacts with the N-terminal prion-forming domain of Sup35 (Bailleul *et al.,* 1999). This interaction is inhibited by alterations to the Sup35 PrD that makes Sup35 unable to propagate as [*PSI*$^+$] and by the absence of Hsp104 (Bailleul *et al.,* 1999). In cells lacking the Sla1 protein curing of [*PSI*$^+$] by Hsp104 overexpression is increased whilst translational readthrough and the *de novo* formation of [*PSI*$^+$] induced by overexpression of Sup35 or Sup35 PrD is decreased (Bailleul *et al.,* 1999). It was

hypothesized that Sla1 assists in prion propagation but further analysis of *sla1* mutants revealed that cells were sensitive to translational inhibitors suggesting that the Sla1/Sup35 interaction may play a role in the functional translation apparatus (Bailleul *et al.,* 1999).

The presence of prion proteins in yeast is a powerful tool for studying the factors that affect the *de novo* appearance and the propagation of prions. Yeast contain many proteins that are highly conserved and present in mammals, indicating that proteins and cellular process regulating prion propagation in yeast may be transferable to the mammalian system which maybe useful in therapeutics and drug development. However further studies are needed to decipher the exact mechanisms of prion propagation in yeast.

1.6 Objectives of this project

The aim of this project is to gain further insight into the involvement of Hsp70 in [*PSI*$^+$] prion propagation. We propose to achieve this by carrying out mutational analysis on the Hsp70 cytosolic members Ssa1-4, which have already been implicated as playing a crucial role in [*PSI*$^+$] propagation (Jung *et al*., 2000; Jones and Masison, 2003; Loovers *et al*., 2007; Sharma and Masison, 2008). We also will carry out further analysis on previously isolated mutants located in the ATPase domain of Ssa1 (Loovers *et al*. 2007) and investigate the role of Ssa1's C-terminal domain in prion propagation and Hsp70 function. Furthermore, we also aim to investigate functional specialization within the cytosolic Hsp70 Ssa family.

2.1.0 Fungal and bacterial growth media

All Chemicals from Sigma-Aldrich Chemical Co. Ltd. U.K, unless otherwise stated.

2.1.1 Yeast peptone dextrose (YPD)

Bacto-yeast extract (10g) (BD), Bacto-peptone (20g) (BD) and glucose (20g) were added to 1000ml of distilled water, and dissolved. The solution was autoclaved and stored at room temperature.

2.1.2 YPD Agar

Bacto- yeast extract (10g) (BD), Bacto-peptone (20g) (BD), glucose (20g) and bacto-agar (20g) were added to 1000ml of distilled water, and dissolved. The solution was then autoclaved and allowed cool. Agar was poured into Petri dishes, under sterile conditions. The plates were allowed set and were stored at 4^{o}C.

2.1.3 Yeast Peptone Glycerol (YPG)

Bacto- yeast extract (10g) (BD), Bacto-peptone (20g) (BD), glycerol (30ml) (Sigma-Aldrich Chemical CO. Ltd. U.K.) and bacto-agar (20g) were added to 1000ml of distilled water, and dissolved. The solution was then autoclaved and allowed cool. Agar was poured into Petri dishes, under sterile conditions. The plates were allowed set and were stored at 4^{o}C.

2.1.4 Yeast peptone Galactose (YPGAl)

Bacto- yeast extract (10g) (BD), Bacto-peptone (20g) (BD), Galactose (20g) and Bacto-agar (20g) were added to 1000ml of distilled water, and dissolved. The solution

was then autoclaved and allowed cool. Agar was then poured into Petri dishes, under sterile conditions. The plates were allowed set and were stored at 4^{o}C.

2.1.5 YPD (G418)

Bacto- yeast extract (10g) (BD), Bacto-peptone (20g) (BD), Glucose (20g) and Bacto-agar (20g) were added to 1000ml of distilled water, and dissolved. The solution was then autoclaved and allowed to cool. Geneticin (20mg/ml) was added. Agar was poured into Petri dishes and allowed cool. The plates were poured and stored at 4^{o}C.

2.1.6 YPD-Copper Sulphate

Bacto- yeast extract (10g) (BD), Bacto-peptone (20g) (BD), Glucose (20g) and Bacto-agar (20g) were added to 1000ml of distilled water, and dissolved. The solution was then autoclaved and allowed to cool. 10mM, 12mM and 14mM of $CuSO_4$ was added to agar, poured into petri dishes under sterile conditions, and stored at 4^{o}C.

2.1.7 YPD-Iron Sulphate

Bacto- yeast extract (10g) (BD), Bacto-peptone (20g) (BD), Glucose (20g) and Bacto-agar (20g) were added to 1000ml of distilled water, and dissolved. The solution was then autoclaved and allowed to cool, 2mM, 3mM and 4mM of $FeSO_4$ was added to agar and poured into petri dishes under sterile conditions and stored at 4^{o}C.

2.1.8 YPD-Sorbitol

Bacto-yeast extract (10g) (BD), Bacto-peptone (20g) (BD), glucose (20g) and 1M sorbitol were added to 1000ml of distilled water, and dissolved. The solution was autoclaved and stored at room temperature.

2.1.9 YPD-Sorbitol (Agar)

Bacto- yeast extract (10g) (BD), Bacto-peptone (20g) (BD), Glucose (20g), Bacto-agar (20g) and 1M Sorbitol were added to 1000ml of distilled water, and dissolved. The solution was then autoclaved and allowed to cool and poured into petri dishes under sterile conditions, and stored at 4^{o}C.

2.1.10 Drop out mixture

Two grams of Alanine, Arginine, Aspargine, Aspartic Acid, Cysteine, Glutamic Acid, Glutamine, Glycine, Isoleucine, Lysine, Methionine, Phenylalanine, Proline, Serine, Threonine, Tyrosine and Valine were ground in a mortar and pestle. This mixture was stored at room temperature.

2.1.11 Amino Acid stocks

Stock solution of the following amino acids were made by adding the amino acid at the following concentrations into distilled water; Adenine sulphate (0.2g/100ml), Uracil (0.2g/100ml), L-Tryptophan (1g/100ml), L-Histidine HCL (1g/100ml) and L-Leucine (1g/100ml). The amino acid stocks were dissolved and filter sterilized using 0.22µm Millex filter units (Millipore Corporation) into sterile tubes. Adenine sulphate and Uracil were stored at room temperature. L-Tryptophan, L-Histidine and L-Leucine were stored at 4^{o}C.

2.1.12 Synthetic complete (SC) and dropout media

Bacto-yeast Nitrogen Base without amino acids or ammonium sulfate (6.7g) (BD), Glucose (20g) and drop out mixture (2g) were added and dissolved in 1000ml

of distilled water. This mixture could then be supplemented with Adenine sulphate (10ml), L-Histidine (2ml), L-Leucine (2ml), L-Trpytophan (2ml) or Uracil (10ml). The solution was autoclaved and allowed to cool and stored at room temperature.

2.1.13 SD

Bacto-yeast Nitrogen Base without amino acids or ammonium sulfate (6.7g) (BD), Glucose (20g), drop out mixture (2g) and Bacto agar (20g) were added to 1000ml of distilled water. The solution was autoclaved and allowed cool. This mixture could then be supplemented with Adenine sulphate(10ml), L-Histidine (2ml), L-Leucine (2ml), L-Trpytophan (2ml) or Uracil (10ml). Agar was poured into Petri dishes, under sterile conditions and plates were stored at 4^{o}C.

2.1.14 5-Fluoro-orotic acid medium

Bacto-yeast Nitrogen Base without amino acids or ammonium sulfate (6.7g) (BD), Dropout mix (2g), Glucose (20g) (Sigma-Aldrich Chemical CO. Ltd. U.K.), Adenine (10ml), Histidine (2ml), Leucine (10ml), Tryptophan (2ml), Uracil (50mg) and 5-FOA were dissolved in 500ml of distilled water. This solution was filter sterilized. Bacto-agar (20g) (BD) was added to 500ml of distilled water and autoclaved. Agar was allowed cool and then mixed with the above mixture. Agar was poured into Petri dishes, under sterile conditions. The plates were stored at 4^{o}C.

2.1.15 Sporulation medium

Potassium acetate (10g) (Fluka), Bacto-yeast extract (1g) (BD), Glucose (.5g), Bacto-agar (20g) (BD) were dissolved in 1000ml of distilled water. The solution was

autoclaved and allowed cool. The agar was then poured into Petri dishes and allowed cool. Plates were stored at 4°C

2.1.16 Minimal sporulation medium

Potassium acetate (10g) (Fluka) and Bacto-agar (20g) (BD) were dissolved in 1000ml of distilled water. The solution was autoclaved and allowed cool. Agar was then poured under sterile conditions and allowed to cool. Plates were stored at 4°C.

2.1.17 LB

LB (25g) (BD) was dissolved in 1000ml of distilled water. The solution was autoclaved, and stored at room temperature.

2.1.18 Ampicillin

Sodium salt of Ampicillin (25mg) was dissolved in 1ml deionised water. The solution was stored at -20°C.

2.1.19 LB Ampicillan Agar

LB (25g) (BD) and Bacto-agar (20g) (BD) was dissolved in 1000ml of distilled water. The solution was autoclaved and cooled to room temperature. Ampicillin (50mg/ml) was added. Agar was poured into Petri dishes under sterile conditions. The plates were allowed set and stored at 4°C.

2.1.20 2YT

Yeast extract (10g) (Oxoid Ltd., Hampshire, England), Tryptone (16g) (Oxoid Ltd., Hampshire, England) and NaCl (5g) (Beijing, Shiji) was dissolved in 1000ml of

distilled water. The solution was autoclaved and cooled and stored at room temperature.

2.1.21 Chloramphenicol stock

Chloramphenicol (30µg/ml) (Inalco spa, milano, Italy) was made as 4000x stock, chloramphenicol (0.15g) was dissolved in molecular EtOH (1.2ml) (Beijing chemical works). Solution was stored at -20^{o}C.

2.1.22 2YT agar Chloramphenicol Ampicillin

Yeast extract (10g) (Oxoid Ltd., Hampshire, England), Tryptone (16g) (Oxoid Ltd., Hampshire, England) and NaCl (5g) (Beijing, Shiji) and agar (20g) was dissolved in 1000ml of distilled water. The solution was autoclaved and allowed cool to room temperature. Chloramphenicol (30µg/ml) and ampicillin (100µg/ml) were added to solution. Agar was pored under sterile conditions into Petri dishes, which were allowed to cool and stored at 4^{o}C.

2.2 Bacterial and yeast Plasmids and strains

2.2.1 Bacterial Plasmids

Plasmid	Description	Origin	Name
pPROEX-HTb SSA1	Bacterial expression.Contains SSA1 fused cleavable His(6X) Tag.	ADEgene	PRO-SSA1
pPROEX-HTb- SSA1^{G73D}	Bacterial expression. Contains SSA1^{G73D} His(6X). Mutation introduced by SDM.	This study	PRO-SSA1 ^{G73D}
pPROEX-HTb- SSA1^{G287D}	Bacterial expression. Contains SSA1 ^{G287D}His(6X). Mutation introduced by SDM.	This study	PRO-SSA1^{G287D}
pPROEX-HTb -SSA1 ^{T295I}	Bacterial expression. Contains SSA1 ^{T295I}His(6X). Mutation introduced by SDM.	This study	PRO-SSA1 ^{T295I}
pPROEX-HTb -SSA1 ^{F475S}	Bacterial expression. Contains SSA1 ^{F475S}His(6X). Mutation introduced by SDM.	This study	PRO-SSA1 ^{F475S}
pPROEX-HTB-SSA1- Δ20	Bacterial expression.Contains SSA1-His(6X) (Δ623-642).	Perret group	PRO- Δ20
pPROEX-HTB-SSA1- Δ40	Bacterial expression.Contains SSA1-His(6X) (Δ603-642).	Perret group	PRO- Δ40
pPROEX-HTB-SSA1- Δ60	Bacterial expression.Contains SSA1-His(6X) (Δ583-642).	Perret group	PRO- Δ60
pPROEX-HTB-SSA1- ΔA	Bacterial expression.Contains SSA1-His(6X) (Δ1-380).	Perret group	PRO- ΔA
pPROEX-HTB-SSA1- ΔC	Bacterial expression.Contains SSA1-His(6X) (Δ525-642).	Perret group	PRO- ΔC
pPROEX-HTB-SSA1- ΔAC	Bacterial expression.Contains SSA1-His(6X) (Δ1-380 + Δ525-642).	Perret group	PRO- ΔAC

Table 2.1: Bacterial plasmids

2.2.2 SSA1 and mutant plasmids

Plasmid	Description	Origin	Name
pC210-*SSA1*	pRS315 carrying *SSA1* under *SSA2* promoter- *LEU2* selection.	YG	pC210-SSA1
pDCM64	pDCM64 is pRS313 carrying *SSA2* gene- *LEU2* selection	Daniel C. Masison	pC210-SSA2
pC210-*SSA3*	pRS315 carrying SSA3 under SSA2 promoter *LEU2* selection	This study	pC210-SSA3
PA4	pRS315 carrying SSA4 under SSA2 promoter *LEU2* selection	Daniel C. Masison	pC210-SSA4
pJ120	pRS315 carrying SSA1 with 500bp 5' and 3' flanking regions *LEU2* selection	Jung *et al*. 2000	pJ120
pJ120 ^{G73D}	G73D introduced by Random mutagenesis- *LEU2* selection	Loovers et al	pJ120 ^{G73D}
pJ120 ^{G287D}	G287D introduced by Random mutagenesis- *LEU2* selection	Loovers et al	pJ120 ^{G287D}
pJ120 ^{T295I}	T295I introduced by random mutagenesis- *LEU2* selection	Loovers et al	pJ120 ^{T295I}
pJ120 ^{F475S}	F475S introduced by Random mutagenesis- *LEU2* selection	Loovers et al	pJ120 ^{F475S}
pJ120 $^{F475S+A394V}$	A394V introduced by random mutagenesis- *LEU2* selection	This study	pJ120 $^{F+A394V}$
pJ120 $^{F475S+P432S}$	P432S introduced by random mutagenesis- *LEU2* selection	This study	pJ120 $^{F+P432S}$
pJ120 $^{F475S+V477I}$	V477I introduced by random mutagenesis- *LEU2* selection	This study	pJ120 $^{F+V477I}$
pJ120 $^{F475S+A519T}$	A519T mutation introduced by SDM- *LEU2* selection	This study	pJ120 $^{F+A519T}$
pJ120 $^{F475S+E540K}$	E540K mutation introduced by SDM- *LEU2* selection	This study	pJ120 $^{F+E540K}$
pJ120 $^{F475S+P636S}$	P636S mutation introduced by SDM- *LEU2* selection	This study	pJ120 $^{F+P636S}$
pJ121	pJ120 carrying SSA1 ^{L483W} introduced by random mutagenesis *LEU2* selection	Daniel C. Masison	pJ120 ^{L483W}
pJ121 ^{A519T}	A519T introduced by random mutagenesis- *LEU2* selection	Daniel C. Masison	pJ120 $^{L+A519T}$
pJ121 ^{E540K}	E540K introduced by random mutagenesis- *LEU2* selection	Daniel C. Masison	pJ120 $^{L+E540K}$
pJ121 ^{P636s}	P636S introduced by random mutagenesis- *LEU2* selection	Daniel C. Masison	pJ120 $^{L+P636S}$
pC210-SSA1-His(6X)	SSA1-His(6X) amplified from PRO-SSA1 and cloned into pC210- *LEU2* selection	This study	pC210-SSA1-H
pC210 -SSA1-His(6X) ^{G73D}	G73D mutation introduced by SDM- *LEU2* selection	This study	pC210-SSA1-H ^{G73D}

Plasmid	Description	Origin	Name
pC210-*SSA1*-His(6X) ^{G287D}	G287D mutation introduced by SDM- *LEU2* selection	This study	pC210-SSA1-H ^{G287D}
pC210-*SSA1*-His(6X) ^{T295I}	T295I mutation introduced by SDM-*LEU2* selection	This study	pC210-SSA1-H ^{T295I}
pC210-*SSA1*-His(6X) ^{F475S}	F475S mutation introduced by SDM *LEU2* selection	This study	pC210-SSA1-H ^{F475S}
SSA2 ^{G73D}	G73D mutation introduced into pDCM64 by SDM	This study	
SSA2 ^{G287D}	G287D mutation introduced into pDCM64 by SDM	This study	
SSA2 ^{T295I}	T95I mutation introduced into pDCM64 by SDM	This study	
SSA2 ^{F475S}	F475S mutation introduced into pDCM64 by SDM	This study	*SSA2* ^{F475S}
pC210-*SSA3* ^{G73D}	G73D mutation introduced into *SSA3*-pC210 by SDM	This study	*SSA3* ^{G73D}
pC210-*SSA3* ^{G287D}	G287D mutation introduced into *SSA3*-pC210 by SDM	This study	*SSA3* ^{G287D}
pC210-*SSA3* ^{T295I}	T95I mutation introduced into SSA3-pC210 by SDM	This study	*SSA3* ^{T295I}
pC210-*SSA3* ^{F475S}	F475S mutation introduced into *SSA3*-pC210 by SDM	This study	*SSA3* ^{F475S}
pC210-*SSA4* ^{G73D}	G73D mutation introduced into PA4 by SDM	This study	*SSA4* ^{G73D}
pC210-*SSA4* ^{G287D}	G287D mutation introduced into PA4 by SDM	This study	*SSA4* ^{G287D}
pC210-*SSA4* ^{T295I}	T95I mutation introduced into PA4 by SDM	This study	*SSA4* ^{T295I}
pC210-*SSA4* ^{F475S}	F475S mutation introduced into PA4 by SDM	This study	*SSA4* ^{F475S}
pRDW10	YCp50 carrying *SSA1* with 500bp 3' and 5' flanking DNA-*URA3* selection	Daniel C. Masison	pRDW10

Table 2.2 SSA1 and mutant plasmids

2.2.3 Truncated SSA1 Mutants

Plasmid	Description	Origin	Name
pJ120-Δ20	pJ120-SSA1 containing a 20 Amino Acid deletion at C-terminus-- *LEU2* selection	Jones group	Δ20
pJ120-Δ40	pJ120-SSA1 containing a 40 Amino Acid deletion at C-terminus- *LEU2* selection	Jones group	Δ40
pJ120-Δ60	pJ120-SSA1 containing a 60 Amino Acid deletion at C-terminus- *LEU2* selection	Jones group	Δ60
pC210- Δ20- His(6X)	Δ20- His(6X) amplified from PRO- Δ20 and cloned into pC210 - *LEU2* selection	This study	Δ20-H
pC210- Δ40- His(6X)	Δ40- His(6X) amplified from PRO- Δ40 and cloned into pC210 - *LEU2* selection	This study	Δ40-H
pC210- Δ60- His(6X)	Δ60- His(6X) amplified from PRO- Δ60 and cloned into pC210 - *LEU2* selection	This study	Δ60-H
pC210- ΔA- His(6X)	ΔA- His(6X) amplified from PRO- ΔA and cloned into pC210 - *LEU2* selection	This study	ΔA-H
pC210- ΔC- His(6X)	ΔC- His(6X) amplified from PRO- ΔC and cloned into pC210 - *LEU2* selection	This study	ΔC-H
pC210- ΔAC- His(6X)	ΔAC- His(6X) amplified from PRO- ΔAC and cloned into pC210 - *LEU2* selection	This study	ΔAC-H

Table 2.3: Truncated SSA1 mutants

2.2.4 Other Plasmids

Plasmid	Description	Origin/Reference	Name
Pzfo	YCp50-based plasmid containing the *SSA1* promoter and the first 30 bases of *SSA1* coding DNA fused in frame to *E.coli lacZ* gene.	Stone and Craig.1990.	pZFO
PUPR	Plasmid containing *UPRE* promoter fused in frame to *E.coli lacZ* gene.	J. Brodsky, University of Pittsburgh	pUPR
pPDR5	Plasmid containing *PDR5* promoter fused in frame to *E.coli lacZ* gene.	Gift from Scott Moye-Rowly, University of Iowa	pPDR5
pDCM90	*URA3*-based single-copy plasmid containing a gene for expression of a thermolabile bacterial luciferase	Daniel C. Masison	pDM90
pRS315	Yeast CEN plasmid containing *LEU2* selection	Sikorski and Hieter 1989	pRS315
BCK	Plasmid constitutively expressing *BCK1*	Levin	BCK
PKC	Plasmid constitutively expressing *PKC1*	Levin	PKC

Table 2.4 Other plasmids used in study

2.2.5 Bacterial Strains

Bacterial strain	Genotype
DH5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*
BL21 (DE3) pLysS	B F– *dcm ompT hsdS* ($r_B^- m_B^-$) *gal* λ(DE3) [pLysS Camr]
BL21 (DE3) pLysS- PRO-SSA1	B F– *dcm ompT hsdS* ($r_B^- m_B^-$) *gal* λ(DE3) [pLysS Camr]/ PRO-SSA1
BL21 (DE3) pLysS- PRO-SSA1^{G73D}	B F– *dcm ompT hsdS* ($r_B^- m_B^-$) *gal* λ(DE3) [pLysS Camr]/ PRO-SSA1^{G73D}
BL21 (DE3) pLysS- PRO-SSA1^{G287D}	B F– *dcm ompT hsdS* ($r_B^- m_B^-$) *gal* λ(DE3) [pLysS Camr]/ PRO-SSA1^{G287D}
BL21 (DE3) pLysS- PRO-SSA1^{T295I}	B F– *dcm ompT hsdS* ($r_B^- m_B^-$) *gal* λ(DE3) [pLysS Camr]/ PRO-SSA1^{T295I}
BL21 (DE3) pLysS- PRO-SSA1^{F475S}	B F– *dcm ompT hsdS* ($r_B^- m_B^-$) *gal* λ(DE3) [pLysS Camr]/ PRO-SSA1^{F475S}
C41 (DE3)	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)
C41 (DE3)-YDJ1	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)-Ydj1

Table 2.5 Bacterial strains. Bacterial strains and genotypes listed that were utilized in this study**.** The DH5α strain was used to amplify plasmid stocks. The BL21 (DE3) and C41 (DE3) were used for purifying Ssa1 and Ydj1 respectively.

2.2.6 Yeast Strains

Strain name	Genotype
G600	(MATA *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3)*
G402	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3*-1f/pRDW10
G402-pJ120	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3*-1f/pJ120
G402-315	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3*-1f/pRS315
Ssa1	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/pC210
Ssa2	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/pDCM64
Ssa3	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/pC210-*SSA3*
Ssa4	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/PA4
$Ssa1^{G73D}$	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ $pJ120^{G73D}$
$Ssa1^{G287D}$	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ $pJ120^{G2877D}$
$Ssa1^{T295I}$	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ $pJ120^{T295I}$
$Ssa1^{V439I}$	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ $pJ120^{V439I}$
$Ssa1^{F475S}$	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ $pJ120^{F475S}$
$Ssa1^{G481D}$	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ $pJ120^{G481D}$
$Ssa1^{L483W}$	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ $pJ120^{L483W}$
$Ssa1^{M515I}$	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ $pJ120^{M515I}$
$Ssa1^{S545F}$	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ $pJ120^{S545F}$
$Ssa1^{L+A519T}$	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ $pJ120^{L+A519T}$
$Ssa1^{L+E540K}$	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ $pJ120^{L+E540K}$
$Ssa1^{L+P636S}$	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ $pJ120^{L+P636S}$
$Ssa1^{F+A394V}$	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ $pJ120^{F+A394V}$
$Ssa1^{F+P433S}$	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ $pJ120^{F+P433S}$
$Ssa1^{F+V477I}$	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ $pJ120^{F+V477I}$

Ssa1 $^{F+P636S}$	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pJ120 $^{F+P636s}$
Ssa2 ^{G73D}	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pDCM64 ^{G73D}
Ssa2 ^{G287D}	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pDCM64 ^{G287D}
Ssa2 ^{T295I}	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pDCM64 ^{T295I}
Ssa2 ^{F475S}	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pDCM64 ^{F475S}
Ssa3 ^{G73D}	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pC210-Ssa3 ^{G73D}
Ssa3 ^{G287D}	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pC210-Ssa3 ^{G287D}
Ssa3 ^{T295I}	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pC210-Ssa3 ^{T295I}
Ssa3 ^{F475S}	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pC210-Ssa3 ^{F475S}
Ssa4 ^{G73D}	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ PA4 ^{G73D}
Ssa4 ^{G287D}	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ PA4 ^{G287D}
Ssa4 ^{T295I}	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ PA4 ^{T295I}
Ssa4 ^{F475S}	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ PA4 ^{F475S}
Δ20-pre	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pRDW10/ pJ120- Δ20
Δ40-pre	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pRDW10/ pJ120- Δ40
Δ60-pre	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pRDW10/ pJ120- Δ60
Δ20	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pJ120- Δ20
Δ40	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pJ120- Δ40
Δ20-His	(*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pJ120- Δ20-His
Δ40-His	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pJ120- Δ40-His
Δ60-His	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pJ120- Δ60-His
Ssa1-His	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pC210-SSA1-H
Ssa1-His ^{G73D}	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pC210-SSA1-H ^{G73D}
Ssa1-His ^{T295I}	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pC210-SSA1-H ^{T295I}
Ssa1-His ^{F475S}	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pC210-SSA1-H ^{F475S}

PDR5-Ssa1 ^{G73D}	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pJ120^{G73D} /PDR5
PZFO- Ssa1 ^{G73D}	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pJ120 ^{G73D} /pZFO
UPR- Ssa1 ^{G73D}	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pJ120 ^{G73D} /pUPR
PDR5-Ssa1 ^{G287D}	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pJ120^{G287D} /PDR5
PZFO- Ssa1 ^{G287D}	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pJ120 ^{G287D} /pZFO
UPR- Ssa1 ^{G287D}	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pJ120 ^{G287D} /pUPR
PDR5-Ssa1 ^{T295I}	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pJ120^{T295I} /PDR5
PZFO- Ssa1 ^{T295I}	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pJ120 ^{T295I} /pZFO
UPR- Ssa1 ^{T295I}	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pJ120^{T295I} /pUPR
PDR5-Ssa1^{F475S}	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pJ120^{F475S} /PDR5
PZFO- Ssa1^{F475S}	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pJ120 ^{F475S}/pZFO
UPR- Ssa1^{F475S}	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pJ120^{F475S} /pUPR
Ssa1-Luc	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/pC210/pDM90
Ssa2-Luc	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/pDCM64/pDM90
Ssa3-Luc	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/pC210-*SSA3/pDM90*
Ssa4-Luc	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/PA4/pDM90
Ssa1^{F475S} -Luc	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/pJ120 ^{F475S} /pDM90
Ssa1-BCK	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/pC210 /BCK
Ssa1-PKC1	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/pC210 /PKC1
Ssa1^{F475S-} BCK	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/pJ120 ^{F475S} /BCK
Ssa1^{F475S-} PKC1	*MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/pJ120 ^{F475S} /PKC1

Table 2.6 Yeast strains. List of all yeast strains and their genotypes used in this study. All Ssa mutant analysis was carried out in the G402 strain

2.3.0 Fungal and bacterial culture conditions

2.3.1 Fungal culture conditions

All yeast stocks were stored in a sterile mixture of 85% YPD and 15% glycerol at -70^{o}C. Working stocks of strains were restreaked onto fresh YPD agar every 3 weeks, grown at 30^{o}C for forty-eight hours and stored at 4^{o}C. For liquid culturing, yeast strains were grown overnight at 30^{o}C in a conical flask to the cell density required, at 200 RPM (Innova 4000) using appropriate media.

2.3.2 Bacterial growth conditions

All DH5α bacterial strains were incubated at 37^{o}C on LB AMP agar for 16 hours and stored at 4^{o}C. For liquid culture, DH5α bacterial strains were cultured overnight in LB AMP media at 37^{o}C in a shaking incubator (Innova 44). All BL21 strains were incubated at 37^{o}C on 2YT agar with chloramphenicol (30µg/ml) and ampicillin (50mg/ml) for 16 hours and stored at 4^{o}C. For liquid culture, BL21bacterial strains were cultured in 2YT media overnight at 37^{o}C in a shaking incubator (Innova 44)

2.4.0 Preparation of component and transformation of Bacterial and Yeast cells

2.4.1 Preparation of competent *E.coli* (DH5α)

20ml of LB was inoculated with a single colony of DH5α. *E.coli* was incubated at 37^{o}C overnight in shaking incubator (Innova 44) at 250 RPM. Cells were transferred to one liter of LB and grown until culture reached an OD_{600} between 0.6-1.0 (≈ three hours). The culture was transferred to 250ml centrifuge tubes and bacterial cells were pelleted at 3913Xg for ten minutes at 4^{o}C. Pellet was resuspended in 10ml of RF1 (KAc 30mM, CaCl2.2H2O 10mM, glycerol 15% and adjust to pH 5.92 with 0.2M acetic acid and RbCl 100mM and MnCl2.4H2O 50mM was added) and incubated on

ice for thirty minutes. *E.coli* was pelleted at 3170Xg for fifteen minutes at 4°C. Pellet was resuspended in 2mlsRF2 (RbCl 100mM and CaCl2.2H_2O 75mM) and incubated on ice. Transfer component *E.coli* to pre chilled 1.5ml microfuge tubes in 50µl aliquots, snap freeze using liquid Nitrogen and store at -80°C.

2.4.2 Transformation of competent DH5α

To amplify plasmids; LB-Amp plates were incubated at 37°C for one hour. Component cells (50µl) were defrosted on ice and 1µl of DNA was added. Cells were incubated on ice for five minutes and were immediately plated onto warm LB-Amp plate under sterile conditions and incubated at 37°C overnight. Transformation of plasmids for site-directed mutagenesis and yeast plasmid preps were carried out by adding 8µl of DNA to 100µl of component cells and incubated on ice for thirty minutes. Cells were then heat shocked at 42°C for fifty seconds and placed on ice for a further two minutes. LB (400µl) was added and cells were incubated at 37°C shaking for one hour. Cells were plated on LB-Amp under sterile conditions and incubated at 37°C overnight.

2.4.3 Preparation of elecrto-competent DH5α

Inoculated 20ml of LB with a single colony of DH5α and incubated at 37°C shaking overnight. Overnight culture was transferred to 1 liter of LB and grown at 37°C shaking until an OD_{600} of 0.8 was reached. Cells were chilled on ice for thirty minutes. Cells were harvested in a GSA rotor at 3913Xg for ten minutes at 4°C. Pellet was resuspended in one liter of ice-cold dH_20 and centrifuged at 3913Xg for ten minutes at 4°C. Pellet was resuspended in 500ml of ice-cold dH_20 and centrifuged at 3913Xg for ten minutes at 4°C. Pellet was resuspended in 250ml of ice-cold dH_20

and centrifuged at 3913Xg for ten minutes at 4°C. Pellet resuspended in 100ml of ice cold dH_20 and centrifuged at 3170Xg for fifteen minutes at 4°C. Pellet resuspended in 1ml 10% glycerol (chilled). Electro-competent cells were transferred to 1.5ml microfuge tubes as 40µl aliquots and stored at -80°C.

2.4.4 Transformation of electro-competent DH5α

Electro-competent DH5α were thawed on ice. Electroporation cuvette (Gene pulser cuvettes, Bio Rad) were chilled on ice. 20µl of cells and 20µl of DNA (concentration)/stH_20 were pipetted into chilled cuvette. Cuvettes were chilled on ice for thirty minutes and then placed in cuvette holder of the MicroPulser electroporator (Bio Rad) which was set to EC2 and cells were pulsed for approximately 3-4 seconds. LB (600µl) was immediately added to cuvette and cells were transferred to microfuge tube and incubated at 37°C shaking for one hour. Following the one hour recovery incubation, cells were plated on LB-AMP under sterile conditions and placed at 37°C overnight.

2.4.5 Preparation of competent BL21 and C41 cells

2YT(50ml) was inoculated (plus chloramphenicol 30µg/ml for BL21) with a single colony of *E.coli*. Cells were grown at 37°C shaking until an OD_{600} of 0.5 is reached. Spin cells at 3913Xg for ten minutes at 4°C. Keep cells on ice. Resuspend in 15ml of 0.1M $CaCl_2$ and centrifuge at 3913Xg for ten minutes at 4°C. Resuspend pellet in 15ml of 0.1M $CaCl_2$ and incubate on ice for twenty minutes. Resuspend pellet in 5ml of 20% glycerol, transfer to pre chilled 1.5ml microfuge tube in 50µl aliquots, snap freeze and store at -80°C.

2.4.6 Transformation of competent BL21 and C41 cells

Competent cells (50µl) were defrosted on ice. DNA (1µl) was added and cells were incubated on ice for thirty minutes. Cells were then placed at 42^{o}C for ninety seconds and immediately plated onto 2YT-Amp- chloramphenicol (BL21) or 2YT-AMP (C41) under sterile conditions. Plate was incubated at 37^{o}C overnight.

2.4.7 Preparation of competent Yeast cells

Inoculated 5ml of YPD or selective media with appropriate yeast strain. Cells were incubated at 30^{o}C shaking overnight. Overnight culture was diluted to a concentration of 5x10^{6} cells/ml in 50ml of YPD. Culture was grown until a concentration of 1x10^{7}-2x10^{7} was reached. Cells were pelleted at 978Xg for five minutes and resuspended in 25ml of dH_20 and spun at 978Xg for 5 minutes. Pellet was resuspended in 1ml of 100mM LiAc and transferred to 1.5ml microfuge tube. Cells pelleted at 17,900 x g for 5 seconds, supernatant was discarded. Pellet resuspended in 500µl of 100mM LiAc. Component cells can be used immediately or stored for up to two weeks at 4^{o}C.

2.4.8 Transformation of competent Yeast cells

Component yeast cells (50µl) were centrifuged at 17,900 x g and supernatant was discarded. The following reagents were added in the order; 240µl 50% PEG, 36µl 1M LiAc, 25µl carrier DNA, DNA mix (200ng DNA mixed with sterile H_20 to a final volume of 50µl). Cells and transformation reagents were mixed thoroughly and placed at 30^{o}C static for thirty minutes. Cells were then placed at 42^{o}C for twenty minutes and then pelleted at 7669Xg for fifteen seconds. Transformed cells were then plated

on appropriate selective media under sterile conditions and incubated at 30^{o}C for forty-eight hours

2.4.8.1 Plasmid shuffle technique

To express mutants of Ssa1 as the sole source of the Ssa family in *S.cerevisiae,* the plasmid shuffle technique was employed. The G402 strain contains the plasmid pRDW10, which contains a Uracil marker and is the sole source of Ssa in the strain. To analyze the affects of mutations in Ssa1 that are expressed from plasmids that contain alternative marker to Uracil, mutant plasmids are transformed into G402 (2.4.8) on media for sufficient selection of mutant plasmid. Transformants were restreaked onto media for sufficient selection of mutant plasmid, and incubated at 30^{o}C for forty-eight hours. The pRDW10 plasmid was eliminated from the strain by replicating transformants onto 5-FOA (2.1.14), which selected against plasmids with Uracil markers.

2.5 Molecular techniques

2.5.1 Preparation of Agarose Gel

Agarose gels (0.8%), were prepared by adding 0.8g of agarose to 50ml of 1XTAE buffer (diluted from 50X TAE buffer; 242g Tris, 100ml 0.5M EDTA ph 8.0 and acetic acid and bring to a final volume of 1000ml with stH20). Solution was heated for approximately one minute until agarose was completely dissolved and poured into gel casting tray. Gel was allowed to cool slightly, 1µl of ethidium bromide was added and mixed with a yellow tip. Comb was placed in gel and gel was allowed set. All gels were run in 1X TAE buffer at 90V for approximately thirty minutes.

2.5.2 Restriction Digestion

All restriction digests were carried out using enzymes and reagents from New England Biolabs inc. A typical digest to prepare pC210 for cloning would be carried out by adding 40µl pC210, 2µl *Nde*1, 2µl *Sph*1, 6µl Buffer 2 (10X) and 10µl molecular H_20. Digestion was incubated at 37^oC for three hours. Diagnostic digests were preformed by adding 5µl of potential clone, 0.5µl *Nde*1, 0.5µl *Sph*1 and 2µl Buffer 2 (10X) and 12µl molecular H_20. Digestions run on a 0.8% agarose gel.

2.5.3 Site-Directed Mutagenesis (SDM)

Mutant alleles were introduced using the appropriate primers with DNA base pair mismatch (Table 2.8). PFU Ultra (Stratagene, La Jolla, CA, USA) was used to amplify the mutant plasmid under the cycle conditions [95^oC for 1 min, 95^oC for 30secs, 55^oC for 1 min, 68^oC extension, 68^oC for 7min, cycle repeated 12 times]. The parental plasmid was digested by 1µl *dpn*1 (Invitrogen) and potential mutant plasmids are transformed into component DH5α (section 2.4.2), mutation confirmed by sequencing (Agowa, LGC)

Primer Name	Primer Sequence 5'-3'
SSA1 G73D- F	CTAAGCGTTTGATTCGGTAGAAACTTCAACGAC
R	GTCGTTGAAGTTTCTACCGAATCAAACGCTTAG
SSA1 G287D F	GACTCTTTGTTCGAAGGTATCGATTTCTACACT
R	AGTGTAGAAATCGATACCTTCGAACAAAGAGTC
SSA1 T295I F	TTCTACACTTCCATCACCAGAGCCAGATTCGAAG
R	CTTCGAATCTGGCTCTGGTGATGGAAGTGTAGAA
SSA1 F475S F	TGTCCCACAAATTGAAGTCACTTCCGATGTCGACTCTAACGG
R	CCGTTAGAGTCGACATCGGAAGTGACTTCAATTTGTGGGACA
SSA1 A519T-F	GAAAGAGTGGTTGCTGAAGCCGAAAAATTCAAGGAAG
R	CTTCCTTGAATTTTTCGGCTTCAGCAACCACTCTTTC
SSA1-E540K-F	CTTCCAAGAACCAATTGGAATCCATTGCTTACTC
R	GAGTAAGCAATGGATTCCAATTGGTTCTTGGAAG
SSA1- P636S-F	CCAGAGGCTGAAGGTTCAACCGTTGAAGAAGTTG
R	CAACTTCTTCAACGGTTGAACCTTCAGCCTCTGG

Table 2.7 SDM primers. Primers designed to insert single base mutations into *SSA1*.

Primer name	Primer Sequence 5'-3'
SSA2-G73D-F	GCTAAGCGTTTGATCGATAGAAACTTTCAATGAC
SSA2-G73D-R	GTCATTGAAAGTTTCTATCGATCAAACGCTTAGC
SSA2-F475S-F	CAAATTGAAGTCACTTCCGATGTCGACTCTAAC
SSA2-F475S-R	GTTAGAGTCGACATCGGAAGTGACTTCAATTTG
SSA3- G73D- F	AAGCGGTTAATTGATCGTAAATTTGATG
SSA3-G73D-R	CATCAAATTTACGATCAATTAACCGCTT
SSA3- G287D- F	GATTCTTTATTTGAAGATATGGATTTCTACACT
SSA3-G287D-R	AGTGTAGAAATCCATATCTTCAAATAAAGAATC
SSA3- T295I- F	TTCTACACTTCGTTAATAAGGGCAAGGTTTGAA
SSA3-T295I-R	TTCAAACCTTGCCCTTATTAACGAATGTAGAA
SSA3- F475S- F	CAAATTGATGTTACCTCTGATATCGACGCTAAT
SSA3-F475S-R	ATTAGCGTCGATATCAGAGGTAACATCAATTTG
SSA4- G73D- F	GCTAAGCGTCTGATCGATCGTAAATTCGATGATC
SSA4-G73D-R	GATCATCGAATTTACGATCGATCAGACGCTTAGC
SSA4- G287D- F	GATTCATTATTTGAGGATATCGATTTCTATACTTC
SSA4-G287D-R	GAAGTATAGAAATCGATATCCTCAAATAATGAATC
SSA4- T295I- F	GGTATCGATTTCTATATTTCCATTACAAGGGCAAG
SSA4-T295I-R	CTTGCCCTTGTAATGGAAATATAGAAATCGATACC
SSA4- F475S- F	CAAATTGAAGTTACATCTGATATCGATGCAAATG
SSA4-F475SR	CATTTGCATCGATATCAGATGTAACTTCAATTTG

Table 2.8 SDM primers. Primers designed to insert single base mutations into *SSA2*, *SSA3* and *SSA4*.

2.5.4 Homologous recombination

2.5.4.1 PCR

DNA was amplified to include flanking regions that are homologous to regions of plasmid/region of genome in which DNA will integrate. Primers were designed to allow for this (Table 2.9) and may include diagnostic restriction sites. The PCR reaction was carried out using High-Fidelity Taq (Invitrogen) under recommended cycling conditions.

Primer name	Primer Sequence 5'-3'
SSA1-his-F	CAAGCAGATTTTATACAGAAATATTTATACATATGCTCGAGCATCACCATCACCATCAC
SSA1-his-R	CTTCCTGATTAAACAGGAAGACAAAGCATGCGGATCCTTAATCAACTTCTTCAACGG
SSA1-China-F	CAAGCAGATTAAACAGGAAGACAAATTAACCGCCTGGAGCACCACCTGCAGC
SSA1-Δ20HR-R	CTTCCTGATTAAACAGGAAGACAAATTAACCGCCTGGAGCACCACCTGCAGC
SSA1-Δ40HR-R	CTTCCTGATTAAACAGGAAGACAAATTACATCATTGGGTTGGCAATGTCTTG
SSA1-Δ60HR-R	CTTCCTGATTAAACAGGAAGACAAATTAGGCAGTGGTGTTGCTGTCTAACC

Table 2.9 Homologous recombination primers. Primers containing 5' and 3' pC210 flanking regions designed to amplify gene of interest.

2.5.4.2 Cloning by homologous recombination

A restriction digest was carried out on pC210 (section 2.5.2) and incubated at 37^{o}C for three hours. Restriction digest was run on an agarose gel (0.8%) and digested plasmid was extracted according to manufacturers' recommendations (Qiagen Gel extraction kit). Yeast transformation preformed as in section 2.4.4 except DNA mix

consists of 45µl PCR products and 5µl of digested pC210. Transformants were screened on selective media. Yeast plasmids were extracted as in section 2.6.1, diagnostic digests and sequencing confirmed successful clones.

2.5.5 Sequencing primers

Sequencing of plasmids was carried out using Agowa genomics sequencing services (100ng DNA, 4µl primer, xµl molecular H_20 made to a total volume of 15µl). Primers were designed to cover complete sequences of *SSA1, SSA2, SSA3* and *SSA4* (Table 2.10).

Primer name	Primer sequence 5'-3'
SP1	CTCATTATACCCAGATCA
SP2	CTTTTCGACGGCGGAAAC
SP3	CATCGACTTGAGATTTG
SP4	CAGCGATTGACCTTGGC
SP5	CGAGAAGGATTGAGTTG
SP6	GGTCTTGGGTAAGATG
SP7	CAGATCTACTTTGGACCC
SP8	CAAAGGCGTGTCCAC
SSA2-1	CCTGTGTTGCTCAC
SSA2-2	CACTTCATCCAAGAATTC
SSA2-3	CTACTTATGCTGACAAC
SSA3-1	GATGATCCTGAAGTGACG
SSA3-2	GATTCGAAGCTGGACAAGTC
SSA3-3	GTAGGCTCTCGAAGGATG
SSA3-7	CTTGTATGTCAATGTTTG
SSA3-8	GACACTCATTTAGGTG
SSA3-9	GGTAGAGAAGGTTTCCG
SSA4-1	GAACGATGCTAAGCATTACC
SSA4-2	GATCTACATTGGAGCCAG
SSA4-3	GAACGTATCTGCCGTTGA
SSA4-4	GCGAAGCATCTAACCAAT
SSA4-5	GTTAAGATGGCAGCCTGTAC
SSA4-6	GATACGAAGAACGTTCAAGC

Table 2.10 Sequencing primers

2.5.6 Random Mutagenesis of pJ120^{F475S}

pJ120^{F475S} was incubated in the mutagen hydroxylamine (20µl EDTA 200mM, 200µl NaCl 1M, 138.98mg of hydroxylamine, 44.6mg of Sodium Pyrophosphate added to 1.8ml of dH_2O) at 70^{o}C for one hour. This reaction was stopped by placing microfuge tube on ice. Excess hydroxylamine was removed from pJ120^{F475S} by performing PCR purification according to manufacturer's recommendations (Qiagen, PCR purification kit). Mutagenized plasmid library was transformed into competent DH5α by electroporation (section 2.4.4). Approximately 20,000 cells are required for this process; therefore, electroporation was repeated twenty times with approximately 1000 colonies on each plate. Mutagenized plasmids were retrieved from *E.coli* by pipetting 1ml of LB onto LB-AMP plates containing mutagenised colonies. Cells were scrapped from plate using a yellow tip into 1ml LB, LB containing cells was removed from plate by pipetting, and added to next plate were the process was repeated twenty times. Plasmid library retrieved from 1ml of LB by carrying out miniprep by manufacturer's recommendations (Qiagen, miniprep kit).

2.5.6.1 Isolation of second-site suppressors of pJ120^{F475S}

The yeast strain G402 was transformed with pJ120^{F475S} mutagenised plasmid library. Second-site suppressors of F475S were screened for by selecting plasmids on media lacking leucine followed by using plasmid-shuffle technique. 5-FOA plates were incubated at 37^{o}C for 48 hours. Colonies with growth at 37^{o}C were selected and restreaked onto media lacking leucine and then replicated onto media lacking Uracil to ensure pRDW10 was not present. Yeast plasmids were retrieved as described in section 2.6.1 and second-site suppressor mutations were located by sequencing analysis (section 2.5.6)

2.5.7 RT-PCR

RNA was extracted from strains using the RNAeasy kit (Sigma) following manufacturers recommendations (section 2.6.3). To ensure that sample was DNA free, samples were treated using the Sigma DNase kit. 1µg of RNA was added to 1µl of 10X buffer and 1µl of DNase, and the reaction was brought to a final concentration of 10µl using stH_20. The reaction was left at room temperature for fifteen minutes and digestion was stopped by adding 1µl of EDTA and incubating at 70^oC for ten minutes. To synthesize cDNA, 8µl of the digestion was added to 1µl of 50µM oligo dT and 1µl 10mM dNTPs and incubate at 65^oC for five minutes and then for one minute on ice. Reaction mixture was prepared by adding 2µl 10RT buffer, 4µl 25mM $MgCL_{2,}$ 2µl 0.1M DTT, 1µl RNase out and 1µl Superscript II . 10µl of reaction mixture was added to 10µl RNA mixture and was mixture was incubated at 50^oC for minutes. Reaction was stopped by incubating mixture at 85^oC for five minutes and chilling it on ice.1µl RNaseH was added to solution and incubated at 37 ^oC for twenty minutes. cDNA was then transferred to a PCR reaction containing 2.5µl of forward and reverse primer (Table 2.8), 2.5µl of PFU ultra buffer (10X) (Agilent technologies), 1µl dNTPS (Promega) and 1µl of PFU ultra (Agilent technologies). RT-PCR was preformed using the PFU cycling conditions; [95^oC for 1 min, 95^oC for 30secs, 55^oC for 1 min, 68^oC extension 2 mins per kb, , 68^oC for 7min,repeated 12 times].

Primer name		Primer sequence 5'-3'
PDR5	F	CATCGGTTTCACTTTCTTC
	R	GCTGCAGACGCGTTGGAG

PDR10	F	GAGTTGGACTGGATGGAAGG
	R	GATGGCCTTTCTCTAGCTTC
PDR15	F	GCAAAGGGATTTGTATGAG
	R	CCATATGTGCCGCTGTTTC
ACT1	F	GGGTTTGGAATCTGCC
	R	CCACCAATCCAGACGGAG

2.6 DNA and RNA extraction from yeast cells

2.6.1 Yeast plasmid prep

Yeast strains containing a specific plasmid were grown in 5ml of YPD or selective growth media overnight. Yeast cells were pelleted at 978Xg for five minutes and resuspended in 250µl of buffer P1 (Miniprep Kit, Qiagen) and transferred to microfuge tube. Approximately half the volumes of 0.5mm soda lime glass beads (Biospec products Inc,) were added, and vortexed at max speed for five minutes (vortex-2 gene, Scientific industries). Liquid was removed and transferred to new eppendorf. 250µl of lysis buffer (P2) and 350µl of Neutralization buffer (N3) (Miniprep Kit, Qiagen) was added to solution and gently mixed and centrifuges at 17,900 x g for ten minutes. Supernatant was removed and transferred to DNA binding column (Miniprep Kit, Qiagen). DNA binding column was centrifuged at 17,900 x g for one minute and flow through was discarded. Wash (750µl) solution was added and column was centrifuged at 17,900 x g for one minute. Flow through was discarded and columns were centrifuged for an additional minute to remove any residual buffer from the binding column. 50µl of elution buffer (Miniprep Kit, Qiagen) was added to column and allowed sit for two minutes. The binding column was transferred to fresh eppendorfs and plasmid DNA was isolated by a final one-minute centrifugation at 17, 900 x g.

To increase the plasmid concentration, 17µl of plasmid, retrieved from yeast was transformed into to *E.coli* as described in section 2.4.2. Plasmids were retrieved from *E.coli* using Qiagen miniprep kit by manufacturer's recommendations.

2.6.2 Isolation of Yeast genomic DNA

Yeast cells cultured in 1.5ml of YPD at 30^{o}C shaking overnight. Cells pelleted at 978Xg for five minutes. Pellets were resuspended in 1M Sorbitol/100mM EDTA. 12µl of Zymolase (5mg/ml) was added and eppendorfs were incubated at 37^{o}C for one hour. The cells were pelleted at 17,900 x g for five seconds and resuspended in 1M Tris/100mM EDTA. SDS 10% (15µl) was added and mixture was incubated at 65^{o}C for thirty minutes. KAc 5M (60µl) was added to and solution was incubated on ice for one hour. The mixture was then centrifuged at 17,900 x g for five minutes. Supernatant was retained, 195µl of 100% Isopropanol was added, and mixture was incubated at room temperature for five minutes DNA pellet was obtained by centrifugation of mixture at 17,900 x g for ten seconds. DNA pellet was resuspended in 45µl of molecular H_20 and stored at -20^{o}C.

2.6.3 RNA extraction

RNA extraction was preformed by using the Sigma RNAeasy kit. Yeast cells cultured in 5mls of YPD overnight. 1.5ml of the overnight culture was removed and transferred onto an eppendorf tube. Cells were centrifuged at 978 x g for five minutes at 4^{o}C and supernatant was removed. The pellet was resuspended in 600µl of buffer RLT. The same volume of glass beads (0.5mm soda lime) (Biospec products) were

added to resuspended cells and cells were vortexed for thirty seconds followed by thirty seconds on ice, five times. Cell lysates were centrifuged at 1308 x g for three minutes and supernatant was transferred into a new sterile tube. One volume of 70% EtOH was added to lysates and mixed well by pipetting, Lysates were then transferred to spin column and centrifuged and spun at 15,652 x g for fifteen seconds. Supernatant was discarded and 700µl of buffer RW1 was added to column, and centrifuged at 15,652 x g for fifteen seconds. Supernatant was discarded and 500µl of buffer RPE was added to column and spun at 15,652 x g for fifteen seconds. Supernatant was discarded and a further 500µl of buffer RPE was added to column and was centrifuged at 15,652 x g for two minutes. Column was then transferred into a new collection tube and spun at 15,652 x g for a further two minutes. Column was transferred into a new sterile 1.5ml tube and 40µl of stH_20 was added. Column was spun at 15,652 x g for one minute. RNA concentrations were determined by using the nanodrop (Nanodrop 1000, Mason).

2.7 Yeast growth assays

2.7.1 Yeast growth curve

Yeast strains were cultured overnight in 5ml of YPD or appropriate selective media at 30^{o}C shaking. Following incubation, the yeast cultures were diluted into 10ml of YPD at OD_{600} of 0.1. OD_{600} measurements were taken every two hours for approximately ten hours. Log values for OD_{600} values were obtained using excel and were plotted against time; the time points which gave the most linear point on graph were used in the following formula to calculate the mean generation time for each yeast strain; (Time (mins) /(LOG T1- LOG T0)/(LOG2). Mean generation times were plotted as minutes on excel graph.

2.7.2 Dot growth assay

Yeast strains were cultured overnight in 5ml of YPD or appropriate selective media at 30^{o}C shaking. Following incubation, the yeast cultures were diluted into 5ml of fresh media at an OD_{600} of 0.15. Cells were grown until they reached a final concentration of $5X10^{6}$ cells/ml; concentration was determined by counting cells on haemocytometer. Cells were pelleted at 978 x g for five minutes and resuspended at a concentration of $3\ X10^{6}$ cells/ml. A 1/5 serial dilution was performed on strains in a 96 well plate under sterile conditions. Using a cell replicator cells were plated onto YPD or appropriate media and incubated at appropriate temperature (25^{o}C, 30^{o}C, 37^{o}C or 39^{o}C) for forty-eight hours.

2.7.3 Thermotolerance assay

Yeast strains were cultured overnight in 5ml of YPD at 30^{o}C shaking. Following incubation, the yeast cultures were diluted into 5ml of fresh media at an OD_{600} of 0.15. Cells were grown until they reached a final concentration of $5X10^{6}$ cells/ml. Cells were transferred to 37^{o}C shaking for one hour, 1ml of cells was then removed from culture and placed on ice. Cells were transferred to 52^{o}C shaking, after fifteen minutes 1ml of cells were removed and stored on ice and after thirty minutes this process was repeated. Dot growth assays (2.7.2) was carried out on aliquots and cells were plated on YPD at 30^{o}C for 48 hours.

2.8 Western Blot

2.8.1 Preparation of cell lysates

Appropriate yeast strain inoculated in 5ml of YPD or selective media at 30^{o}C shaking overnight. Cells were diluted into 25ml of fresh YPD to an OD_{600} between 0.1-0.2 and incubated until an OD_{600} 0.6-0.8 was reached. Cells were harvested by centrifugation at 978Xg for five minutes at 4^{o}C and pellets were immediately placed on ice. Pellet was resuspended in 1ml of lysis buffer (Tris-Hcl pH 7.5 (250 µl), 1M Kcl (125µl), 1M $MgCl_2$ (50µl), 1.25M DTT (4µl), RNase A 10µg/ml (100µl), 100mM PMSF (50µl), PIC (10µl) were added to 9.46ml sterile water and stored on ice) and transferred to a 2ml screw cap tube. Glass beads were added to approximately 75% of 2ml tube. Cells were then vortexed (vortex-2 gene, Scientific industries) for twenty seconds and placed on ice, vortexed again and then centrifuged at 7669 x g for ten minutes. Supernatant was then transferred to a pre-chilled 1.5ml microfuge tube. Protein concentrations determined by adding 10µl of protein to 900µl of Bradford reagent (Quickstart Bradford dry reagent 1X, Biorad) and concentrations measured on nanodrop (Nanodrop 1000, Mason) following manufacturer's recommendations. Protein samples (80µl) were mixed with 20µl 4X protein sample buffer (Tris-HCL (0.25M) adjusted to pH 6.8, SDS (1g) , Glycerol (5ml) Bromophenol blue (5mg) and DTT (755mg) dissolved in 10ml of sterile water and stored at room temperature). Samples were boiled on heat block at 100^{o}C for five minutes.

2.8.2 Preparation of SDS-PAGE gels

The reagents used for making 12.5% SDS-PAGE gels are listed in Tables 2.12 and 2.13. Gels were prepared by adding various components and mixing gently, running gel was pipetted between two clean glass plates to about 70% capacity of glass plate.

Isopropanol (100%) was pipetted on top of running gel to 100% capacity of glass plates. Running gel was allowed to set and isopropanol was discarded. Stacking gel components were mixed and pipetted on top of running gel to 100% capacity of glass plates. Comb was placed and stacking gel was allowed set.

No. of gels	**1**	**2**
Volume	10ml	15ml
4X Running Buffer(34.75g Tris base and 4g SDS dissolved in 500ml distilled H_20 and adjusted to pH8)	2.5ml	3.75ml
stH_20	3.3ml	4.95ml
Protogel	4.15ml	6.225ml
10% APS	100µl	150µl
TeMed	10µl	15µl

Table 2.12 Composition of polyacrylamide running gel. All components of running gel stored at room temperature with the exception of 10% APS (4^{o}C).

No. of gels	**1**	**2**
Volume	10mls	15mls
2X Stacking buffer (15g Tris Base and .2g SDS dissolved in 500ml d H_20 and adjust to pH 6.8	3.3ml	4.95ml
H_20	0.825 ml	1.65ml
Protogel	0.4ml	0.8ml
10% APS	25µl	50µl
TeMed	2.5µl	5µl

Table 2.13 Composition of polyacrylamide stacking gel. All components of stacking gel stored at room temperature with the exception of 10% APS (4^{o}C)

2.8.3 SDS-PAGE

Protein gels placed in electrophoresis tank and which was filled to appropriate level with 1X protein gel buffer (10X protein gel buffer; Tris Base (30.2g), Glycine for electrophoresis (18.8g), and 10% SDS (100ml) dissolved in 1000ml of distilled water and stored at room temperature). Comb was gently removed from gel and protein samples (10µg/lane) were loaded onto gel along with protein ladder (Bioline, hyperladder I). Electric current (100V for 15minutes and 200V one hour) was applied to gels.

2.8.4 Transfer to PVDF membrane

PVDF membrane and four sheets of whatman paper were cut to a similar size of protein gel. PVDF membrane was soaked in MeOH for a few seconds and then soaked in dH_20 for one minute. Membrane, whatman paper and sponges were all soaked in Transfer buffer (Tris (3.03g) , Glycine for electrophoresis (14.4g) and 200ml of Methanol were added to 800ml of distilled water and stored at -20^oC for approximately one hour) for approximately five minutes. Sponges were placed on each side of transfer case, followed by two sheets of Whatman paper on each side, membrane was placed on clear side of transfer case and gel was placed on top of membrane. Transfer case was closed, ensuring that no air bubbles were present and placed in transfer tank, with clear side of transfer tank facing red side of transfer apparatus; tank was filled with transfer buffer, an ice block and a magnetic stirrer bar. Transfers were run at 200V/ 400mA for one hour exactly on a magnetic stirrer.

2.8.5 Blocking

Membranes were transferred to blocking solution (Marvel (1.25g) was dissolved in 25ml of 1X TBS-T (10X TBS; Tris (100mM) and NaCl (1M) and 1ml of tween-20, dissolved in distilled water), for approximately one hour shaking at room temperature.

2.8.6 Primary Antibody and secondary Antibody

Primary antibodies were diluted in 10ml of TBS-T according to manufacturer's recommendations (Table 2.14). The PVDF membrane was probed with primary antibody overnight shaking at room temperature. Membrane was then washed with 5ml TBS-T for 5 minutes three times. Alkaline phosphatase labeled secondary antibodies were diluted in 10ml of TBS-T according to manufacturer's recommendations (Table 2.15). Membrane was probed with secondary antibody for approximately one-two hours hour shaking at room temperature. Following this, the membrane was washed with 5ml TBS-T for 5 minutes three times.

Primary Antibody name	Animal of origin	Source	Dilution
SPA-822	Mouse	Stressgen (Victoria, BC, Canada)	1/2000
Ssa1/2 (yT-14)	Goat	Santa Cruz, biotechnology, inc.	1/200
Ssa3/4 (60591)	Mouse	Elizabeth Craig (University of Wisconsin)	1/5000
Hsp104	Rabbit	John Glover (University of Toronto)	1/150,000
Anti-His	Rabbit	Santa Cruz, biotechnology, inc	1/200

Table 2.14 Primary antibodies. Primary antibodies diluted in 10ml of TBS-T and incubated overnight at room temperature.

Secondary Antibody	Animal of origin	Source	Dilution
Anti-Mouse IgG (Fab specific)– Alkaline Phosphatase antibody	Goat	Sigma-Aldrich Chemical CO. Ltd. U.K	1/2000
Anti-Goat IgG (whole molecule)– Alkaline Phosphatase antibody	Rabbit	Sigma-Aldrich Chemical CO. Ltd. U.K	1/200
Anti –Rabbit- Alkaline Phosphatase antibody	Mouse	Sigma-Aldrich Chemical CO. Ltd. U.K	1/2000

Table 2.15 Secondary antibodies. Secondary antibodies diluted in 10ml of TBS-T and incubated for two hours at room temperature.

2.8.7 Chemiluminescence and developing blot

The membrane was covered in 5ml of Chemiluminescence substrate CDP-star (PerkinElmer), for five minutes at room temperature. Membrane was placed between two acetate sheets and air bubbles were removed. The remainder of the developing process was carried out in dark room. Membrane was placed on X-ray cassette and film was placed on top Kodak film (PerkinElmer). X-ray cassette was shut for varying times depending on Antibody. Film was immediately transferred to developer (25ml Kodak developer and 100ml of dH_20) for ten seconds. Film was then washed in H_20 and transferred to fixer (25ml Kodak fixing solution and 100ml of dH_20) for approximately thirty seconds. Film was washed in H_20 and then allowed to dry.

2.8.8 Stripping membrane

Membranes were firstly washed twice for five minutes with 5ml of dH_20, followed by 5ml of TBS-T. The membrane was stripped by adding it to 0.2M Sodium hydroxide for five minutes, shaking at room temperature. Sodium hydroxide was removed by washing in 5ml of dH_20 for five minutes.

2.8.9 Coomassie staining of protein gels

Following electrophoresis polyacrylamide gels were transferred to Coomassie brilliant blue stain shaking, overnight at room temperature. Gels were washed with dH20 and then in destain solution (10% Ethanol, 10% Acetic acid and 80% dH_20) for approximately four hours.

2.8.10 Amido black staining of membrane

PVDF membranes stained in 10ml of Amido black (0.1% Naphtol blue black dissolved in 2% acetic acid and 45% MeOH for one minute. Membrane was then washed with stH20 and washed with destain (2% acetic acid and 45% Methanol)

2.9 Hsp70 protein purification

2.9.1 Transformation of BL21

BL21 cells were transformed with the plasmids pPRO-*SSA1*, pPRO-*SSA1*^{G73D}, pPRO-*SSA1*^{G287D}, pPRO-*SSA1*^{T295I} and pPRO-*SSA1*^{F475S}. BL21 component cells were defrosted on ice. The plasmid (1µl) was added to 100µl BL21 component cells and this was incubated on ice for thirty minutes. Cells were then heat shocked at 42^{o}C for ninety seconds and under sterile conditions plated on 2YT-AMP-CHL- agar and incubated at 37^{o}C for sixteen hours.

2.9.2 Scaling of BL21

One single colony from BL21 transformants was added to 4ml 2YT-AMP-CHL and grown at 37^{o}C in a shaking incubator for four hours until an OD_{600} of 0.8 is reached. Culture is diluted (1/100) into 50ml of 2YT-AMP-CHL and grown at 37^{o}C in a

shaking incubator for three hours until an OD_{600} of 0.8 is achieved. Culture was then diluted (1/100) into 1000ml (x4) of 2YT-AMP-CHL and grown for four hours at 37°C in a shaking incubator for three hours until an OD_{600} of 0.8nm is reached. To induce protein expression, 200µM of IPTG was added and culture is incubated at 16°C in a shaking incubator for 14 hours.

2.9.3 Cell collection

Collect cells by centrifugation at 3170 x g for 30 mins at 4°C. Resuspend pellet in 35ml of dH_20 by vortexing and transfer to 50ml falcon tube (pellets from 2 liters/ falcon). Centrifuge at 3170 x g for one hour at 4°C. Discard supernatant and store pellets at -80°C.

2.9.4 Protein extraction and Nickel column purification

Buffers prepared for Nickel column purification; MCAC-0 (Metal-Chelate Affinity Chromatography) (50mM Na_2HP0_4, 50mM NaH_2P0_4, 0,2M Kcl and 5% Glycerol) this should be approximately pH 7.4 and MCAC-500 (50mM Na_2HP0_4, 50mM NaH_2P0_4, 0,2M Kcl, 5% Glycerol and 500mM Imidazole) adjust to same pH as MCAC-0 and store at 4°C. *E. coli* pellets thawed on ice and resuspended in 40ml of MCAC-10 (490ml MCAC-0 and 10ml MCAC-500). *E. coli* cells were disrupted by using a high-pressure homogenizer and cell crusher (JNBIO- JN3000). Cells were then centrifuged at 17, 562 x g for thirty mins at 4°C (Sorvall), supernatant transferred to sterile falcon and stored on ice. Nickel column (chelating sepharose fast flow, GE healthcare) washed with approximately 100ml molecular H_20, column is then washed with MCAC-10 (250ml/sample). Supernatant was run through Nickel

column. Column is then washed with MCAC-10, followed by washing with MCAC-100. Protein was eluted by washing with MCAC-350.

2.9.5 Dialysis

Supernatant collected from MCAC-350 elution is transferred to Dialysis bag (www.biodee.net). Dialysis bag is then transferred to 2 liters of Dialysis buffer (Tris Base 2.36g, Tris Hcl 12.7g, 150mM Kcl, 5mM $MgCl_2$, 5% glycerol and 1mM DTT dissolved in 2 liters of molecular H_20) stored at 4^oC stirring overnight. Supernatant is then transferred to protein condensing tubes (Millipore) to condense volume to less than 2.2ml.

2.9.6 Gel Filtration

Gel filtration column (HiLoad superDex 200pg, GE Health care) stored in 20% Ethanol. Using ACTA, column washed with approximately 150ml filter sterilized molecular H_20. Column then equilibrated with 150ml Dialysis buffer (RT). Sample applied to column, and fractions 1 (aggregated Ssa1) and fractions 2 (purified Ssa1) were collected and applied to a 12.5% SDS-PAGE gel.

2.10 YDJ1 protein purification

2.10.1 Transformation of C41 *E.coli*

YDJ1 plasmid was transformed to C41 as described in section 2.4.6.

2.10.2 Scaling of transformed C41 *E.coli*

One single colony from C41 transformants is added to 4ml 2YT-AMP and grown at 37^oC in a shaking incubator for four hours until an OD_{600} of 0.8 is reached. Culture is

diluted (1/100) into 50ml of 2YT-AMP and grown at 37°C in a shaking incubator for three hours until an OD_{600} of 0.8 is achieved. Culture is then diluted (1/100) into 1000ml (x2) of 2YT-AMP and grown for four hours at 37°C in a shaking incubator for three hours until an OD_{600} of 0.8 is reached. To induce protein expression, 200µM of IPTG is added and culture is incubated at 16°C in a shaking incubator for fourteen hours.

2.10.3 Cell collection

Collect cells by centrifugation at 3170Xg for thirty minutes at 4°C. Resuspend pellet in 35ml of dH_20 by vortexing and transfer to 50ml falcon tube (pellets from 2 liters/ falcon). Centrifuge at 3170 x g for one hour at 4°C. Discard supernatant and store pellets at -80°C.

2.10.4 Protein extraction and Nickel column purification

Buffers prepared for Nickel column purification; MCAC-0 (Metal-Chelate Affinity Chromatography) (50mMTris-base, 0.3M NaCl and 5% Glycerol) this should be approximately pH 7.5 and MCAC-200 (50mM Tris-base, 0.3M NaCl, 5% Glycerol and 200mM Imidazole) adjust to same pH as MCAC-0 and store at 4°C. *E. coli* pellets thawed on ice and resuspended in 40ml of MCAC-10 (490ml MCAC-0 and 10ml MCAC-500). *E. coli* cells were disrupted by using a high-pressure homogenizer and cell crusher (JNBIO- JN3000). Cells were then centrifuged at 17,800 x g for thirty mins at 4°C (Sorvall), supernatant transferred to sterile falcon and stored on ice. Nickel column (chelating sepharose fast flow, GE healthcare) washed with approximately 100ml molecular H_20, column is then washed with MCAC-10 (250ml/sample). Supernatant is run through Nickel column. Column was washed with

MCAC-10, followed by washing with MCAC-70. Protein is eluted by washing with MCAC-200.

2.10.5 Dialysis

Supernatant collected from MCAC-200 elution is transferred to Dialysis bag (www.biodee.net). Dialysis bag is then transferred to 1 liter of Dialysis buffer (Tris Base 2.36g, Tris Hcl 12.7g, 150mM Kcl, 5mM $MgCl_2$, 5% glycerol and 1mM DTT dissolved in 2 liters of molecular H_20), after four hours sample was transferred to 1 liter of fresh dialysis buffer stored at 4^oC stirring overnight. Supernatant is then stored at -80^oC.

2.11 ATPase assay

Measuring ATPase activity depends on a coupled enzyme assay, which is based on the conversion of phosphoenolpyruvate (PEP) to pyruvate by pyruvate kinase (PK); this depends on the hydrolysis of ATP to ADP. This reaction is coupled to the conversion of pyruvate to lactate by lactate dehydrogenase (LDH), which requires NADH being oxidized to NAD^+. NADH is strongly absorbed at OD_{340} but NAD^+ is not. This enables reduction of NADH over time to be monitored by decrease in absorbance value. Assay carried out with Dialysis buffer, PK (2U/ml), LDH (10U/ml), PEP (4mM), NADH (0.15mM), ATP (1mM) and 2µM of purified protein in a total volume of 900µl. Dialysis buffer, PK, LDH, PEP, NADH and ATP incubated at 30^oC for 10 minutes. Mixture is then transferred to a cuvette and measured at 340nm, after 40 seconds 2µM of protein is added to cuvette and mixed and absorbance is read for a further six minutes. Rate (min^{-1}) is calculated by the formula; slope*60/Є (3440)/ protein concentration (0.000002).

2.12 Circular dichroism spectroscopy

Spectra were recorded over the range 200–250 nm in a Pistar-180 Spectrometer (Applied Photophysics). Spectra of 4µM native Ssa1 and mutants were measured in dialysis buffer in a 0.1mm path length thermostatted cuvette.

2.13.0 Assay of ß-Galactosidase in yeast

2.13.1 Preparation of crude extracts

A 5ml culture of yeast was grown overnight in YPD. The culture was diluted to a concentration of $5x10^6$ (cells/ml) in 5ml of YPD and were then grown to a concentration of $1x10^7$-$2x10^7$ (cells/ml). Cells were chilled on ice and then harvested at 4^oC and kept on ice for remainder of the experiment. Supernatant was discarded and pellet was resuspended in breaking buffer (100mM Tris-CL pH8, 1mM dithiothreitol and 20% Glycerol) and transferred into a 1.5ml microfuge tubes. Glass beads (0.5mm soda lime) (Biospec products) were added until the beads reached a level just below meniscus of the liquid. 12.5µl of PMSF stock solution (40mM PMSF in 100% isopropanol) was then added to liquid. Cells were then vortexed six times at top speed in fifteen second bursts, and chilled on ice between bursts. 250µl of breaking buffer was added to liquid and mixed well. The liquid is then clarified by centrifuging for fifteen minutes at 17,900 x g in a microfuge. Supernatant was then transferred into a sterile 1.5ml microfuge tube.

2.13.2 ß-Galactosidase assay

To perform ß-Gal assay, 100µl of extract was added to 900µl Z buffer (16.1g $Na_2HPO_4 .7H_20$, 5.5g of $NaH_2PO_4. H_20$, 0.75g KCL, 0.246g $MgSO_4.7H_20$, 2.7ml ß-mercaptoethanol made up to one liter with distilled H_20. The buffer was adjusted to

pH 7.0 and was stored at 4°C). The mixture was then incubated at 28°C for five minutes. The reaction was initiated by adding 200µl of o-nitrophenyl- ß-D-galactoside (ONPG) (4mg/ml in Z Buffer and stored at -20°C). The time was noted at the exact moment ONPG was added into mixture. The mixture was then watched until a pale yellow color was observed. The reaction was terminated by adding 500µl of Na_2CO_3 stock solution (1M Na_2CO_3 in distilled water). The precise time of adding Na_2CO_3 was noted. The optical density of solution was measured at OD_{420}.

2.13.3 Determination of protein concentration

The extract (10µl) was added to 1ml of Bradford solution (Quickstart Bradford dry reagent 1X, Biorad). This was left at room temperature for twenty minutes. Protein concentration was read on a nanodrop (nanodrop 1000, Mason) at OD_{595}. A standard curve was prepared by using several dilutions (0.125µg-2000µg/ml) of BSA (New England Biolabs)

2.13.4 Expressing specific activity of extract

ß-Gal units of each extract were determined by using the formula; (OD_{420} x 1.7) / (.0045x protein concentration x extract volume x time). OD_{420} is the optical density of the product, σ-nitrophenol, at 420nm. The factor 1.7 corrects for the reaction volume. The factor 0.0045 is the optical density of a 1 nmole/ml solution of σ-nitrophenol. Protein concentration is expressed as mg/ml. Extract volume is the volume assayed in a ml. Time is in minutes. Specific activity is expresses as nmoles/minute/mg of protein.

2.14 Luciferase assay

Yeast cultures containing the pDCM90 plasmid, that were grown overnight in SD media lacking Uracil at 30^{o}C, were diluted to an OD_{600} of 0.2. Diluted cultures were incubated at 37^{o}C shaking for thirty minutes to induce expression of heat shock proteins. Luciferase activity of three 200µl aliquots was measured to get a reading for 100% activity. Cells were then shifted to 42^{o}C and incubated with shaking for one hour. Cycloheximide was added to culture fifty minutes after shifting to 42^{o}C to prevent synthesis of new luciferase, activity of luciferase was measured of three 200µl aliquots. To allow recovery, cells were shifted to 25 ^{o}C for thirty minutes; luciferase activity of three 200µl aliquots was measured. Luciferase recovery was calculated as a percentage of the 100% activity.

2.15 Structural analysis

Carried out by Dr. Gemma Kinsella (The Marie Curie Laboratory for Membrane Proteins, National University of Ireland, Maynooth). The models created are based on the bovine Hsc70 (PDB: 3C7N chain B, 3.12 Å) (Schuermann. *et al.,* 2008) which was the top BlastP hit (sequence identity ~76%). However, only residues 3-557 were modeled.

2.16 Next Generation Sequencing

Next Generation sequencing was carried out on the Illumina GAII. Yeast genomic DNA was isolated from the strains; G600, G402 and S288C as in section 2.6.2. Strains were sequenced by the Trinity sequencing group.

2.17 Microarray analysis

A 3-D^{TM} competition award was granted by Toray industries, Inc. Japan to enable microarray analysis to be carried out. RNA was isolated (2.6.3), from [*psi*$^{-}$] yeast strains Ssa1, Ssa2, Ssa3, Ssa4, Δ20 and Ssa1^{F475S}. Microarray analysis was carried out by Toray industries in Japan.

3.0 Introduction

The aim of this chapter is to provide insight into the influence of the Hsp70s peptide binding domain (PBD) on [*PSI*$^+$] propagation and determine its importance with regards to other cellular functions. We generated mutants in the PBD of *SSA1* that have impaired [*PSI*$^+$] propagation. Propagation of yeast prions requires the activity of molecular chaperones. Heat shock proteins (HSPs) are a group of molecular chaperones that are involved in protein folding and dissaggregation.

Hsp70 is a ubiquitous and essential protein chaperone involved in many processes such as protein folding and is a critical component of chaperone machinery. It is highly conserved in archaea, eubacteria, eukaryotes and organelles (mitochondria, chloroplasts) (Karlin and Brocchieri, 1998)**.** Hsp70 proteins are abundant under cellular stress such as heat shock, osmolarity and acidity. In yeast, the Hsp70 family comprises of many 70 kDa chaperones, which exist in various compartments of the cell. The main member of Hsp70 family in yeast cytosol is the Ssa family (stress seventy subfamily A), which comprises of Ssa1-4, which collectively provide essential cellular functions. The function of Hsp70 depends on the binding and hydrolysis of ATP. Hsp70 contains a conserved N-terminal ATPase (44-kDa), a peptide-binding domain (18-kDa) and a C-terminal domain lid domain (10-kDa). ATP binds to the ATPase domain in a cleft, which is between two lobes (Flaherty *et al*., 1990). Most of the structural and biochemical analysis on Hsp70 has been carried out using the *E.coli* Hsp70 homologue DnaK (Mayer and Bukau, 2005). Structural analysis of the DnaK PBD highlights the importance of three elements for binding of

substrates; the α-helical lid, the arch enclosing the substrate peptide and the central hydrophobic pocket (Mayer and Bukau, 2005).

The function of Hsp70 relies on many co-chaperones and other factors involved in the regulation of the ATPase cycle. The Hsp40 chaperone binds unfolded proteins and presents them to Hsp70, which then refolds them into the correct confirmation. The Hsp70 family member, Hsp110 (Sse1), acts as a nucleotide exchange factor, peptide release can be triggered by NEF's, by helping liberate bound ADP and there facilitating ATP rebinding. The Hsp104 chaperone also works in conjunction with Hsp70; it is a member of the Hsp/100ClpB family of hexameric AAA^+ ATPases. Hsp104 acts as a protein disaggregase; it shears high molecular weight proteins into smaller aggregates that are then dealt with by the Hsp70/40 system.

Recent studies have implicated many of these chaperones as having an essential role in the propagation of [*PSI*$^+$]. Overexpression or deletion of Hsp104 causes loss of the [*PSI*] in yeast cells, this is thought to happen due to the prion remodeling activities of Hsp104 (Shorter 2008). Recent studies have found Sse1 is required for *de novo* [*PSI*$^+$] formation and efficient prion propagation (Kryndushkin and Wickner, 2007); Fan. *et al*., 2007). Biochemical studies using Sup35 show that the most abundant proteins to co-purify with [*PSI*$^+$] cells are Ssa1 and Ssa2 (Bagrianstsev *et al*., 2008). The isolation of *SSA1* mutants that impair [*PSI*$^+$] propagation has also implicated Hsp70 as having an essential role in prion propagation. The *SSA1* mutant L483W (Ssa1-21), located in the PBD, shows impairment of prion propagation but exhibits no effect on stress tolerance or cell growth (Jung *et al*., 2000). The majority of *SSA1* mutants, that affect prion propagation (approximately ninety percent), are located in the ATPase

domain of Ssa1 (Jones and Masison, 2002; Loovers. *et al.,* 2007), which suggests the regulation of chaperone activity may be more important than direct binding with substrates in relation to [*PSI*$^+$] propagation.

The main objective of this chapter was to:

- Isolate mutants in the PBD of Ssa1 that impair [*PSI*$^+$] propagation.
- Genetically and biochemically characterize isolated mutants.
- Investigate mutants with respect to other cellular functions
- Isolate second -site suppressors of any PBD mutants, which may provide further insight into Ssa1's role in prion propagation.

3.1 Strategy for isolating Ssa1 PBD mutants that impair [*PSI*$^+$] propagation.

Random mutagenesis of Ssa1 to isolate mutations that affect [*PSI*$^+$] propagation has resulted in a predominance of mutations being located in the ATPase domain of Ssa1 (Jones and Masison, 2002; Loovers. *et al.,* 2007). Therefore, we employed a strategy that would yield mutants located in the PBD or C-terminal domain that affect [*PSI*$^+$] propagation. To isolate PBD mutants we employed a modified version of the plasmid shuffle technique (Figure 3.1). Colonies harboring potential PBD mutants that impair [*PSI*$^+$] had plasmids isolated and were transformed back into G402 to verify phenotype, mutant plasmids were isolated and sent for sequencing (Figure 3.1).To isolate mutants located in the PBD, a slightly modified version of plasmid shuffle technique was employed. The pJ120 plasmid was subjected to hydroxylamine treatment for one hour at 70^{o}C, the PBD and C-terminal were amplified from plasmid library and cloned *in vivo* into a *SexA*1 and *Sph*1 digested pJ120 by transforming G402 yeast cells with the PCR product and digested vector on SD media lacking leucine (Figure 3.2). Transformants were replicated onto 5-FOA to select against the

pRDW10 plasmid which expresses wildtype Ssa1. To determine whether colonies appearing red or dark pink were harboring a *SSA1* PBD mutant allele that impairs [*PSI*$^+$] propagation and supports growth, the potential mutant plasmids were isolated and re-transformed into G402 to verify phenotypes. Plasmids were extracted and sequenced; mutations were located by aligning sequencing results with wildtype Ssa1 sequence by BLAST analysis.

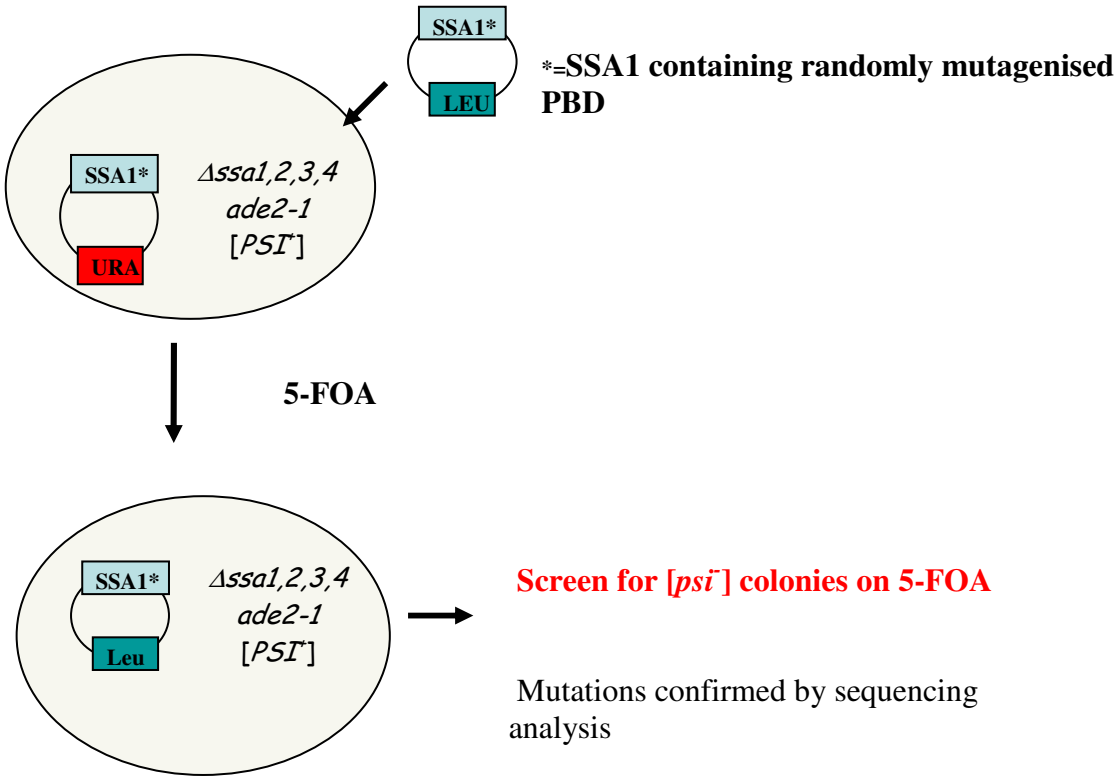


Figure 3.1 Proposed Strategy for isolating SSA1 mutations. To create a mutant library, *SSA1* was mutagenized by hydroxylamine at 70^{o}C for one hour. The mutant library was amplified by transformation to *E.coli* and the plasmid library was isolated and transformed into the [*PSI*$^+$] yeast strain G402 onto SD media lacking leucine. The transformants were replicated on to 5-FOA to eliminate the pRDW10 plasmid which contains wildtype *SSA1*. Mutant plasmids were retrieved from [*psi*$^-$] colonies and sequenced.

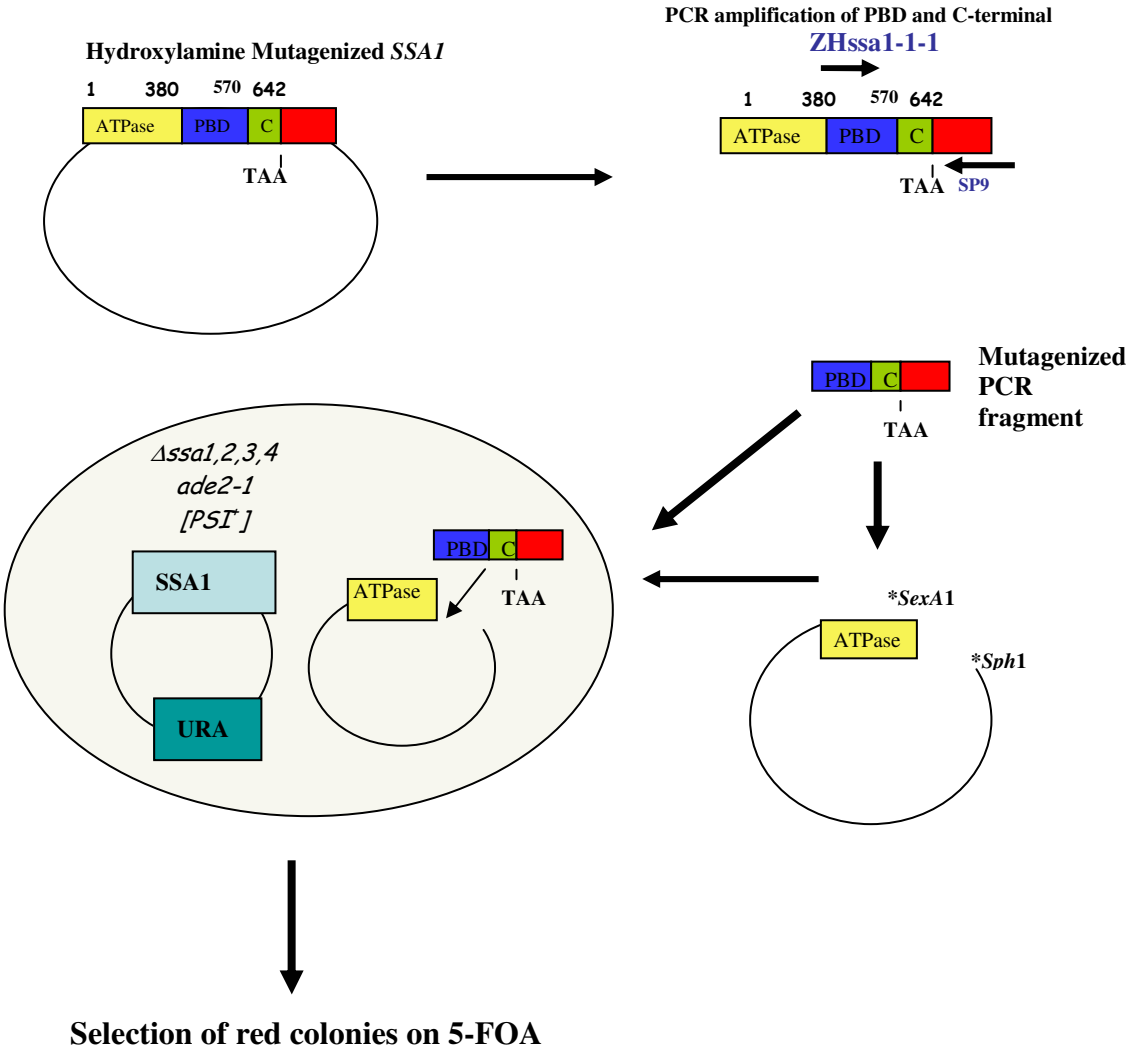


Figure 3.2 Strategy for isolation of PBD mutants. *SSA1* was randomly mutagenized by hydroxylamine at 70°C for one hour. The potentially mutagenized PBD and C-terminal of *SSA1* was amplified using the primers ZHssa1-1-1 and Sp9. A restriction digest was preformed on the pJ120 PBD plasmid using *SexA*1 and *Sph*1. The digested plasmid and PCR fragment were transformed simultaneously into the yeast strain G402 in which the PCR fragment ligated into the digested plasmid by homologous recombination. Mutant plasmids were identified as described in Figure 3.1.

3.1.1 Isolation of *SSA1* PBD mutants that impair [*PSI*$^+$] propagation.

To isolate potential cells harboring mutations of SSA1 that impair [*PSI*$^+$] propagation, the plasmids from colonies that appeared red on 5-FOA were isolated. A secondary screen to confirm [*psi*$^-$] phenotypes was preformed by retransforming mutant plasmids into the [*PSI*$^+$] strain G402. *SSA1* mutations were confirmed by sequencing and BLAST analysis (Table 3.1).The Ssa1 mutagenesis screen revealed four novel and two previously categorized Ssa1 PBD mutations that impair prion propagation. Color of mutants in G402 was scored subjectively by applying a numerical scale, zero being [*PSI*$^+$] (white) and ten being [*psi*$^-$] (red). The novel mutant F475S and previously categorized mutant L483W (Jung. *et al.,* 2000) appear to have the strongest [*psi*$^-$] phenotypes

Plasmid name	*SSA1* nucleotide change	Amino Acid change	Times isolated	Color in G402
V439I	Position 1315- G-A	V439I	2	7
F475S	Position 1424- T-C	F475S	3	9
G481D*	Position 1442-G-A	G481D*	1	9
L483W*	Position 1448- T-G	L483W*	1	9
M515I	Position 1545 G-C	M515I	1	6
S545F	Position 1634 C-T	S545F	1	5

Table 3.1 Ssa1 PBD mutants that impair [*PSI*$^+$] propagation. The screen identifies four novel PBD; V439I, F475S, M515I and S545F and two previously characterized mutants G481D (Loovers *et al*. 2007) and L483W (Jung *et al*. 2000). This Table also shows how many times each PBD mutant was isolated in genetic screen and also numerically categorizes mutants subjectively according to their color, zero representing white [*PSI*$^+$] and nine representing red [*psi*$^-$].
*-previously characterized mutant

3.2 Phenotypic analysis of Ssa1 PBD mutants

Four novel PBD mutants that were identified as impairing [*PSI*$^+$] prion propagation. We further characterized mutants with respect to prion propagation and other cellular processes. Phenotypic analysis for the four novel PBD mutants; V439I, F475S, M515I and S545F was carried out, mutant phenotypes were reconfirmed by growing mutants on YPD along with controls Ssa1 [*PSI*$^+$] and [*psi*$^-$] (Figure 3.3).

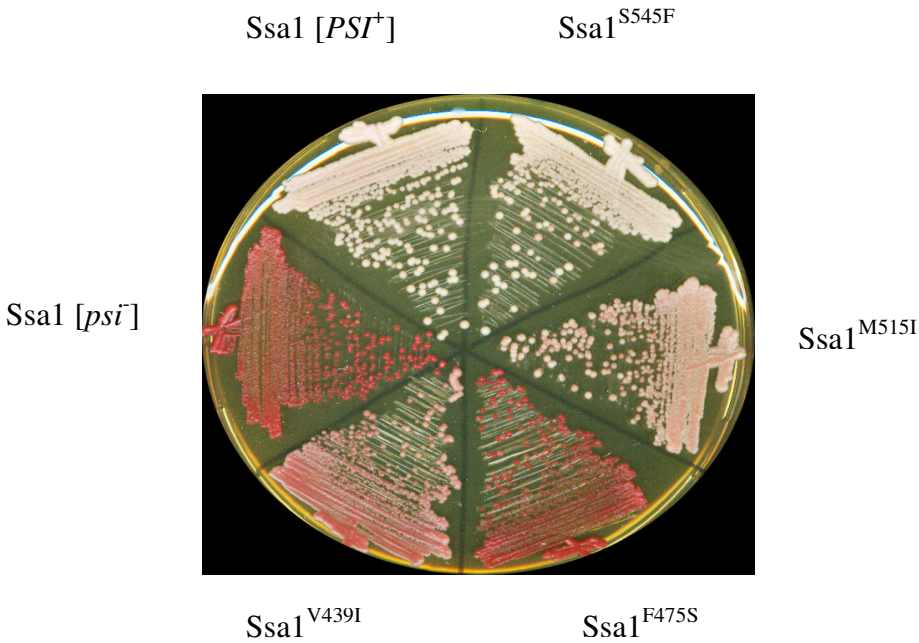


Figure 3.3 Phenotypes of yeast cells expressing Ssa1 mutants in G402. Cells were streaked onto YPD and incubated at 30^{o}C for 48 hours. WT Ssa1 [*PSI*$^+$] (white) and [*psi*$^-$] (red) were used as controls.

3.2.1 Comparison of growth rates of wildtype and PBD mutants

To further compare the phenotypic differences between wildtype Ssa1 and PBD mutants, growth rates were assessed. A substantial difference in growth rates may be indicative that PBD mutants are not just impairing prion propagation but are also affecting other cellular functions The mean generation time (MGT), is the time (minutes) in which the yeast cells double. From Table 3.2 it can be deduced that the PBD mutations do not have a major effect on the growth rate of *S. cerevisiae*. The mutant with the biggest difference in growth rate is $Ssa1^{F475S}$ which is approximately 12% slower in comparison to wildtype. This highlights that mutants in the PBD do not have a significant effect on cell growth when expressed in yeast.

Mutations in SSA1	MGT (Mins)	MGT % of WT
SSA1	111.5	100%
V439I	119.5	93% ±0.7%
F475S	126.5	88% ±0.7%
M515I	115.5	96% ±2.12%
S545F	109	102% ±2.82%

Table 3.2 Comparison of PBD growth rates. Growth rates were obtained by diluting overnight 30°C cultures which expressed Ssa1 or PBD mutants, to an OD_{600} 0.1, the OD_{600} of cultures was, measured every two hours for approximately ten hours (section 2.7.1)

3.2.2 Investigation of PBD mutant's temperature sensitivity.

Hsp70 functions as a heat shock protein, refolding misfolded or aggregated proteins at higher temperatures. Therefore measuring the ability of cells expressing mutated Hsp70 growing at higher temperatures such as 37°C or 39°C is indicative of altered Hsp70 function. The ability of the PBD mutants to grow at higher temperatures; 37°C and 39°C was tested. Previously isolated PBD mutants L483W (Jung. *et al.,* 2000) and G481D (Loovers. *et al.,* 2007) had no effect on growth at higher temperatures. Testing mutant temperature sensitivity reveals $Ssa1^{F475S}$ is temperature sensitive (t.s) at 37°C whereas other PBD mutants grow similarly to wildtype (Figure 3.4). This may be due to the F475S mutation causing Ssa1 to become unstable. All mutants, with the exception of S545F appear t.s at 39°C (Figure 3.4). This indicates that the ability of Hsp70 to rescue aggregated proteins at elevated temperatures is effected by mutations in the PBD.

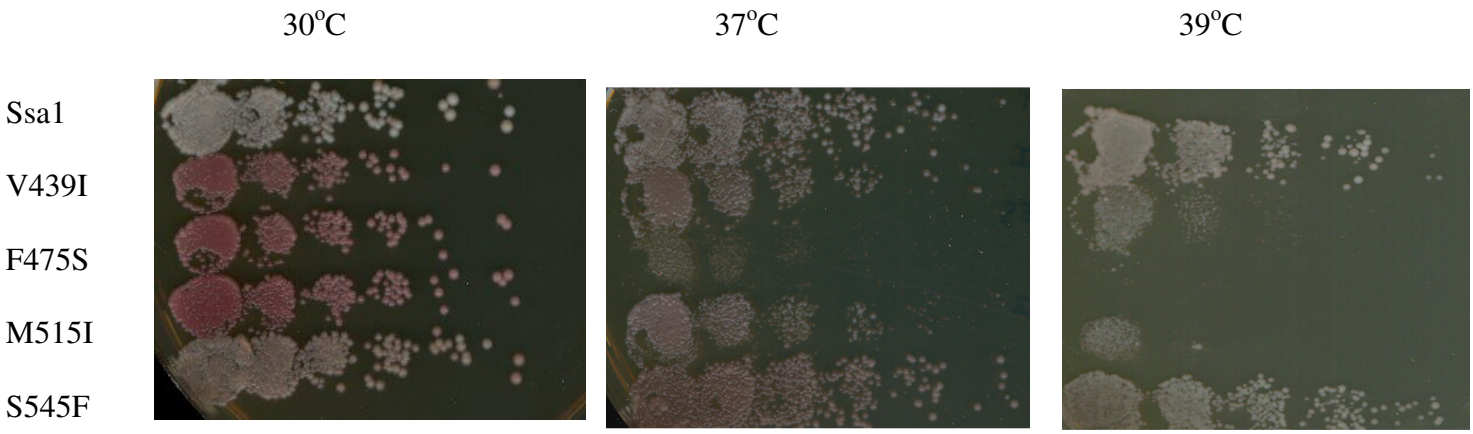


Figure 3.4 Temperature sensitivity of PBD mutants. Cells incubated at specific temperatures for 48 hours on YPD. Cells were diluted by a 1/5 serial dilution. Spots labeled accordingly. Wildtype [PSI^+] and mutants expressed in G402 as sole source of Ssa in strain.

3.2.3 Analysis of cell growth and cell morphology of Ssa1^{F475S}

The phenotypical analysis carried out so far on the PBD mutants has highlighted that Ssa1^{F475S} is the only PBD mutant to date that exhibits impairment of prion propagation and a 37^{o}C t.s phenotype. Due to Ssa1^{F475S} possessing both phenotypes we decided to further analyze the growth and morphology of this mutant. *S.cerevisiae* is a budding yeast, i.e. reproduces asexually by a small outgrowth forming from parental cell. The nucleus of the parental bud divides and the daughter nucleus migrates to the bud, which increases in size and eventually breaks off. Due to the growth of yeast cells being concentrated in the bud, the bud gives us an indication of the cells position in the cell cycle. For example, when yeast cells are exponentially growing one third of cells will have small buds, one third will have large buds and one third will contain no buds. This allows comparative analysis between wildtype and mutant strains in relation to their stage in the yeast cell cycle.

To compare cell growth of wildtype Ssa1 and Ssa1^{F475S}, overnight yeast cultures expressing either Ssa1 or Ssa1^{F475S} were diluted to an OD_{600} 0.2 and then grown to an OD_{600} 1.0. Cells from both strains were examined by light microscopy at both stages. Images (A) and (B) indicate that both strains are exponentially growing with roughly one third of cells with small buds, one third of cells with large buds and one third of cells with no buds. Images (C) and (D) indicative that cells have stopped dividing and may both be in stationary phase. There appears to be no difference between wildtype Ssa1 and Ssa1^{F475S} with respect to their growth rates or cellular morphology (Figure 3.5).

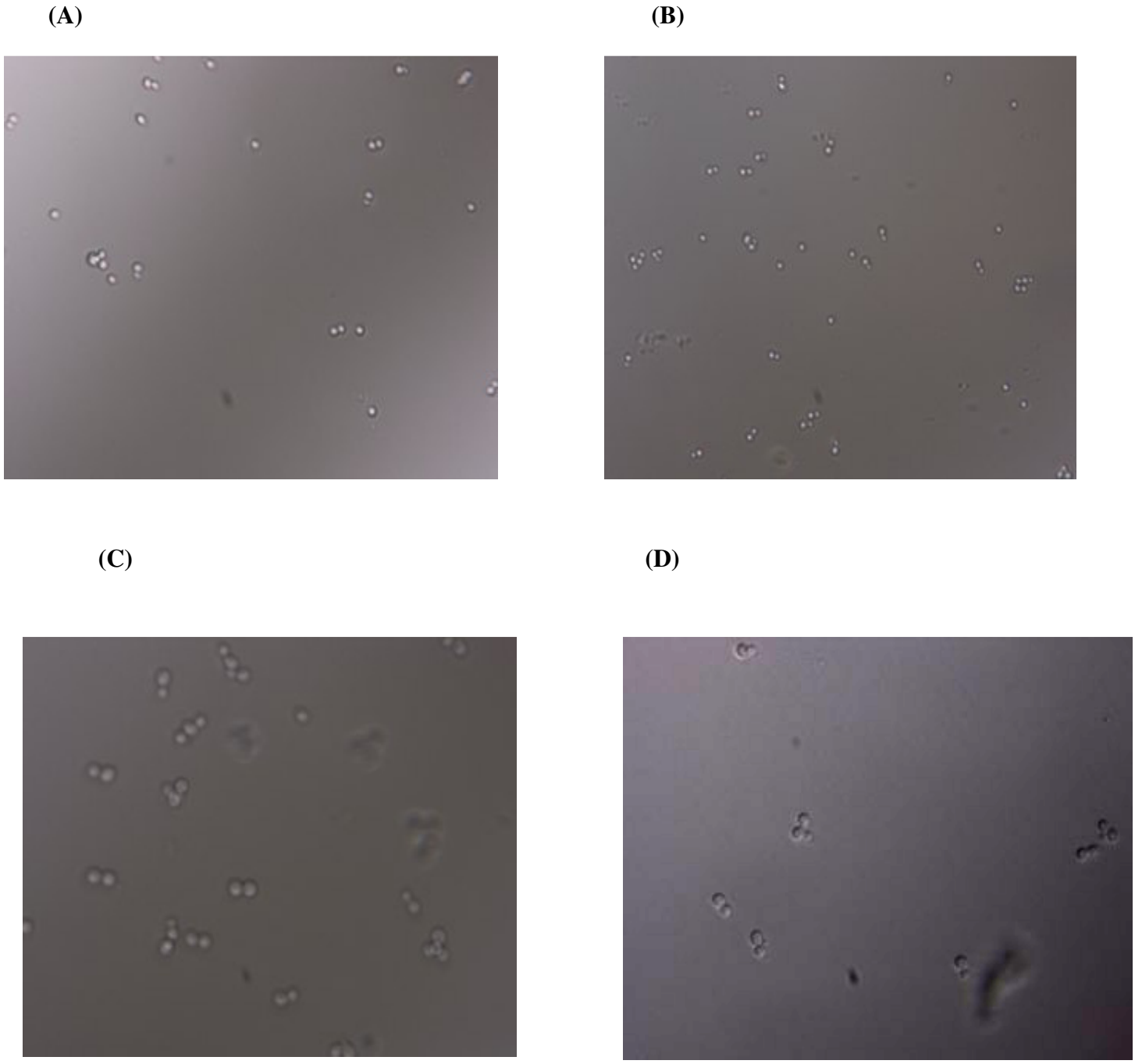


Figure 3.5 Comparison of growth and morphology between Ssa1 and Ssa1^{F475S}. Cells that expressed Ssa1 or Ssa1^{F475S} were cultured in 5ml of YPD at 30^{o}C overnight. They were then diluted to an OD_{600} of 0.2 and grown at 30^{o}C until an OD_{600} of 1.0 was reached. Cells were observed by microscopy (x40) at both stages. Images (A) and (B) represent Ssa1 and Ssa1 ^{F475S} respectively at an OD600 of 0.2, images (C) and (D) represent Ssa1 and Ssa1 ^{F475S} respectively at an OD_{600} of 1.0.

3.3 Analysis of Hsp70 and Hsp104 expression of PBD mutants

It is clear that protein chaperones play an essential role in prion propagation. Therefore analysis of Hsp70 and Hsp104 levels is an essential step whilst investigating impairment of [*PSI*$^{+}$] propagation; as their levels can influence propagation of the prion. Protein levels for wildtype and mutant alleles were observed by Western blotting. Total protein was extracted from cells and probed with Hsp70 (SPA-822) and Hsp104 antibodies. The majority of the mutant alleles have a similar abundance of Hsp70 in comparison to wildtype with the exception of Ssa1^{F475S} appearing to have lower levels (Figure 3.6). Reduced amount of Hsp70 in the presence of the F475S mutation may be due to protein stability or an altered gene expression. Hsp104 does not seem to be increased in cells expressing Ssa1^{F475S} suggesting that cells are not stressed.

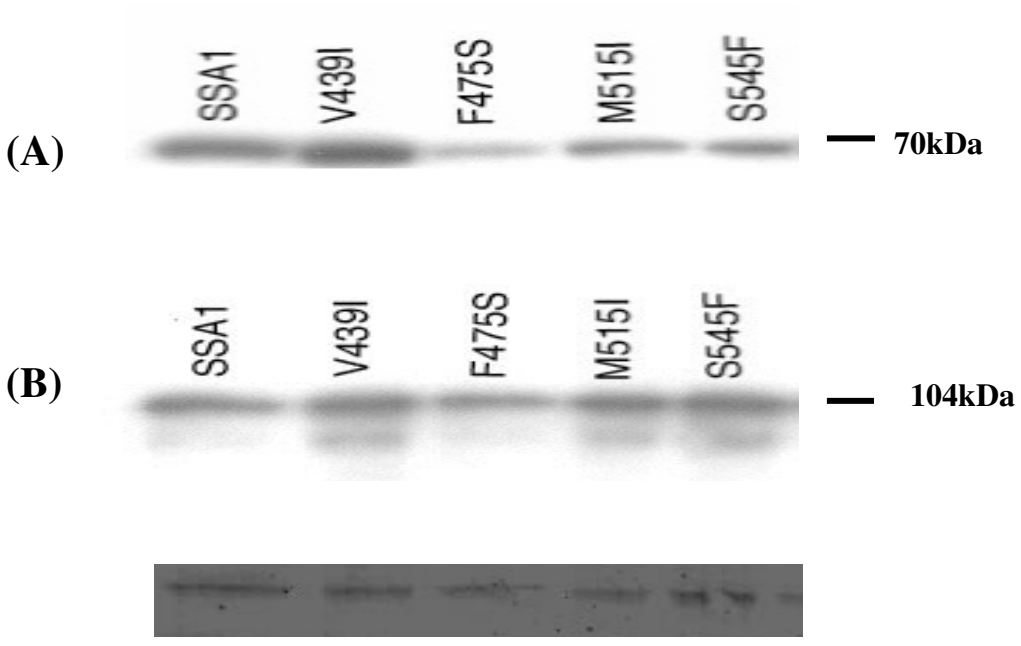


Figure 3.6 Basal levels of Hsp70 and Hsp104. Lysates prepared from overnight 30^{o}C cultures. Aliquots of proteins (10µg) were separated on two polyacrylamide gels and each gel was immunoblotted with antibodies either specific for (A) Hsp70 (SPA-822, 1/2000 dilution) or (B) Hsp104 (HSP104, 1/150,000 dilution). PVDF membrane stained with amido black representative of sample loading. Mutant alleles are as indicated.

3.4 Protein refolding activity of Ssa1^{F475S}

A major function of Hsp70 is refolding proteins that have become misfolded due to heat stress. The ability of chaperones to refold misfolded proteins can be measured by performing a luciferase assay. The luciferase enzyme is unstable at high temperatures and emits light in the presence of its substrate decanal, which can be measured on a luminometer. Cells expressing wildtype Ssa1 or Ssa1^{F475S} were transformed with a plasmid (pDCM90), expressing the luciferase protein. Exposing cells to elevated temperatures causes unfolding of proteins to occur. Hsp104 is critical for survival at elevated temperatures and its function is dependent on Hsp70 and Hsp40. To monitor luciferase activity, exponentially growing cells were shifted from 30^{o}C to 37^{o}C, inducing the expression of heat shock proteins. Cells were then shifted to 42^{o}C for one hour to induce unfolding of the luciferase protein, after fifty minutes of the 42^{o}C incubation cycloheximide was added to prevent synthesis of new luciferase during recovery period. Cultures were shifted to 25^{o}C to allow recovery of unfolded luciferase by the heat shock proteins. Luciferase levels were measured using a luminometer. In wildtype cells 81% of luciferase activity was restored compared to 72% restoration in cells expressing Ssa1^{F475S} (Figure 3.7), therefore we can conclude that luciferase refolding activity of Ssa1 is not significantly affected by the F475S mutation.

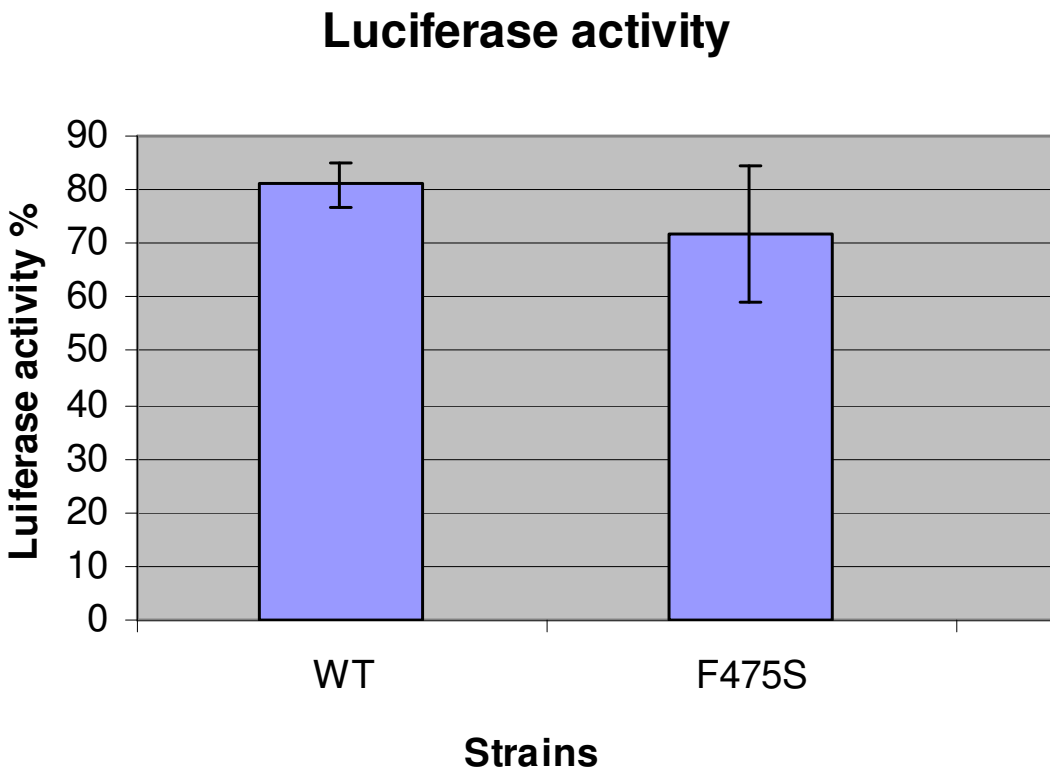


Figure 3.7 Refolding activity of Ssa1^{F475S}. Cultures of strains expressing luciferase and Ssa1 or Ssa1^{F475S} were shifted from 30^{o}C to 37^{o}C for 30 minutes and then shifted to 42^{o}C for one hour. Cycloheximide was added 50 minutes after shifting to 42^{o}C to prevent synthesis of luciferase during the recovery period. Luciferase activity, expressed as a percentage of pre-heat shock activity, was measured after allowing cells to recover for 30 minutes at 25^{o}C. Experiment was repeated twice in triplicate. There appears to be no significant difference between WT and F475S.

3.5 Location of PBD mutants on Hsp70 crystal structure

Previously, structural analysis of mutations in Ssa1 that affected prion propagation was carried out using the *E.coli* Hsp70 homologue, DnaK, model (Loovers *et al*., 2007; Jones and Masison., 2003). The DnaK model has 62.6% similarity to yeast Hsp70. More recently, the bovine homologue of Hsp70, Hsc70, PBD has been modeled. The Hsc70 model has 87.3% homology to yeast Hsp70. Therefore, it was decided, the locations of the PBD mutations are revealed by mapping mutant residues onto a 3-D crystal structure modeled from the 3C7N template (chain B) (Schuermann. *et al*., 2008).This model revels that a majority of the mutants, with the exception of M515I and S545F, are located closely to one another (Table 3.3). Although the F475 residue appears to be closely located to the other mutated residues, none of the other mutants exhibit a 37^{o}C t.s phenotype. Both V439I and F475S are located in a ß-strand, G481D is in a turn region, L483W is located at the top of the strand and M515I is located in the helix (Figure 3.8).

	V439I	G481D	L483W	F475S
V439I	-	22.17	16.69	12.97
G481D	-	-	5.9	17.62
L483W	-	-	-	13.36

Table 3.3 Cα**-C*α* distances (Å) for residues at the base of the PBD**. Modeled from the 3C7N template (chain B) (Schuermann. *et al*. 2008).

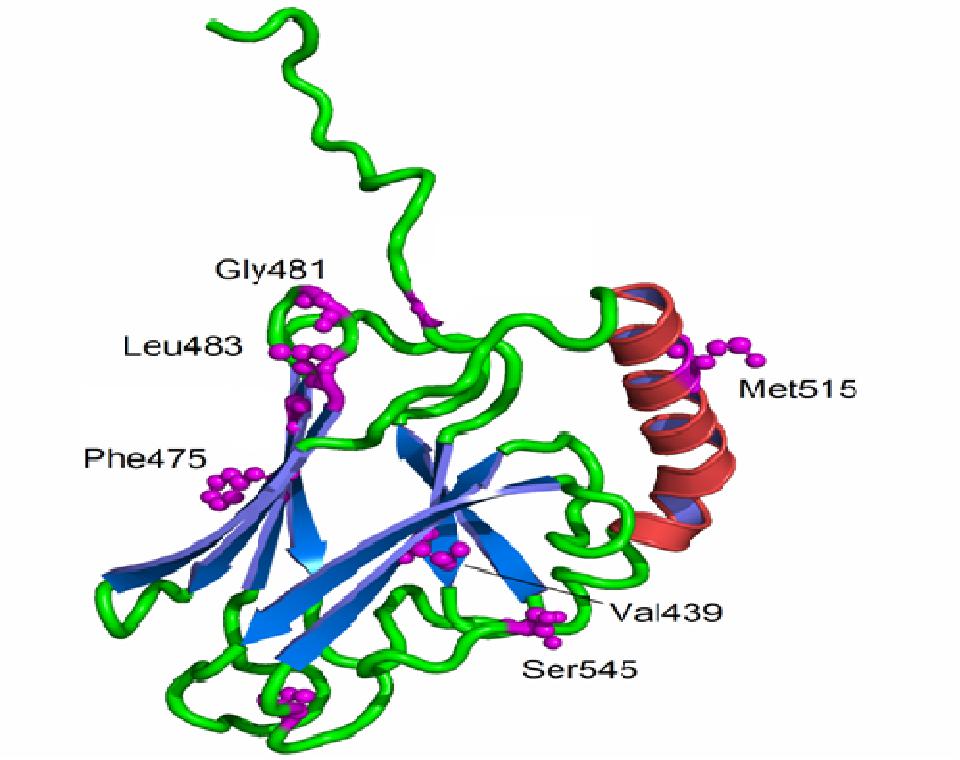


Figure 3.8 Substrate binding domain (380-570) of yeast: Hsp70 modeled from the 3C7N template (chain B) (Schuermann *et al*., 2008). Images were generated using Pymol (DeLano. 2002).

3.6 Comparative analysis of PBD mutants Ssa1^{L483W} and Ssa1 ^{F475S}

Mapping the PBD mutants onto the 3D crystal structure reveals that Ssa1 ^{F475S} is located closely to the previously isolated mutant Ssa1^{L483W} (*SSA1-21*) (Jung *et al.*, 2000). Further studies on the *SSA1-21* revealed that the [*psi*$^-$] phenotype could be suppressesed by the introduction of second site suppressors of the L483W mutation (Jones and Masison., 2002). In order to determine whether Ssa1^{F475S} impairs prion propagation in a similar manner to *SSA1-21,* three second site suppressors (A519T, E540K and P636S) were introduced to the pJ120 ^{F475S} plasmid by site-directed mutagenesis. F475S in combination with P636S was the only combination that restored the [*PSI*$^+$] phenotype, A519T and E540K in combination with F475S proved detrimental (Figure 3.9). This result shows that F475S is slightly similar to L483W, but also behaves differently.

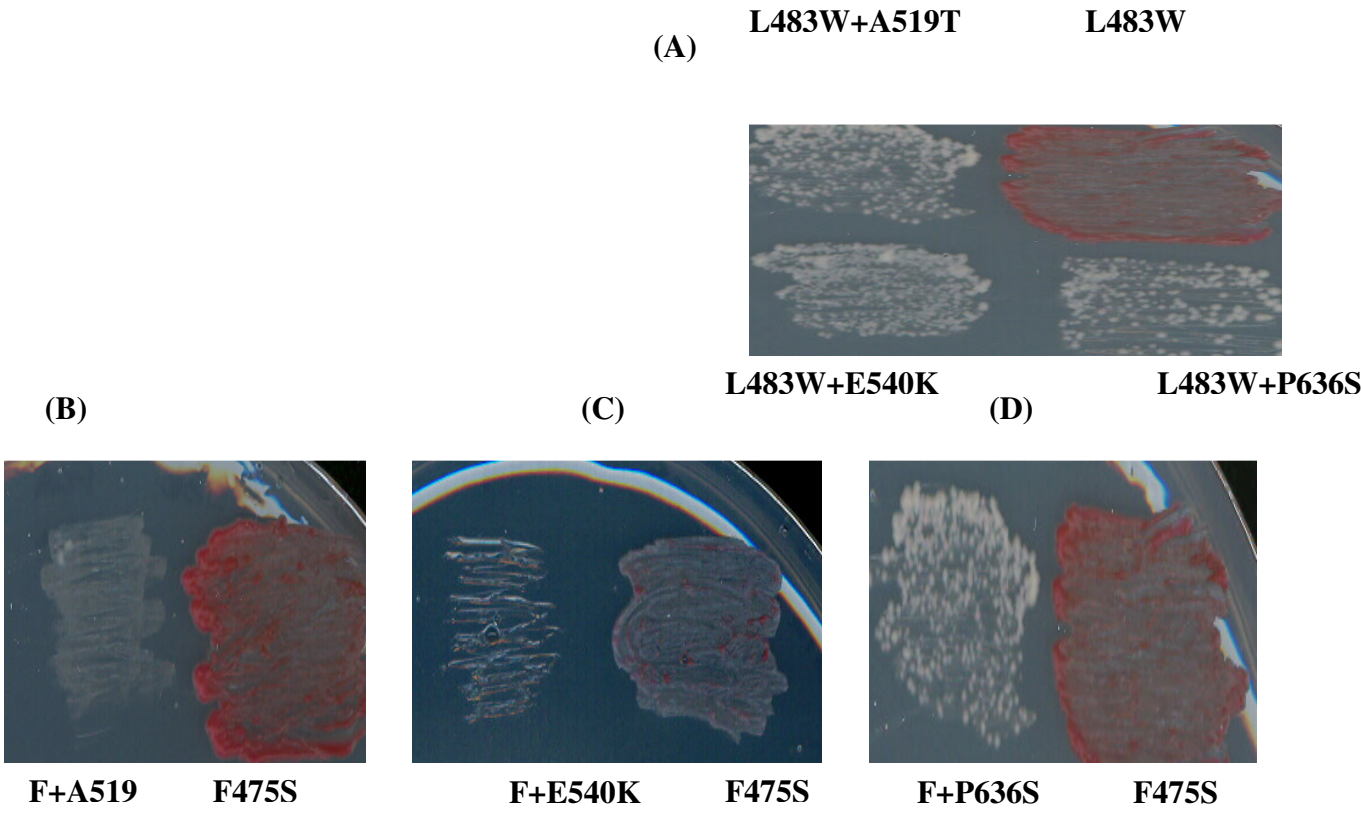


Figure 3.9 Phenotypes of PBD mutants with second site suppressors. Cells replicated to 5-FOA from SD minus leucine plates and incubated at 30^{o}C for 48 hours. (A) SSA1 ^{L483W} and second site suppressors that restore [*PSI*$^+$] phenotype when in combination with L483W. (B-D) Ssa1^{F475S} and second site suppressors labeled accordingly. [*PSI*$^+$] is restored when the F475S mutation is combined with P636S.

The comparative analysis between *SSA1-21* and Ssa1^{F475S} lead us to conclude that Ssa1$^{F+P636S}$ restore the **[***PSI*$^{+}$] phenotype. However, Ssa1^{F475S} differs from *SSA1-21 as* phenotypical analysis revealed Ssa1 ^{F475S} is t.s at 37^{o}C. We therefore tested whether Ssa1F^{+P636S} could also restore the t.s phenotype or was it just unique to [*PSI*$^{+}$] restoration. From Figure 3.10 it can be concluded that Ssa1$^{F+P636S}$ cannot restore the 37^{o}C t.s phenotype of Ssa1^{F475S}, in fact it appears to worsen the phenotype. The P636 residue is in a highly conserved motif of Ssa1's C-terminal (GPTVEEVD), which mediates interactions with tetratricopeptide repeat (TPR) Cochaperones such as Sti1 (Scheufler *et al*., 2000). This suggests that interactions with TPRs may be more significant for prion propagation than other cellular functions.

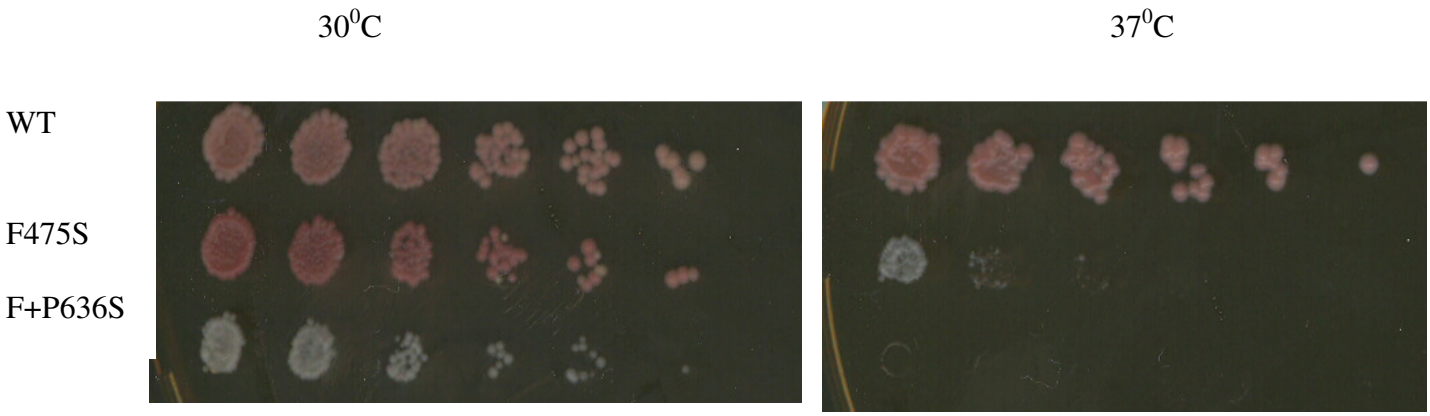


Figure 3.10 Temperature sensitivity assay of Ssa1$^{F+P636S}$
Cells incubated at 30^{o}C and 37^{o}C on YPD for 48 hours. Spots labeled accordingly. Cells diluted by a 1/5 serial dilution. Wildtype [*PSI*$^{+}$] and mutants expressed in G402 therefore are sole source of Ssa in strain.

3.7 Second- site suppressors of Ssa1^{F475S} that restore 37^{o}C t.s phenotype

Comparative studies between *SSA1-21* and Ssa1^{F475S} lead us to conclude that there are a number of similarities and differences between these PBD mutants. The major difference is Ssa1^{F475S} is t.s at 37^{o}C. To provide further insight on how Ssa1^{F475S} is affecting [*PSI*$^{+}$] propagation and the Ssa1 heat shock function, a random mutagenesis screen was carried out to isolate second-site suppressors of the F475S mutation. The mutagenesis screen was employed to isolate mutants that were [*PSI*$^{+}$] or were viable at 37^{o}C (Figure 3.11). This screen identified three second-site suppressors of F475S that restored growth the t.s 37^{o}C phenotype; A394V, P432S and V477I, all of which are located in the PBD (Table 3.4). The screen yielded no second-site suppressors that restored [*PSI*$^{+}$] propagation, highlighting another difference with *SSA1-21* which yielded many second-site suppressors from a random mutagenesis screen (Jones and Masison, 2003). Interestingly cells expressing Ssa1^{F475S} in combination with second-site suppressors P432S and V477I appear to remain [*psi*$^{-}$] (Figure 3.12), whereas cells expressing A394V appear [*PSI*$^{+}$] leading us to conclude that certain regions of Ssa1 may be involved with suppressing particular cellular functions whereas other regions e.g. the conserved motif of Ssa1's C-terminal (GPTVEEVD) may be exclusively involved with [*PSI*$^{+}$] propagation mediated through co-chaperones.

3.7.1 Phenotypical analysis of all second-site suppressors of F475S

To further our knowledge of how these second-site suppressors are affecting Ssa1 function, phenotypical analysis was carried out on all Ssa1^{F475S} suppressors; A394V, P432S, V477I and P636S, [*PSI*] status was analyzed by streaking mutants onto YPD

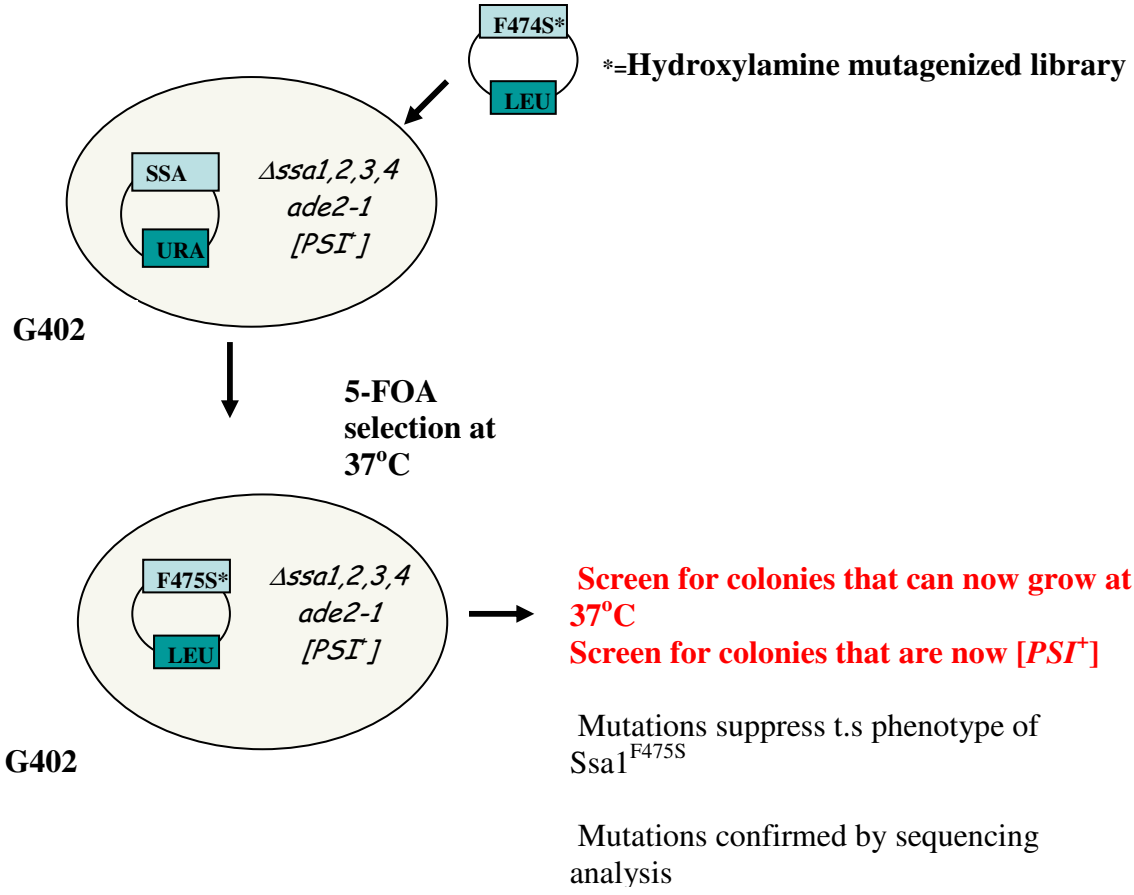


Figure 3.11 Strategy for isolating second site suppressor mutations of SSA1^{F475S} T.S phenotype. To create a mutant library of second site suppressors of Ssa1 ^{F475S} mutation, pJ120^{F475S} was randomly mutagenized by hydroxylamine at 70^{o}C for one hour. The mutant library was transformed into G402 and mutants were selected for by the plasmid shuffle technique by selecting for growth on 5-FOA at 37^{o}C after 48 hours. Potential mutant plasmids were isolated and retransformed into G402 and a secondary screen was preformed, mutations were confirmed by sequencing.

Plasmid name	*SSA1* nucleotide change	Amino Acid change	Screen results	Color in G402
2-20	Position 1182 C-T	A394V	1	5
	Position 1424- T-C	F475S		
2-16	Position 1294 C-A	P432S	3	9
	Position 1424- T-C	F475S		
2-8	Position 1438 G-A	V477I	1	9
	Position 1424- T-C	F475S		

Table 3.4 Second-site suppressors of Ssa1^{F475S} that restore t.s phenotype. The screen identified three mutations; A394V, P432S and V477I that in combination with F475S suppress the t.s phenotype i.e. enable strains to grow at 37°C. All three mutations are located in the peptide-binding domain of Ssa1. Each mutant was isolated once with the exception of P432S, which was isolated, three times. Color of strains expressing Ssa1 mutants were subjectively scored; zero representing [*PSI*$^+$] (white) and nine representing [*psi*$^-$].

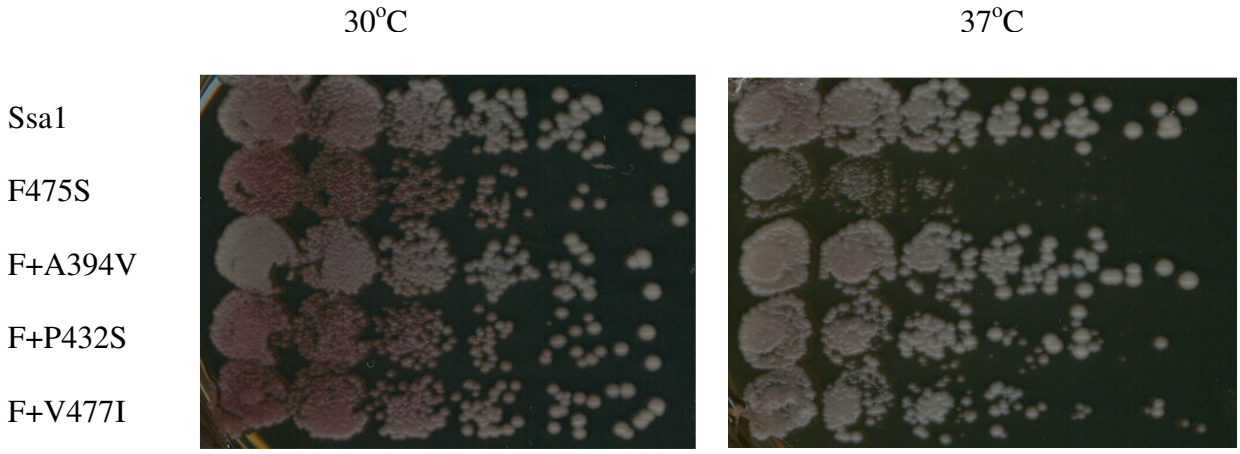


Figure 3.12 Confirmation of restored T.S phenotype by second-site suppressors. Mutants expressed in G402 were incubated at 30°C and 37°C on YPD for 72 hours. Cells diluted by a 1/5 serial dilution. Mutants as indicated. Second-site suppressors exhibit recovery of t.s phenotype at 37°C.

and incubating at 30°C for 48 hours. Ssa1 $^{F+P432S}$ and Ssa1 $^{F+V477I}$ (t.s suppressing mutants) remained [*psi*$^{-}$] whereas Ssa1 $^{F+P636S}$ and Ssa1$^{F+ A394V}$ have [*PSI*$^{+}$] restored (Figure.3.13). Ssa1$^{F+P432S}$ and Ssa1$^{F+V477I}$ have the strongest [*psi*$^{-}$] phenotypes whereas Ssa1$^{F+A394V}$ appears to be [*PSI*$^{+}$] phenotype (Figure 3.13).

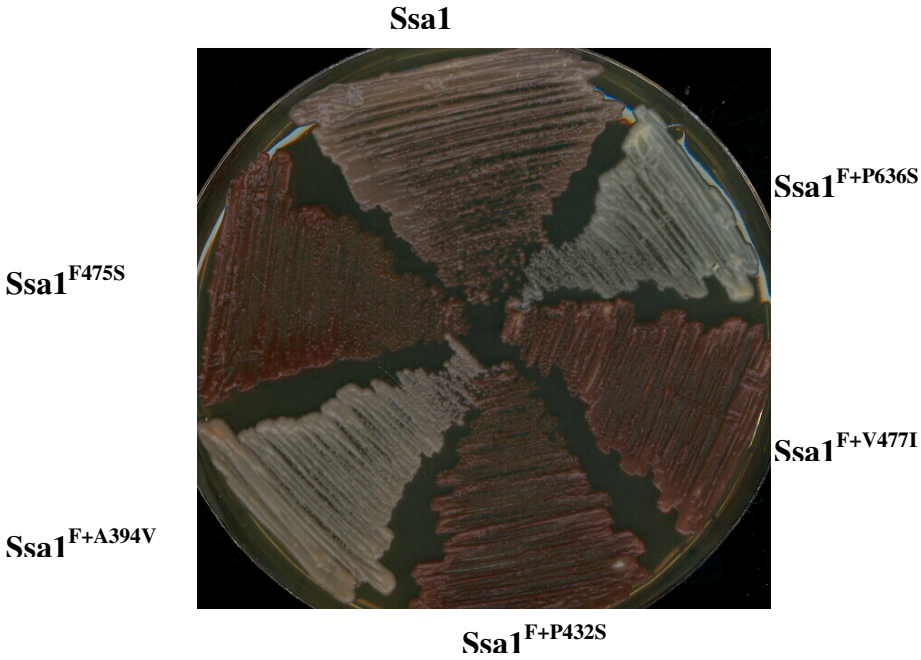


Figure3.13 [*PSI*] status of second-site suppressors. Phenotypes of second-site suppressors being expressed in yeast as only source of the Ssa. Cells streaked onto YPD plate and incubated at 30°C for 48 hours. Mutants are as indicated. White color indicated the presence of [*PSI*$^{+}$] prion and pink and red indicates presence of [*psi*$^{-}$] prion.

3.7.2 Relative effects of second-site suppressors on cell growth

To provide further insight of how second-site suppressors are restoring phenotypes relating to the Ssa1^{F475S} mutation, growth rates of second-site-suppressors were measured. In section 3.2.3, it is observed that the growth rate of Ssa1^{F475S} is 88% of the MGT of wildtype Ssa1. Ssa1$^{F+A394V}$ appears to restore growth rate similar to that of wildtype, Ssa1 $^{F+P432S}$ and Ssa1 $^{F+V477I}$ appear to have no effect on growth rates

remaining similar to that of Ssa1^{F475S}. Ssa1$^{F+P636S}$ appears to have the greatest effect, with the growth rate being reduced to almost half of Ssa1 (Table 3.5). This is indicative that alteration of the conserved motif of Ssa1's C-terminal (GPTVEEVD) in combination with the F475S mutation does have a severe effect on cell growth.

Second-site suppressor mutations	MGT (Mins)	MGT % of WT
SSA1	105.66	100%
SSA1^{F475S}	119.33	112.9% ±6.02%
SSA1$^{F+A394V}$	103.33	98.7% ±1.53%
SSA1$^{F+P432S}$	112.67	107% ±3.51%
SSA1$^{F+V477I}$	120	113.5% ± 1.73%
SSA1$^{F+P636S}$	202.67	191% ± 13.5%

Table 3.5 MGT of second-site suppressors. This table represents the mean generation time of second-site suppressors of the Ssa1^{F475S} mutation. Overnight 30^{o}C cultures diluted to an OD_{600} 0.1. Cultures grown at 30^{o}C, OD_{600} measured every two hours for approximately ten hours (section 2.7.1)

3.8 Basal levels of Hsp70 and Hsp104 in yeast expressing Ssa1^{F475S} second-site suppressors

Abundance of Hsp70 in yeast strains expressing Ssa1^{F475S} appeared lower in comparison to those expressing wildtype Ssa1 when immunoblotted with an Hsp70 antibody (Figure 3.14). Lower levels of Hsp70 in Ssa1^{F475S} could lead to the t.s phenotype observed at 37^{o}C and the impairment of [*PSI*$^{+}$] propagation. To test this hypothesis, abundance of Hsp70 in yeast cells expressing second-site suppressors of Ssa1^{F475S} was observed by western blotting. As previously observed the abundance of Hsp70 in yeast cells expressing Ssa1^{F475S} is reduced in comparison to wildtype. Hsp70 levels in yeast cells expressing Ssa1$^{F+A394V}$ and Ssa1$^{F+P433S}$ are restored to levels similar to wildtype. Whereas the abundance of Hsp70 in yeast cells expressing Ssa1$^{F+V477I}$ and Ssa1$^{F+P636S}$ appears slightly reduced in comparison to Ssa1^{F475S}. This result indicates that abundance of Hsp70 in Ssa1^{F475S} is not the cause of the t.s or prion phenotype and A394V and P432S may be suppressing the F475S mutation in a different manor to V477I.

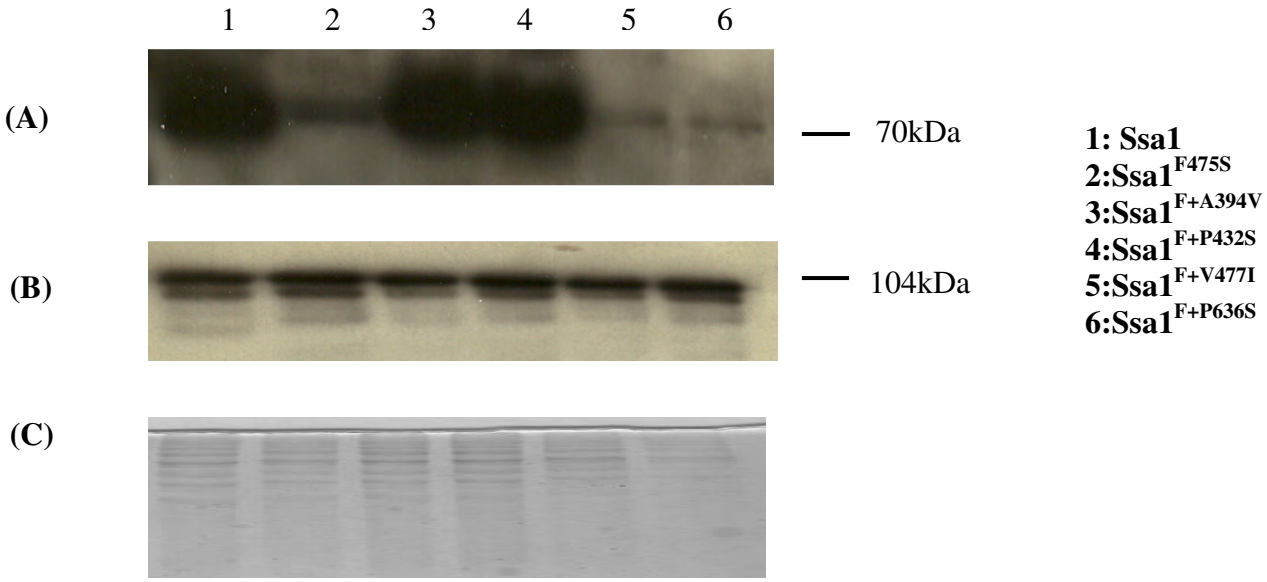


Figure 3.14. Abundance of Hsp70 in Ssa1^{F475S} second-site suppressors. Lysates prepared from overnight 30^{o}C cultures. Aliquots of proteins (10µg) were separated on three polyacrylamide gels, gels were either immunoblotted with antibodies either specific for (A) Hsp70 (SPA-822, 1/2000 dilution) and (B) Hsp104 (Hsp104, 1/1000 dilution) or stained (C) coomassie brilliant blue stained overnight and destained the following day. Mutant alleles are as indicated.

3.9 Structural analysis of second-site suppressors

Carrying out structural analysis on F475S and the second-site suppressors, will give us an indication of how closely located suppressors are to the F475 residue and this maybe indicative as to how they restore phenotypes caused by F475S. From Table 3.6 we can infer that V477I is located in the ß-strand and is the closest suppressor located to F475S. A394V is located in the flexible linker region between domains and P432S is located at top of the ß-strand (Figure 3.15). As A394V and P432S appear to have similar affects, with respect to abundance of Hsp70 and effects on growth rates, it seems these mutants are affecting Hsp70 in the same manor. As A394V is located within the linker region between the ATPase domain and PBD, this may infer that F475S is affecting inter-domain communication, which could affect the heat shock function of Hsp70, and the A394V and P432S mutations are restoring the function.

	A394	**P433**	**V439**	**F475**	**V477**	**G481**	**L483**
A394	-	39.46	21.88	17.69	12.97	10.02	11.91
P433		-	20.52	23.24	29.16	39.48	33.98
V439			-	12.73	12.56	22.17	16.69
F475				-	6.40	17.62	13.36
V477					-	11.29	7.73
G481						-	5.9
L483							-

Table 3.6 Cα-Cα distances (Å) for residues at the base of the PBD. Those closest (< 15 Å) are indicated in red. Modeled from the 3C7N template (chain B) (Wiederstein *et al.,* 2007).

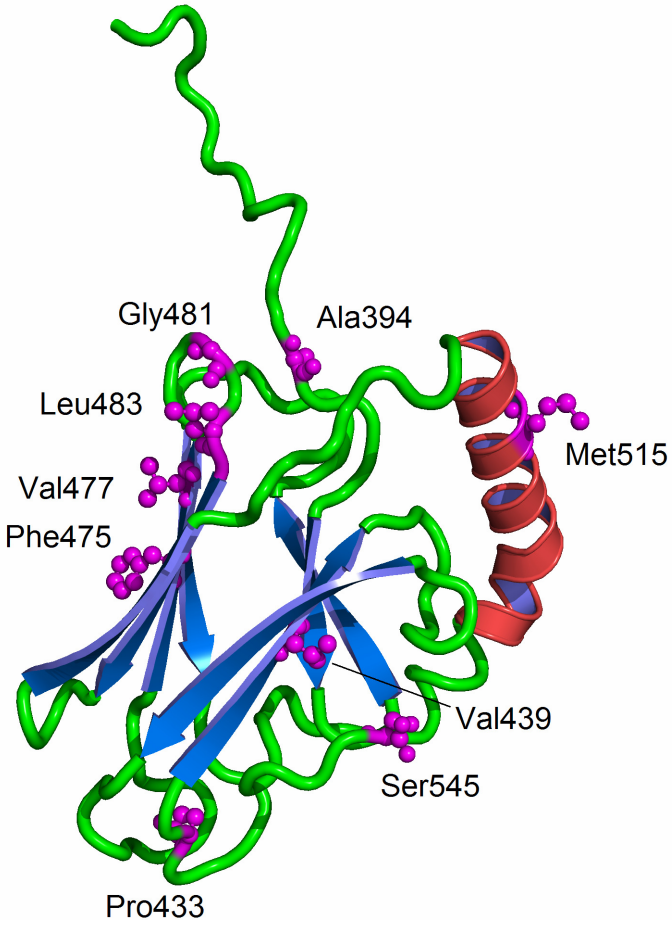


Figure 3.15 Substrate binding domain (380-570) of yeast Hsp70 modeled from the 3C7N template (chain B). Locations of PBD mutants and F475S second-site suppressors mapped. Images were generated using Pymol (DeLano. 2002).

3.10 Biochemical analysis of Ssa1 and PBD mutant

To further our understanding of the role of Hsp70 and PBD mutants in prion propagation it was essential to gather biochemical data in addition to *in vivo* genetic data. Originally our goal was to purify Ssa1 using the *Pichia pastoris* expression system (Invitrogen, San Diego). The *P. pastoris* expression system is frequently used as *Pichia* does not require complex growth media, genetic manipulation is relatively simple and it has a eukaryotic protein synthesis pathway. To achieve expression, *SSA1* was cloned into a *P. pastoris* expression vector under the alcohol oxidase (*AOX1*) promoter, which is a methanol inducible promoter. This was digested by a *Sac*1 restriction digest and the linear plasmid was integrated into *P. pastoris* by homologous recombination. Expression of recombinant *Pichia* proteins was induced by methanol and proteins were purified by ion exchange chromatography, hydrophobic interaction chromatography and size exclusion chromatography (Wegele *et al*., 2003). This method of Hsp70 purification has been used to determine the function of yeast Hsp70 and its isoforms and the involvement of Sti1 as an activator of Ssa1 (Wegele *et al.,* 2003; Tutar *et al.,* 2006). However, Western blot analysis of purified Hsp70 revealed no signal when probed with the Hsp70 specific antibody SPA-822, yet the purified protein exhibited ATPase activity. Investigative analysis of this purified protein by the Perrett group in Beijing revealed that the purified protein was actually Aox1. Interestingly Aox1 had not previously been identified as an ATPase and expression of Aox1 in *S. cerevisiae* appears to impair the propagation of the yeast prion [*URE3*] (Zhang. *et al.,* 2009).

Due to evidence that *P. pastoris* expression system was not suitable for Hsp70 purification, we decided to purify Ssa1in *E.coli.* The Ssa1 expression plasmid was provided by Prof. Susan Lindquist (Adegene plasmid 1231).

3.10.1 Viability of His(6X) tagged Ssa1^{F475S} *in vivo*

Fusing a His (6X) tag to the N- terminal of a protein of interest may lead to functional changes, for example, fusing a His (6X) tag to L483W caused the mutant to become non-functional *in vivo* (G. Jones personal communication). The ATPase assays and CD spectroscopy were carried out *in vitro*, therefore it was necessary to examine the viability of the His (6X) tagged versions of Ssa1 and Ssa1^{F475S}. Similarly, to L483W, fusing a His tag to the N-terminus of Ssa1^{F475S} causes the mutant to become non-functional *in vivo* (Figure 3.16)

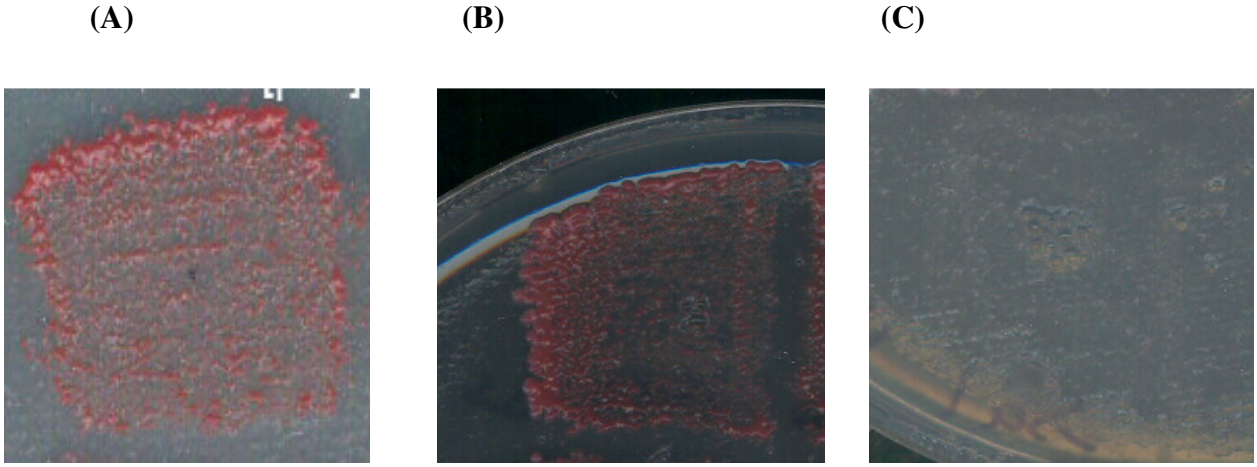


Figure 3.16 Viability of His tagged Ssa1^{F475S}. (A)Ssa1 (His6X) (B) Ssa1^{F475S} and (C) Ssa1^{F475S} with N-terminus fused His (6X) tag, were transformed into G402. Cells replicated from SD media lacking Leucine onto 5-FOA. Cells incubated at 30^{o}C for 48 hours. The His (6X) tags appears not to affect wildtype Ssa1 but causes Ssa1^{F475S} to become non-functional *in vivo.*

3.10.2 Protein Purification Hsp70 and PBD mutant

We chose to purify Ssa1^{F475S} as it is a unique PBD; it is the first PBD mutant to exhibit (both impairment of [*PSI*] propagation and temperature sensitivity at 37^{o}C. The F475S mutation was introduced by site-directed mutagenesis into a bacterial expression vector that expresses Ssa1 which has an N-terminus fused Histidine (6X) tag (Figure 3.17). Ssa1 and Ssa1^{F475S} were purified under native conditions by Ni^{2+} affinity chromatography, dialysis and size exclusion chromatography (chapter 2.6). To condense large volumes of protein supernatant collected from dialysis and size exclusion chromatography, protein samples were condensed using Millipore condensing tubes. Previous results in the Perrett lab have identified two protein fractions of interest following Ssa1 purification by size exclusion chromatography. Peak one has been shown to be aggregated Hsp70 whereas peak two is native Hsp70. Therefore two protein fractions were collected from size exclusion chromatography; peak one and peak two which is native Hsp70 (Figure 3.18).

3.10.3 Confirmation of Ssa1 purification

Following purification, protein purity was assessed by SDS-page and coomassie staining (Figure 3.19). Fractions pre-purification and post Ni^{2+} affinity chromatography, dialysis and size exclusion chromatography were collected and boiled in protein sample buffer and run on a 12.5% polyacrylamide gel. A majority of Hsp70 is lost during size exclusion chromatography but this is an essential step as protein samples are not pure after Ni^{2+} affinity chromatography and dialysis (Figure 3.19). To further confirm the protein purified was Hsp70, P1 protein samples collected from size-exclusion chromatography were analyzed by western blot.

Samples were probed with antibodies specific for Hsp70 and His6X tag (Figure 3.20).

Western blot confirms presence of both Hsp70 and His (6X) tag.

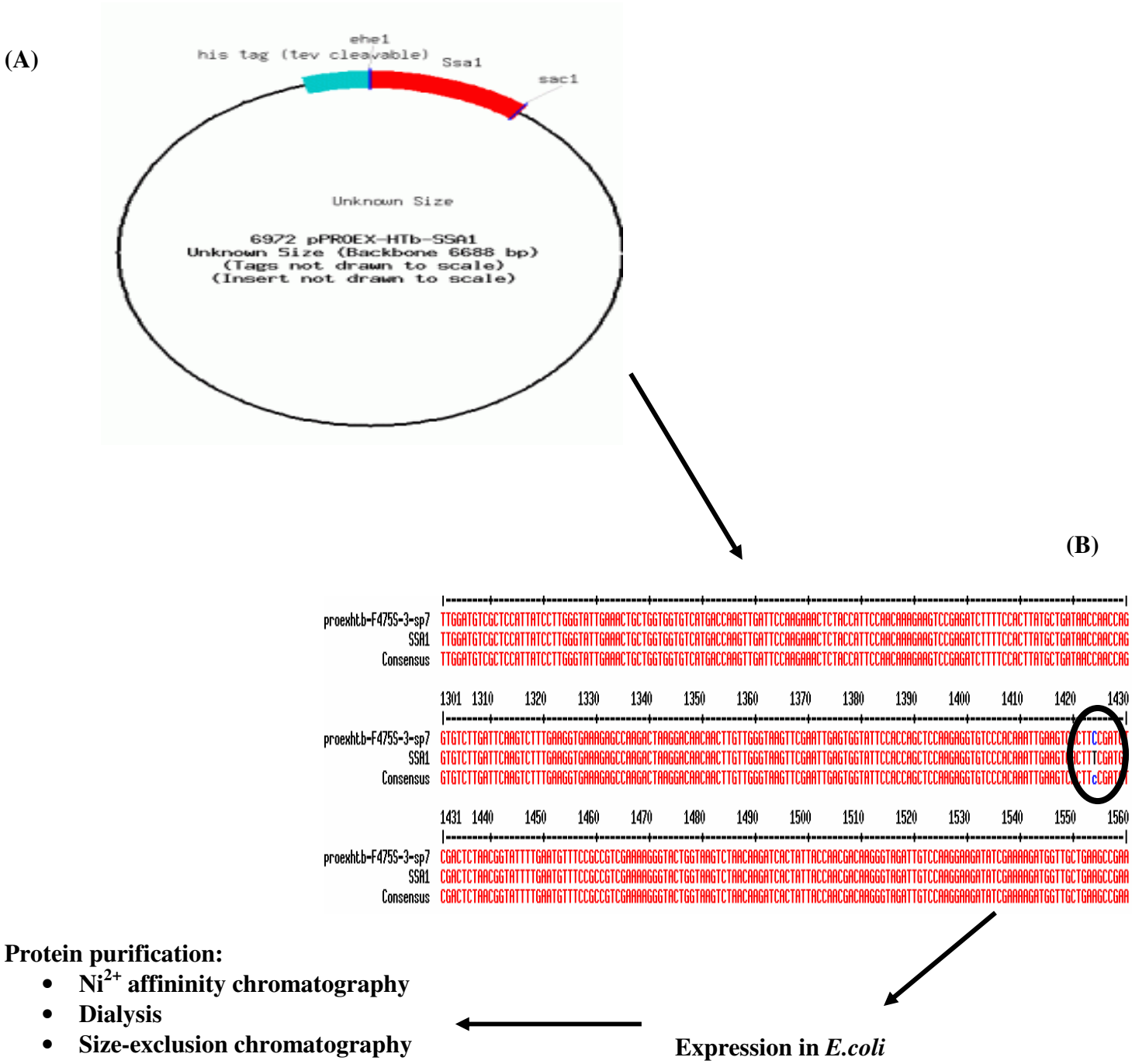
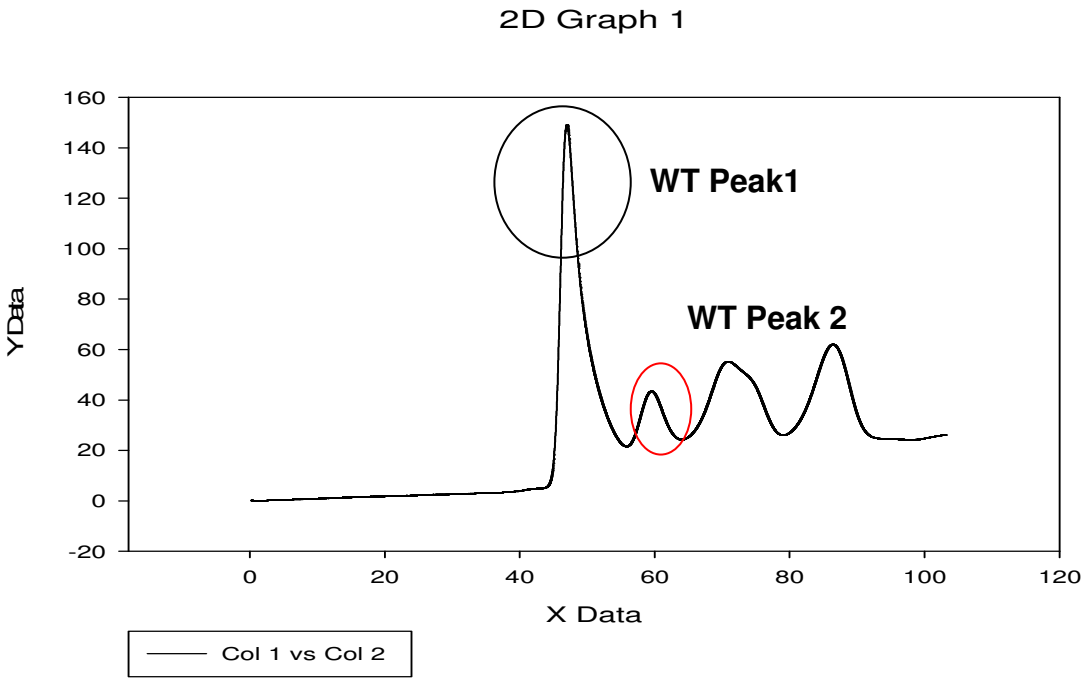


Figure 3.17 Methodology for protein purification in *E.coli.* The F475S mutation was introduced to the pPROEX-Htb-SSA1 plasmid (A) by SDM. Introduction of the TTT-TCT mutation was confirmed by sequencing (B). The PRO-SSA1^{F475S} plasmid was expressed in *E.coli* (BL21-DE3). Proteins were extracted and purified by Ni^{2+} affinity chromatography, dialysis and size exclusion chromatography.

(A)



(B)

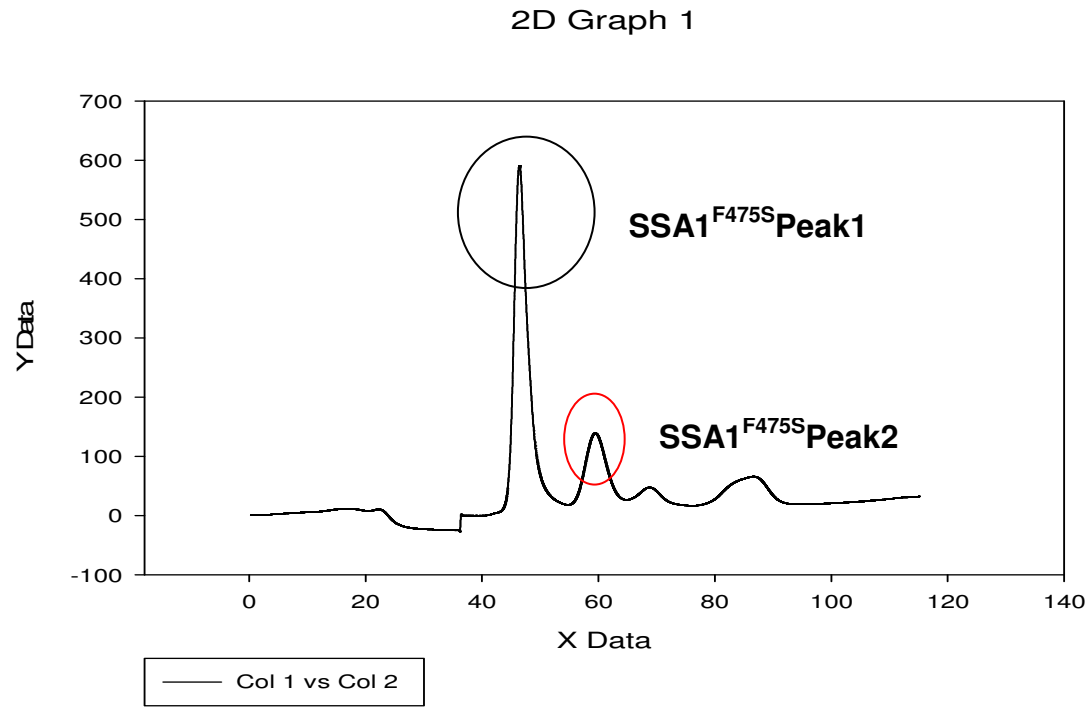


Figure 3.18 Size- exclusion chromatography chromatogram. Gel filtration column (HiLoad superDex 200pg, GE Health care) loaded with (A) Ssa1 and (B) Ssa1^{F475S} supernatant. Peak one represents aggregated Ssa1 and peak two represents native Ssa1. Both fractions were collected and stores at -70^{o}C.

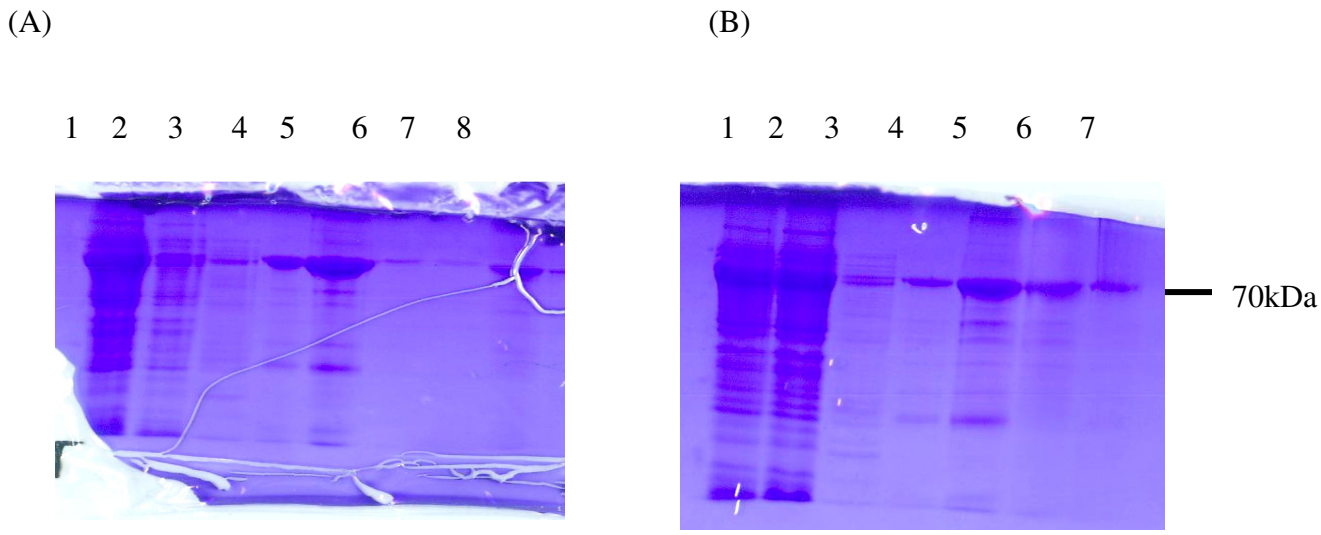


Figure 3.19 Determination of purity of Ssa1 and Ssa1^{F475S} by SDS-page
Protein fractions run on 12.5% polyacrylamide gel at 200V for one hour. Gels stained in coomassie brilliant blue for one hour and destained overnight. Gel (A) represents purification of Ssa1; Lanes 1 represents supernatant pre purification, lanes 2-4 represents fractions collected from Ni column eluted with MCAC-10, MCAC-100 and MCAC-350 respectively, lanes 5 represent post condensing of proteins post dialysis, lanes 6 and 7 represent peak one (P1) and peak two (P2) post size exclusion chromatography and lane 8 represents P1 post condensing. Gel (B) represents purification of Ssa1^{F475S}, lane 1 is loaded with supernatant pre purification, lanes 2-4 loaded as gel (A), lane 5 is protein post dialysis and lanes 6 and 7 peak one and peak two post size exclusion chromatography post condensing.

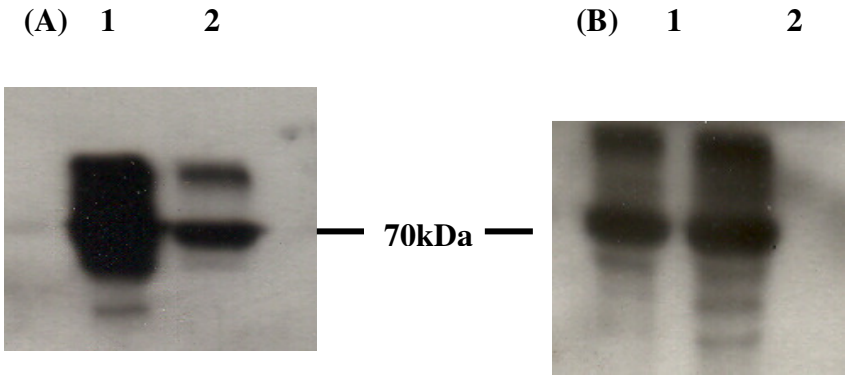


Figure 3.20 Detection of Hsp70 and His6X tag by western blotting. Peak one purified Ssa1 and Ssa1^{F475S} separated by SDS-page. Gels were immunoblotted with antibodies specific for Hsp70 (SPA-822, 1/2000 dilution) and His6x tag (anti His-Santa Cruz 1/200 dilution). Gel (A) shows Ssa1 (1) and Ssa1^{F475S} (2) probed with SPA-822 and gel (B) exhibits Ssa1 (1) and Ssa1^{F475S} (2) probed with an anti-His tag antibody.

3.11 Measuring ATPase activity of Hsp70

The binding of Hsp70 to its substrates is dependent on the binding and hydrolysis of ATP. In an ATP bound state there is rapid substrate exchange. When ATP is hydrolyzed to ADP, the substrate is trapped by Hsp70, this cycle is influenced by co-chaperones such as Hsp40 (Ydj1). One hypothesis of how Ssa1 mutants impair [*PSI*$^+$] is alteration of ATPase cycle to favor ADP bound form appears to impair prion propagation. Previous ATPase analysis on the PBD mutant *SSA1-21* showed nearly a ten fold increase of ATPase activity in comparison to wildtype. The 483 residue is closely located to two important regions related to inter-domain regulation. A change to the 483 residue could result in an inappropriate stimulatory signal to the NBD (Needham and Masison., 2008). Therefore testing ATPase activity of mutants may aid in gaining insight into how mutants such as Ssa1^{F475S} are impairing [*PSI*$^+$] propagation. The method employed for measuring ATPase activity depends on a coupled enzyme assay which is based on the conversion of phosphoenol pyruvate (PEP) to pyruvate by pyruvate kinase (PK) which is dependent on ATP hydrolysis (Figure 3.21). This reaction is coupled to the conversion of pyruvate to lactate by lactate dehydrogenase (LDH), which requires NADH being oxidized to NAD^+. NADH is strongly absorbed at OD_{340} but NAD^+ is not (Figure 3.21). This enables reduction of NADH over time to be monitored by decrease in absorbance value (Ferard *et al*., 1975). This assay can be used to indirectly measure the ATPase activity of Hsp70 through measuring absorbance of NADH.

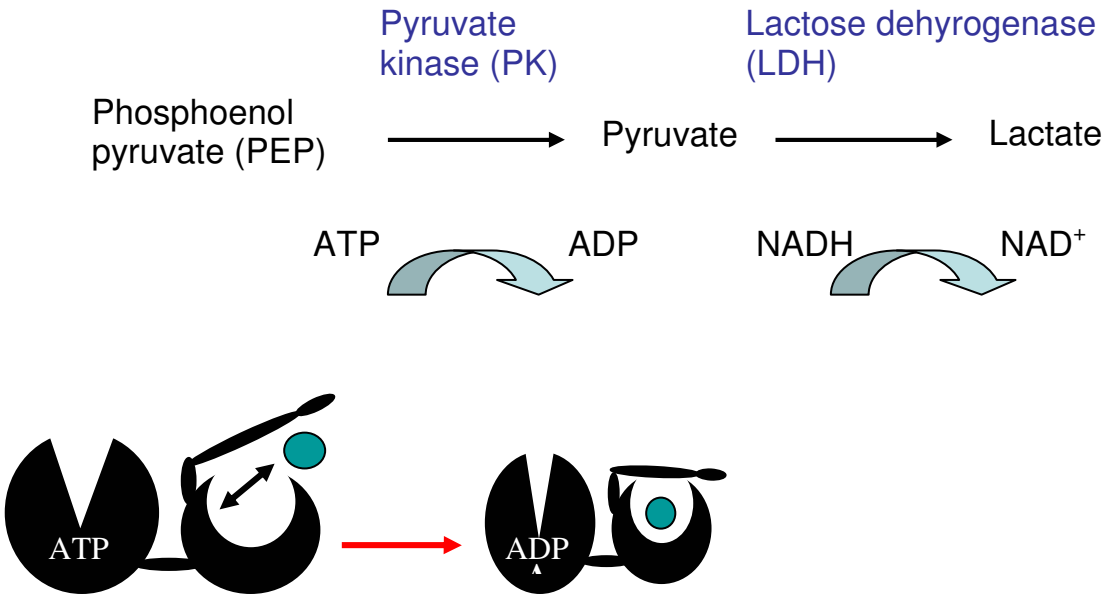


Figure 3.21 Measuring ATPase activity of Hsp70. Measuring the ATPase activity of Hsp70 using coupled enzymes assay which involves the conversion of PEP to pyruvate by the enzyme PK that is dependent on the hydrolysis of ATP to ADP. This reaction is coupled to the conversion of Pyruvate to lactate by LDH, which is dependent on the conversion of NADH to NAD^+. NADH is strongly absorbed at 340nm whereas NAD^+ is not. As the function of Hsp70 is dependent on hydrolysis of ATP, the ATPase activity of Hsp70 can be measured indirectly by this assay.

3.11.1 Investigation of ATPase activity of Ssa1^{F475S}

Alterations to ATPase activity of Ssa1^{F475S} were measured as described in section 3.10. Previous studies on the ATPase cycle of the PBD mutant *SSA1-21* revealed a ten fold increase of intrinsic ATPase activity in comparison to wildtype (Needham and Masison., 2008). Similarly, Ssa1^{F475S} appears to have increased ATPase activity, which is nearly three fold higher in comparison to that of wildtype (Figure 3.22).

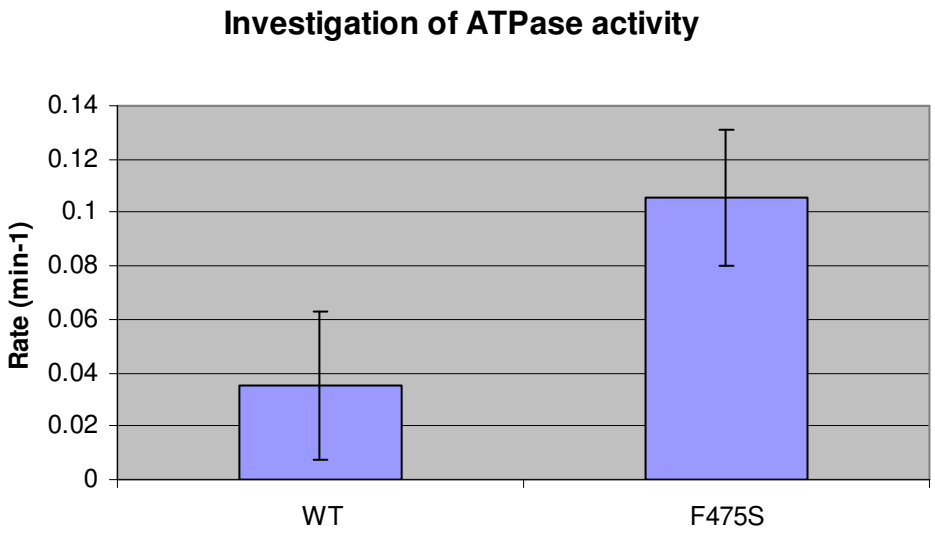


Figure 3.22 Investigation of ATPase activity Ssa1^{F475S}. Comparison of ATPase activity between wildtype and Ssa1^{F475S} was measured. ATPase assay carried out using 2µM purified wildtype Ssa1 and Ssa1^{F475S}. The x-axis represents proteins being assayed and y-axis represents rate of ATP turnover per minute. Assays repeated three times in triplicate. Ssa1^{F475S} appears to have increased ATPase activity almost three fold higher in comparison to wildtype.

3.11.2 Stimulation of ATPase activity by Hsp40 (Ydj1)

The hydrolysis of ATP is essential for the functioning of Hsp70; hydrolysis is stimulated by the Hsp40 family of co-chaperones. In yeast Ydj1 is a major Hsp40 involved in this process, increasing wildtype ATPase activity by seven fold (Needham and Masison., 2008). A recent study predicted that Hsp70 with increased ATPase activity would be less dependent on co-chaperones such as Ydj1 (Hu *et al*., 2006). This hypothesis was observed with the *SSA1-21*, the mutant's ATPase activity was stimulated two fold by Ydj1, suggesting the mutant might not be regulated by Hsp40 as efficiently as wildtype (Needham and Masison., 2008). Ydj1 was purified as described in chapter 2.7, by Ni^{2+} affinity chromatography and dialysis (Figure 3.23). The stimulation of ATP hydrolysis of $Ssa1^{F475S}$ was increased by less than two fold by Ydj1, suggesting that the function of $Ssa1^{F475S}$ may not be as efficiently regulated by Hsp40 as wildtype Ssa1 (Figure 3.24).

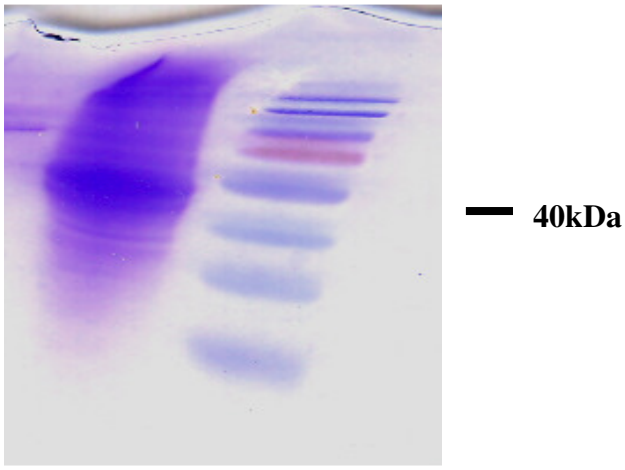


Figure 3.23 Purified Ydj1. Ydj1 was expressed and extracted from *E.coli.* The expressed protein with an N-terminal His6 tag was purified using Ni^{2+} affinity chromatography and dialysis. Purified protein was run on a 12.5% polyacrylamide gel and stained with coomassie overnight and destained the following day.

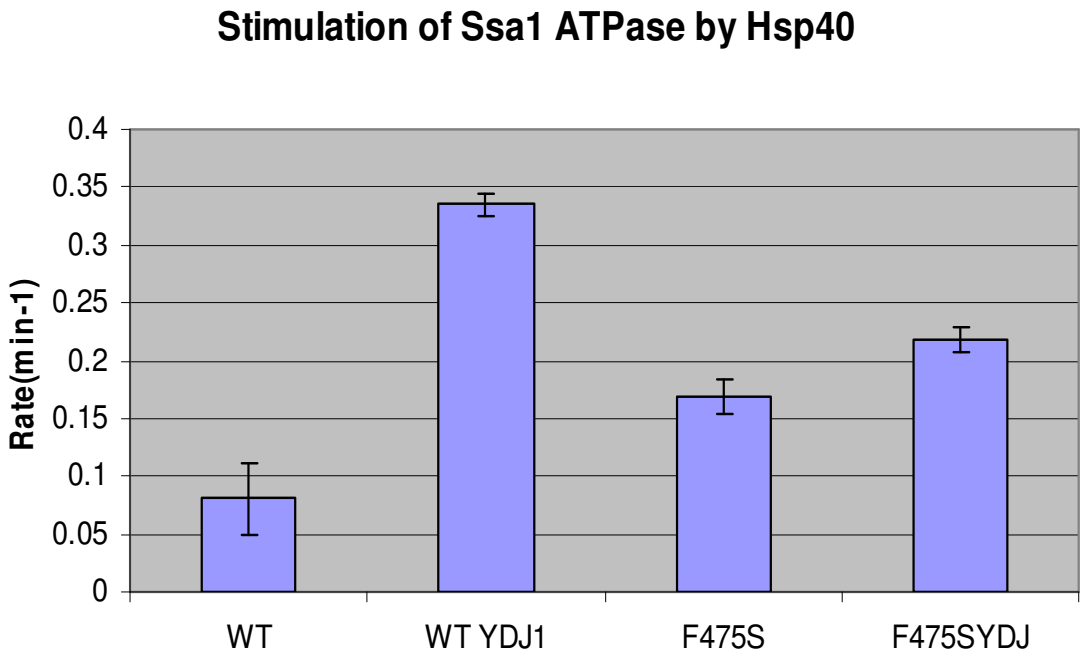


Figure 3.24 Stimulation of Ssa1 ATPase by Hsp40. This graph represents the stimulation of ATPase activity of Ssa1 and Ssa1 ^{F475S} by Hsp40. ATPase assay carried out using 2µM of purified proteins. The x-axis represents proteins being assayed and y-axis represents rate of ATP turnover per minute. Assay carried out once in triplicate. Wildtype ATPase activity increases by four fold in presence of Hsp40 whereas there is only a modest increase with Ssa1 ^{F475S}.

3.12 Analysis of protein structure of Ssa1^{F475S}

The increase in the rate of ATP turnover in Ssa1^{F475S} may be due to the F475S mutation causing a conformational change in the structure of the Ssa1p. Comparing the secondary structure of wildtype Ssa1 and Ssa1^{F475S} by Circular Dichroism (CD) spectroscopy may elicit changes to secondary structure to Ssa1$^{F475S.}$ CD spectroscopy measures differences in the absorption of left-handed polarized light versus right-handed polarized light which arise due to structural asymmetry. Alpha-helix and beta sheet each give rise to a characteristic shape and magnitude on CD spectrum; alpha-helix structures absorb at and OD_{222} whereas beta sheet structures absorb at OD_{216} (Figure 3.25)

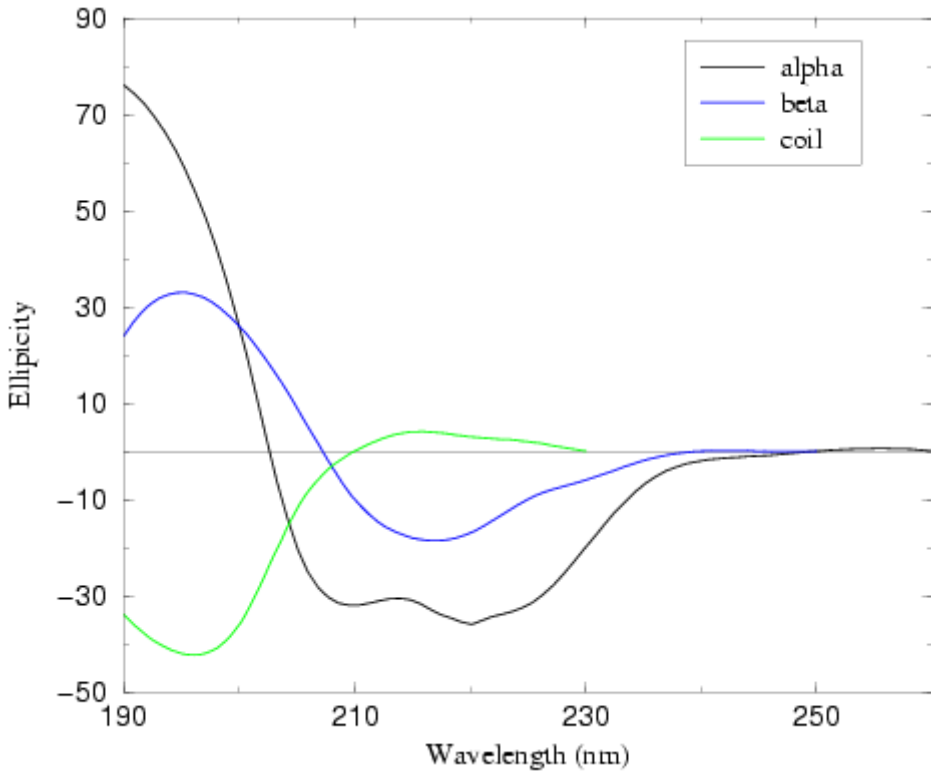


Figure 3.25 Example of CD spectroscopy. Alpha- Helices absorb at OD_{208} and OD_{222} as represented by black line. Beta-sheets absorb at 216nm, the absence of B-sheet is represented by a bell shape between OD_{208} and OD_{222}, the presence of B-sheet is represented by a dip in line between OD_{208} and OD_{222} this is represented by blue line (http://besley.chem.nottingham.ac.uk/research/cdcurves.gif).

CD spectroscopy analysis of Ssa1^{F475S} revealed no major structural changes to the secondary structure of Ssa1 (Figure 3.26), indicating that the increased rate of ATP turnover is probably not correlated with a change in protein structure caused by the F475S mutation.

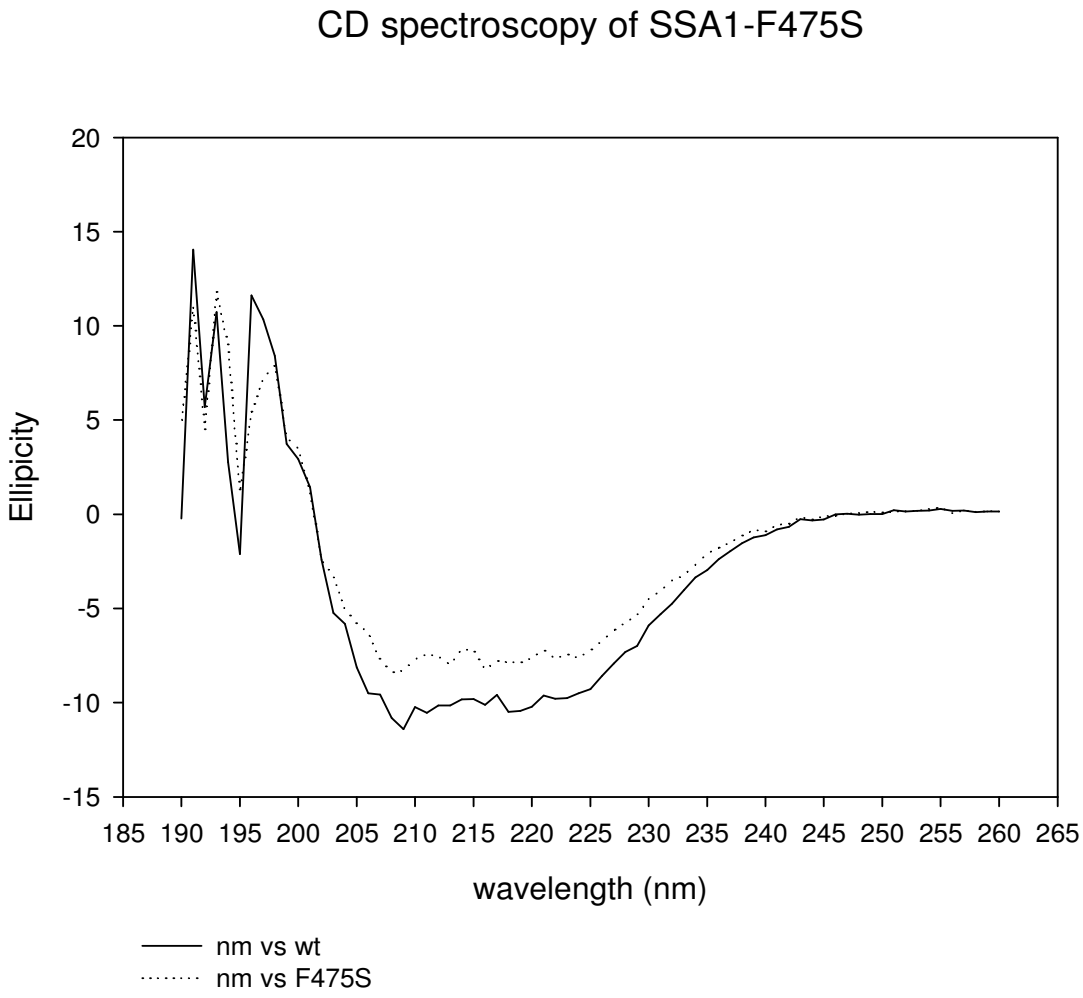


Figure 3.26 CD spectroscopy analysis of Ssa1^{F475S}. CD spectroscopy was preformed measuring the absorbance of 4µM of purified Ssa1p and Ssa1^{F475S} in a total volume of 350µl of dialysis buffer on a Pistar CD stopped flow spectrophotometer (Applied biophysics limited).

3.13 Functional conservation of F475S across the Ssa family

Ssa1 and Ssa2 are constitutively expressed and have 97% homology, Ssa3 and Ssa4 have 87% homology and are induced when the cell encounters stress, stationary phase or during sporulation. To assess functional conservation, the F475S mutation was introduced into *SSA2*, *SSA3* and *SSA4* genes by site-directed mutagenesis. Mutated plasmids were transformed into G402 and by the plasmid shuffle technique; each mutant was expressed in yeast as the sole source of the Ssa family. To compare each mutant to $Ssa1^{F475S}$ the prion impairing and t.s phenotype for each mutant was assessed. $Ssa2^{F475S}$ and $Ssa4^{F475S}$ behaved in the same manner as $Ssa1^{F475S}$; exhibiting a [*psi*$^-$] phenotype and temperature sensitivity at 37^{o}C (Figure 3.27- A). Interestingly when the F475S mutation was introduced into Ssa3, the protein became non-functional (Figure 3.27- B). Therefore, in Ssa2 and Ssa4 the F475 residue also appears to be important for prion propagation and heat shock functions. For an unknown reason, the mutation has a different effect in Ssa3.

3.14 Affect of Hsp70 in cell wall integrity signaling

Damage to the cell wall occurs when yeast encounter heat and chemicals such as caffeine or congo red. When such encounters occur a MAP kinase pathway, which is a signal transduction pathway is activated; cell wall damage is transmitted by protein kinase C to the MAP kinase pathway, this is a linear pathway comprised of many components that

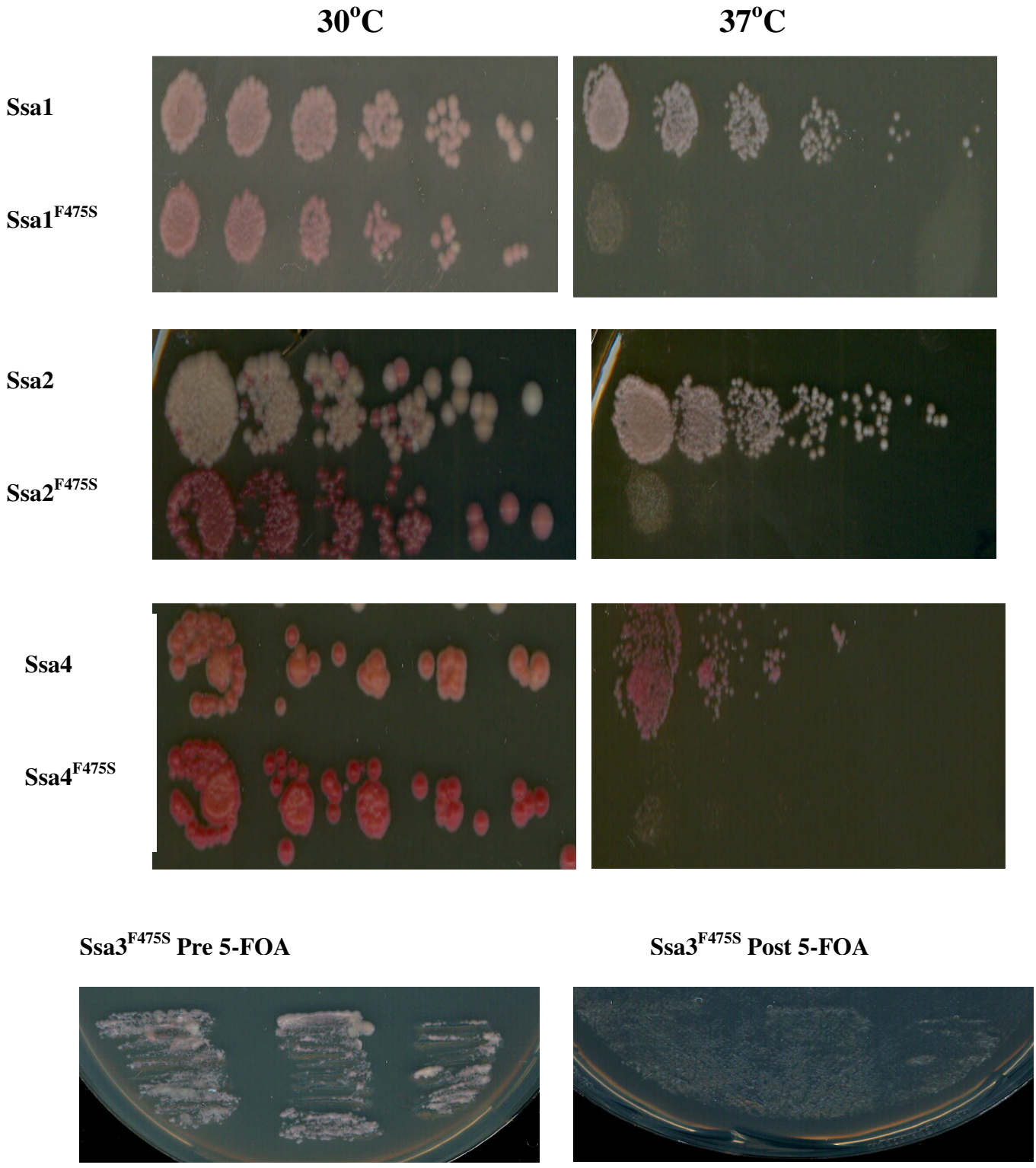


Figure 3.27 (B)

Figure 3.27 Analysis of F475S mutation in Ssa1-4. (A) Ssa1, 2 and 4 mutants, as indicated, expressed in G402 as sole source of Ssa. Cells diluted by a 1/5 serial dilution and incubated for 48 hours.Ssa2^{F475S} and Ssa4 ^{F475S} exhibited the same phenotypes as Ssa1 ^{F475S} **(B)** Ssa1 ^{F475S} expressed in G402, pre and post 5-FOA.

terminates with the phosphorylation of the transcription factors Rlm1 and SBF, which then activate the expression of genes involved in cell wall repair and synthesis (Figure 3.28). Introducing mutations to components of the cell wall integrity (CWI) signaling pathway results in common phenotypes such as temperature sensitivity at 37^{o}C and sensitivity to drugs that damage the cell wall. Both phenotypes can be rescued by osmotic support (e.g. sorbitol) in growth media. Hsp90 and the Hsp70 nucleotide exchange factor Sse1 have been implicated in the cell wall integrity signaling pathway. Hsp90 was identified as a Slt2 binding partner (Millson. *et al.,* 2005) and Sse1 genetically interacts with the SBF and MBF cell cycle-regulated transcription factors to support growth. (Shaner *et al*., 2008). The Ssa1^{F475S} mutant may implicate Ssa1 as playing a role in the cell wall integrity signaling pathway as Ssa1^{F475S} exhibits the temperature sensitivity phenotype at 37^{o}C. To test this hypothesis the 37^{o}C t.s mutants Ssa1^{F475S}, Ssa2^{F475S} and Ssa4^{F475S} were incubated at 37^{o}C on YPD containing 1M sorbitol. Recovery of the 37^{o}C t.s mutants on 1M sorbitol is indicative that Ssa1 is a component of the cell wall integrity signaling pathway (Figure 3.29).

3.14.1 Effect of 1M sorbitol on expression of Ssa1^{F475S}

To further our inverstigation of Ssa1's involvment in the cell wall integrity signalling pathway we examined the abundance of Ssa1^{F475S} at 30^{o}C and 37 ^{o}C in the presence of 1M sorbitol. This was achieved by making lysates from yeast cultures expressing Ssa1^{F475S} that were grown at 30^{o}C and 37^{o}C in YPD supplemented with 1M sorbitol (YPDS). Abundance of Hsp70 and Hsp104 was observed by western blot analysis.

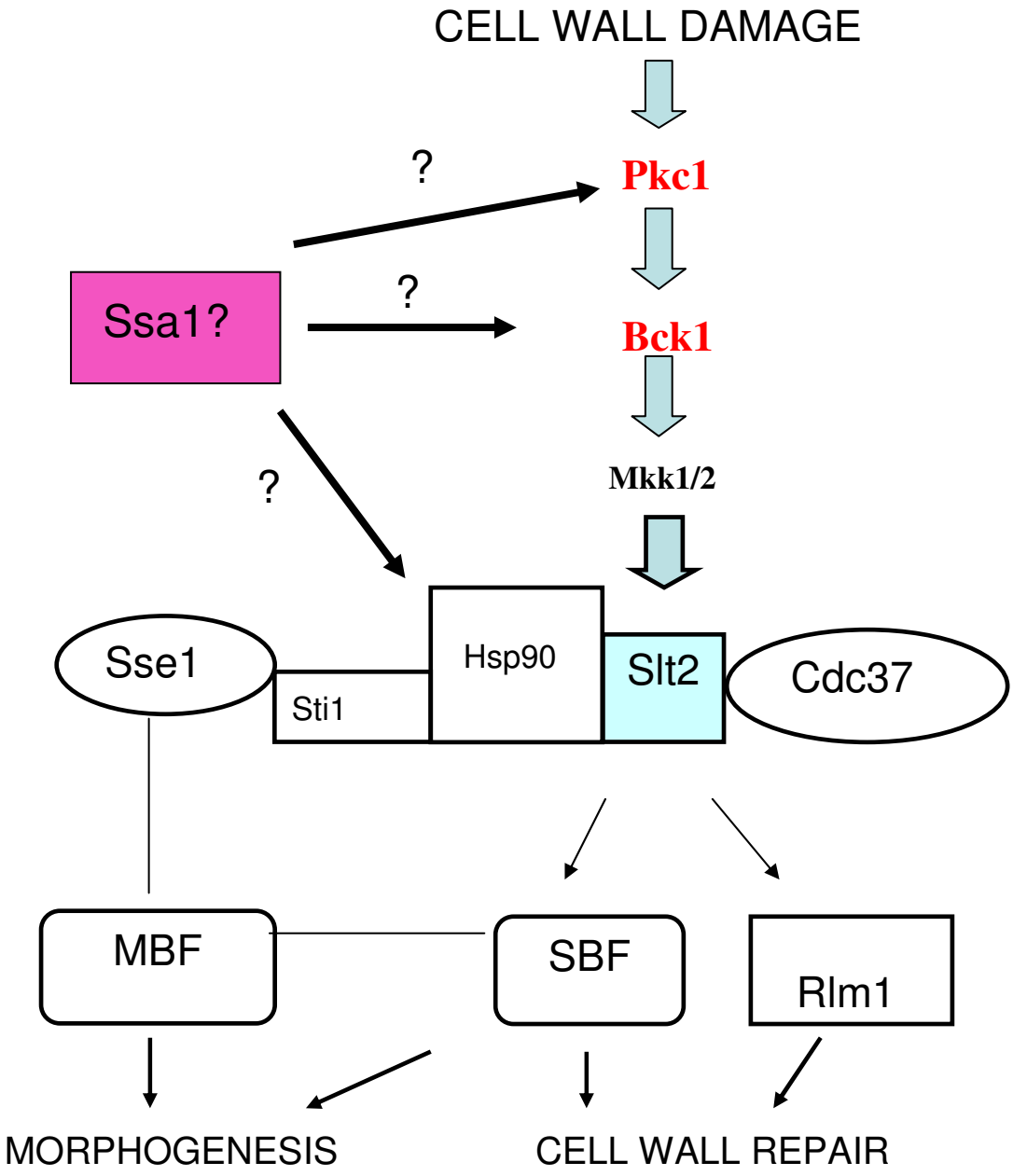


Figure 3.28 Cell wall integrity signaling pathway. A linear MAP Kinase signaling cascade is responsible for the amplification of damage to the cell wall. It has been established that Pkc1 activates Bck1 which then activates Mkk1/2, which then phosphorylates Slt2. Slt2 phosphorylates and activates the transcription factors Rlm1 and SBF which activate genes involved in cell wall synthesis and repair. Hsp90 and the Hsp70 nucleotide exchange factor Sse1 have been implicated as part of this pathway via a partnership with Slt2.

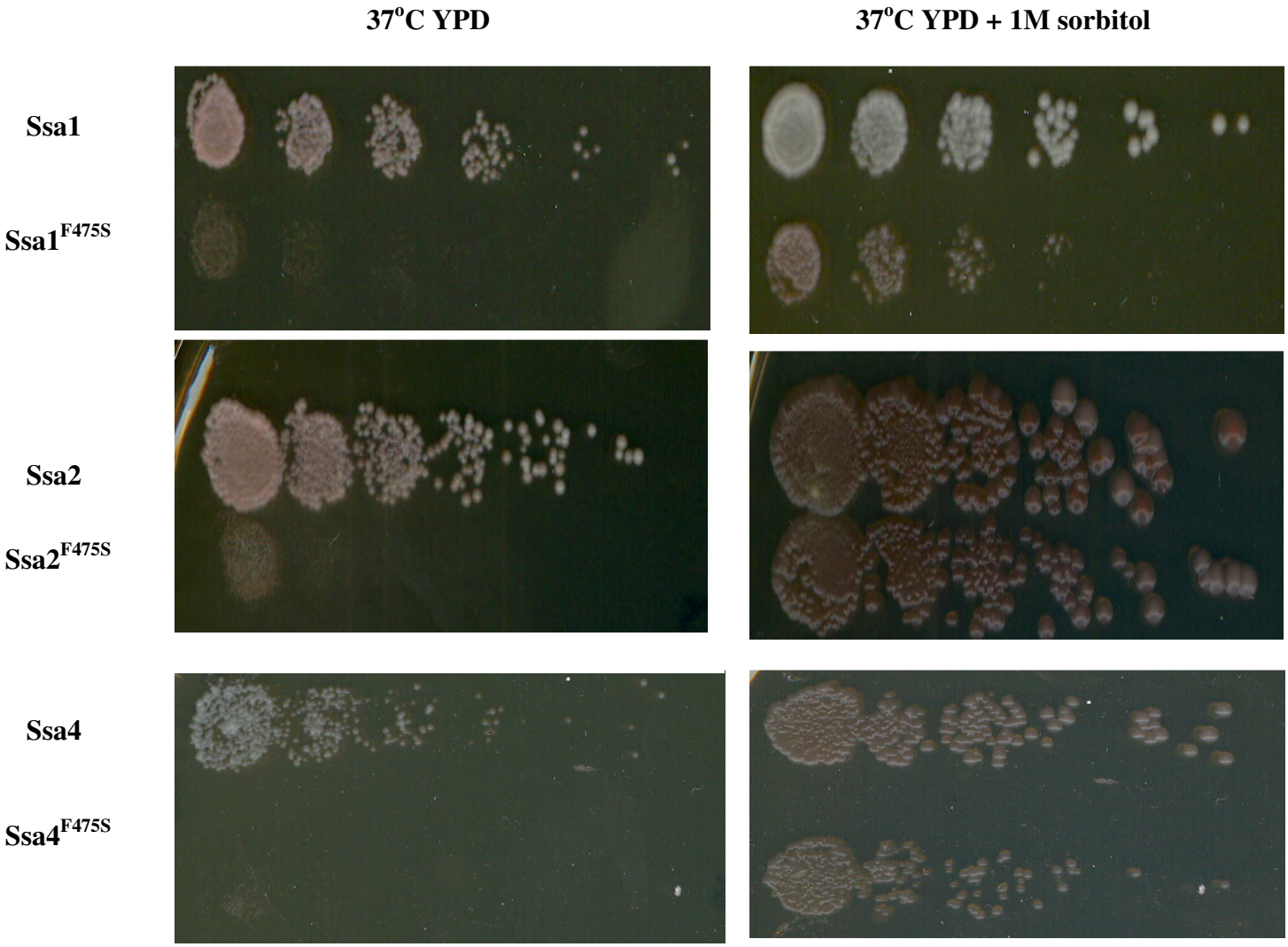


Figure 3.29 Recovery of 37°C t.s phenotype by 1M sorbitol. Cells incubated at 37°C on YPD and YPD supplemented with 1M sorbitol for 48 hours. Cells diluted by a 1/5 serial dilution. Mutants as indicated. The 37°C t.s phenotype of $SSA1^{F475S,}$ $Ssa2^{F475S}$ and $Ssa4^{F475S}$ were recovered by growing cells at 37°C on YPD supplemented with 1M sorbitol.

Ssa1^{F475S} was unable to grow in YPD 37 ^{o}C but was recovered when grown in YPDS. Growing wild type in YPDS seems to have no effect on the abundance of Ssa1 at 37^{o}C , whereas growing Ssa1^{F475S} in YPDS the protein level appears to increase at 30^{o}C and recovers at 37^{o}C (Figure 3.30).

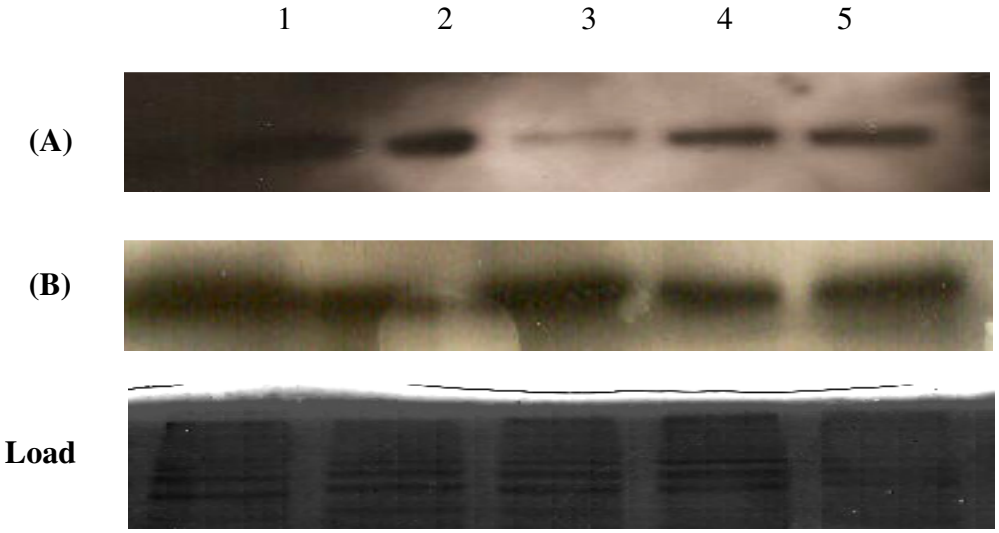


Figure 3.30 Effect of 1M sorbitol on abundance of Hsp70 and Hsp104. Lysates prepared from overnight 30^{o}C and 37^{o}C cultures grown in YPD and YPD supplemented with 1M sorbitol (YPDS). Aliquots of proteins (10µg) were separated on three polyacrylamide gels, gels were either immunoblotted with antibodies either specific for (A) Hsp70 (SPA-822, 1/2000 dilution) and (B) Hsp104 (HSP104, 1/150,000 dilution) or stained (C) coomassie brilliant blue stained overnight and destained the following day. Lanes 1-2 contain wildtype Ssa1 grown at 37^{o}C in YPD and YPDS, lanes 3-4 contain Ssa1^{F475S} grown at 30^{o}C in YPD and YPDS, lanes 5 contains Ssa1^{F475S} grown at 37^{o}C in YPDS.

3.14.2 Expression of constitutively active components of CWI pathway

It has been well established that chaperones and cochaperones such as Hsp90 and Sse1 play a role in CWI signaling pathway. Or results may implicate Hsp70 as also playing a role. To further test this hypothesis, we assessed the effects of expressing constiuitevly active components of the CWI signaling pathway, Bck1 and Pkc1. Loss of function of any of the componets below Pkc1 results in a 37^{o}C t.s phenotype. Cells harboring the Ssa1^{F475S} mutation were transformed with plasmids expressing either constitutively active Bck1 or Pkc1 and phenotypes were observed at 37^{o}C. Recovery of Ssa1^{F475S} by the expression of Bck1 and Pkc1 is indicative that the 37^{o}C t.s phenotype is due to a disruption in the CWI signaling pathway (Figure 3.31).

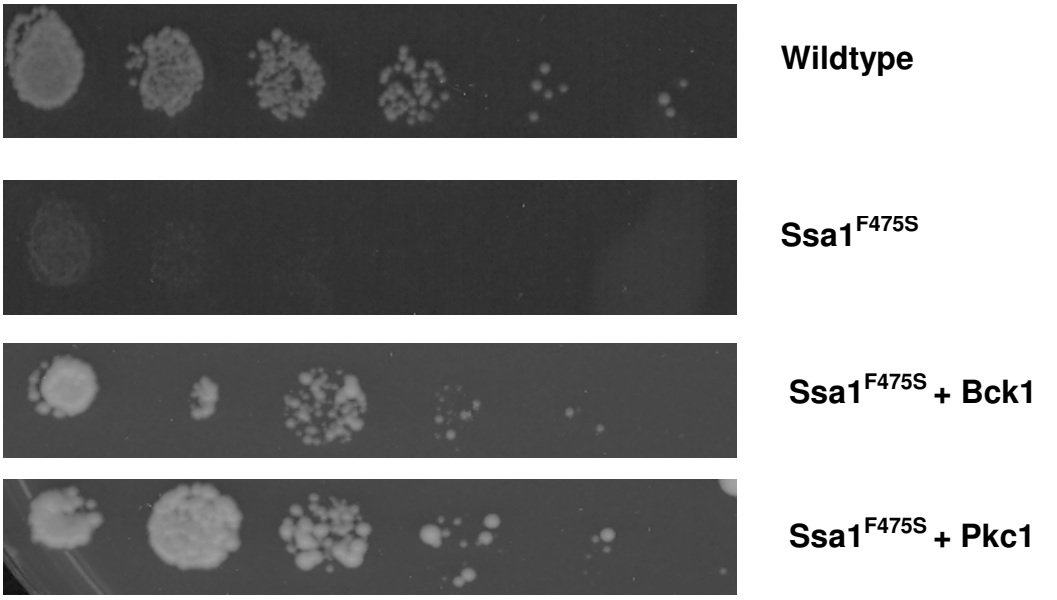


Figure 3.31 Recovery **of Ssa1^{F475S} 37^{o}C t.s phenotype by components of CWI signaling pathway.** Plasmids expressing components of the CWI signaling pathway, Bck1 and Pkc1 were expressed in cells harboring Ssa1^{F475S}. Cells incubated at 37^{o}C for 48 hours on YPD. Cells diluted by a 1/5 serial dilution. Mutants as indicated. The 37^{o}C t.s phenotype of Ssa1^{F475S} was recovered in the presence of Bck1 and Pkc1.

3.15 Effects of PBD mutations on other cellular functions.

We have observed the Hsp70 PBD mutant Ssa1^{F475S} as impairing [*PSI*$^{+}$] propagation and have implicated Hsp70 as playing a role in CWI signalling pathway through investigation of the Ssa1^{F475S} 37^{o}C t.s phenotype. To gain further insight of the role of Hsp70 we decided to investigate the effect of Ssa1^{F475S} on other cellular functions. The three cellular functions we assessed in *S.cerevisiae* were; pleiotropic drug resistance (Pdr), Hsp70 self- regulation and the unfolded protein response.

3.15.1- Investigation of the effect of Ssa1^{F475S} on PDR

Pleiotropic drug resistance (Pdr) is a major problem in the treatment utilizing antifungal drugs. Isolation of *S. cerevisiae* containing single nuclear mutations have exhibited resistance to a wide range of toxic compounds, these cells are refered to as having a Pdr phenotype. In *S. cerevisiae* a complex network of genes mediates the Pdr response. Pdr1 and Pdr3 are two major transcription factors which control the Pdr response by activating genes such as Pdr5, Pdr10 and Pdr15 which encode ATP-binding cassette (ABC) transporters which operate as drug efflux pump. Ssa1 was found to co-purify with the transcription factor Pdr3 and further studies revealed that binding of Ssa1 to Pdr3 keeps this protein in a low activity state (Shahi. *et al*. 2007). To analyze the effect of Ssa1^{F475S}, we received a ß-galactosidase reporter plasmid which expresses the *PDR5* promoter fused to the *lac*Z reporter gene. The bacterial *lac*Z gene encodes the ß-galactosidase enyme which cleaves the compond *o*-nitrophenol- ß-D-galactoside (ONPG) to produce a coloured compound *o*-nitrophenol, which can be quantified by measuring its absorbance at OD_{420} (Figure 3.32). In order for us to assess the effect of Ssa1^{F475S} on Pdr5 expression, the Pdr5 ß-galactosidase

reporter plasmid was transformed into G402 yeast cells expressing wildtype Ssa1 or Ssa1^{F475S}. The ß-galactosidase activity was measured as decribed in chapter 2.8. The F475S mutation appears to increase the activity of the Pdr5 promoter by more than six fold (Figure 3.33). This may be indicitave that the F475S mutation affects binding of Ssa1 to Pdr3 and this may impair the negative regulation of Pdr3 by Ssa1.

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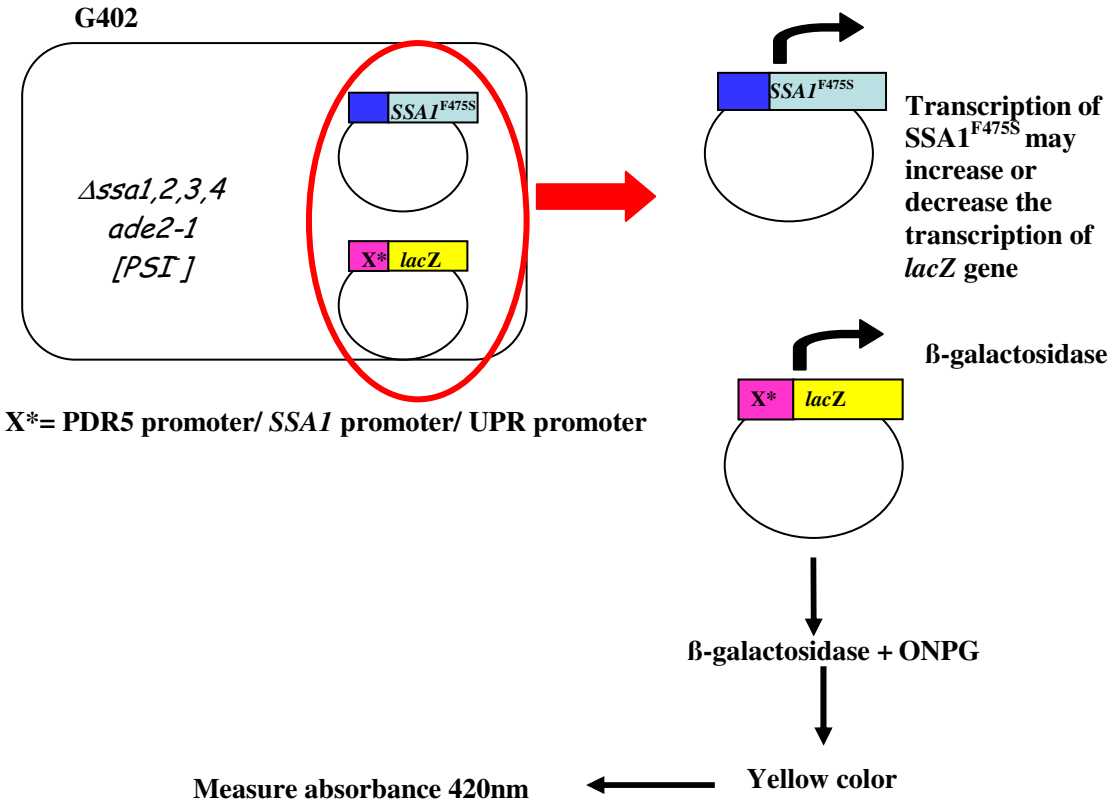


Figure 3.32 Strategy for performing ß-galactosidase assays in yeast. The *lacZ* reporter plasmid was transformed into the yeast strain G402 which was expressing Ssa1^{F475S} as the sole source of Ssa. The transcription of Ssa1^{F475S} may affect levels of expression of *lacZ* depending on promoter fused to the reporter gene. When *o*-nitrophenol- ß-D-galactoside (ONPG) is added the reaction will turn yellow over a period of time, depending on how strong/weak the reaction is. Reaction is measured at OD_{420}.

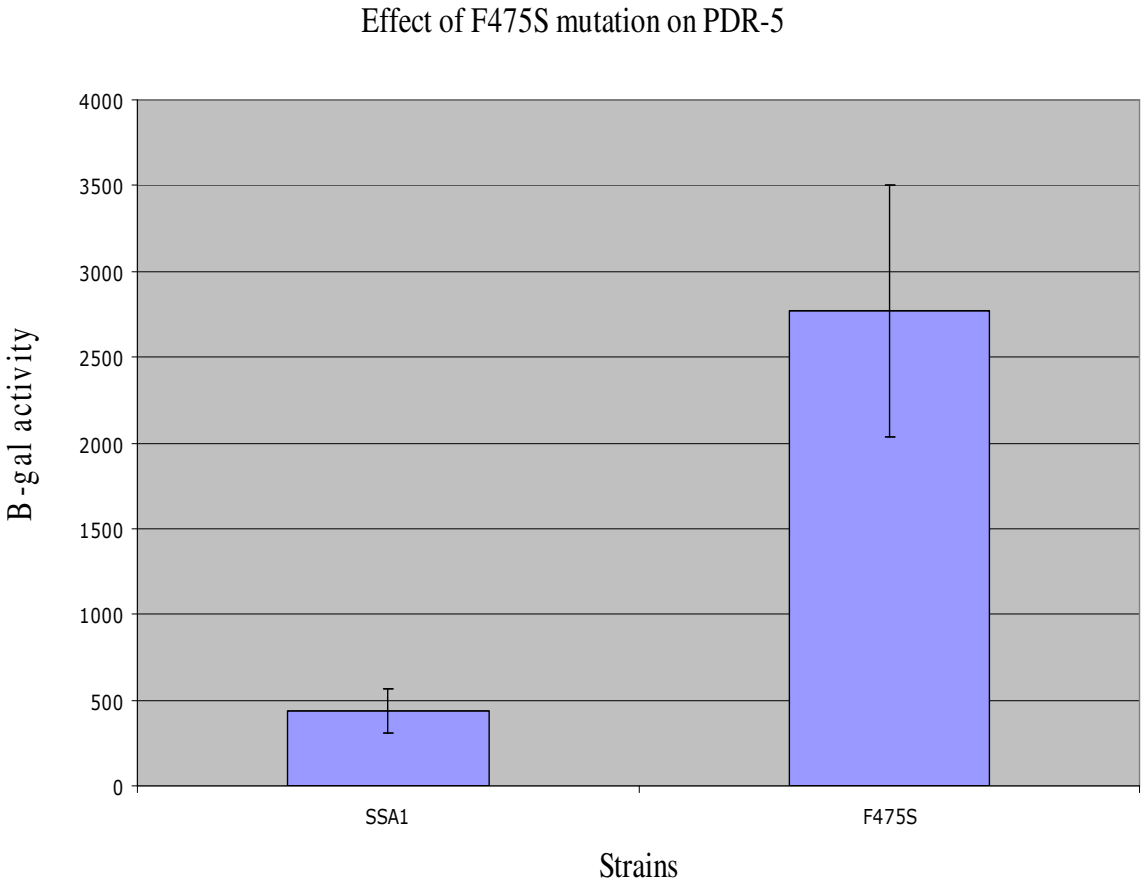


Figure 3.33 Effect of Ssa1^{F475S} on *PDR5* expression. Yeast cells expressing wildtype Ssa1 or Ssa1^{F475S} were transformed with the Pdr5 ß-galactosidase reporter palsmid. Transformed strains were grown overnight in 5ml of YPD at 30^{o}C to a concentration of 5x10^{6} (cells/ml). Cells were then grown to a concentration of 1x10^{7}-2x10^{7} (cells/ml). Cells were chilled on ice and then harvested at 4^{o}C. Protein was extracted and reaction was initiated by adding ONPG, once a pale yellow color was observed the reaction was terminated by adding 1M Na_2CO_3. *o*-nitrophenol was quantified by measuring absorbance at OD_{420}. Units of ß-galactosidase activity (Y axis) were defined as (OD_{420}) X (1.7)/ (.0045) X (volume) X (time) X (protein concentration). Ssa1^{F475S} appears to have more than a six fold increase in *PDR5* promoter activity in comparison to the effect of wildtype Ssa1 on *PDR5* promoter activity.

3.15.2 Effect of Ssa1^{F475S} on Ssa1 expression

In yeast the Ssa family consists of four highly conserved proteins, Ssa1-Ssa4. Ssa1 and Ssa2 are constitutively expressed but only expression of Ssa1, Ssa3 and Ssa4 are induced by shock. The induction of heat shock proteins by stress depends on heat shock factors (HSF), which bind to heat shock elements (HSE) in the promoters of heat shock genes. The *SSA1* promoter contains HSE's, HSE2 has proven to be necessary for basal and heat inducible expression (Slater and Craig, 1987). Ssa1 is autoregulatory, i.e. it negatively regulates its own expression (Stone and Craig, 1990).

To test the effect of Ssa1^{F475S} on *SSA1* expression, the *SSA1* promoter was fused to the *lac*Z gene and the plasmid was transformed into cells expressing wildtype Ssa1 or Ssa1 ^{F475S} and activity was measure by ß-galactosidase assay (section 3.12.1). Expression of Ssa1 is increased by more than two fold when the F475S mutation is present (Figure 3.34). This indicates the the F475S mutataion may have an effect on the transcriptional levels of the *SSA1* gene.

3.15.3 Effect of Ssa1^{F475S} on the unfolded protein response

As Hsp70 is a chaperone that is involved in folding of nascent proteins and the refolding of misfolded proteins, we decided to analyze the effect of Ssa1^{F475S} on the unfolded protein response in yeast. Biosynthesis and maturation of secretory proteins occurs in the endoplasmic reticulum (ER). Stress in the ER inhibit these events and trigger the unfolded protein response (UPR). The type I transmembrane protein Ire1 is a key component of the UPR, it detects elevated unfolded protein levels in the ER.

Figure 3.34 Effect of SSA1^{F475S} on *SSA1* expression. Yeast cells expressing wildtype Ssa1 or Ssa1^{F475S} were transformed with the *SSA1* promoter ß-galactosidase reporter plasmid (pZFO) . ß-galactosidase assays were carried out as described in Figure 3.33. The F475S mutation appears to increase transcription of the *SSA1* promoter by more than two fold in comparison to wildtype.

The C-terminus of Ire1 possesses RNase activity, which acts to splice *HAC1* precursor mRNA. The spliced *HAC1* mRNA is translated into the transcription factor Hac1, which targets promoters of various genes containing the unfolded protein response element (UPRE), which encode protein chaperones and protein folding catalysts (Figure 3.35). The gene *KAR2* that encodes the ER Hsp70, Bip, contains UPRE and is thought to be involved in the UPR (Kohno. *et al.,* 1993). An additional function of the Hsp70 protein, Bip, is the repression of the UPR by interacting with Ire1 (Okamura. *et al.,* 2000).

To assess whether the $Ssa1^{F475S}$ mutation affects the UPR, we measured the activity of an unfolded protein response element. This was achieved by utilizing a ß-galactosidase reporter plasmid which expressed the UPRE promotor fused to the *lac*Z gene. The reporter plasmid was transformed into yeast cells expressing wildtype Ssa1 or $Ssa1^{F475S}$. To ensure measurments of the UPR were accurate, the assay was preformed with and without 5mM of the reducing agent dithiothreitol (DTT). Therefore DTT should increase protein unfolding and there should be a higher rate of the UPR in assays containing DTT.

We observed that in yeast cells expressing $Ssa1^{F475S}$, the expression of the UPRE had increased nine fold in comparison to cells expressing wildtype Ssa1 in the presence of 5mM DTT (Figure 3.36). The addition of DTT had a modest affect on the UPRE promoter activity; this may be due to strain background. This may implicate cytosolic Hsp70 as playing a part in the UPR. One predication may be that Bip utilizes cytosolic

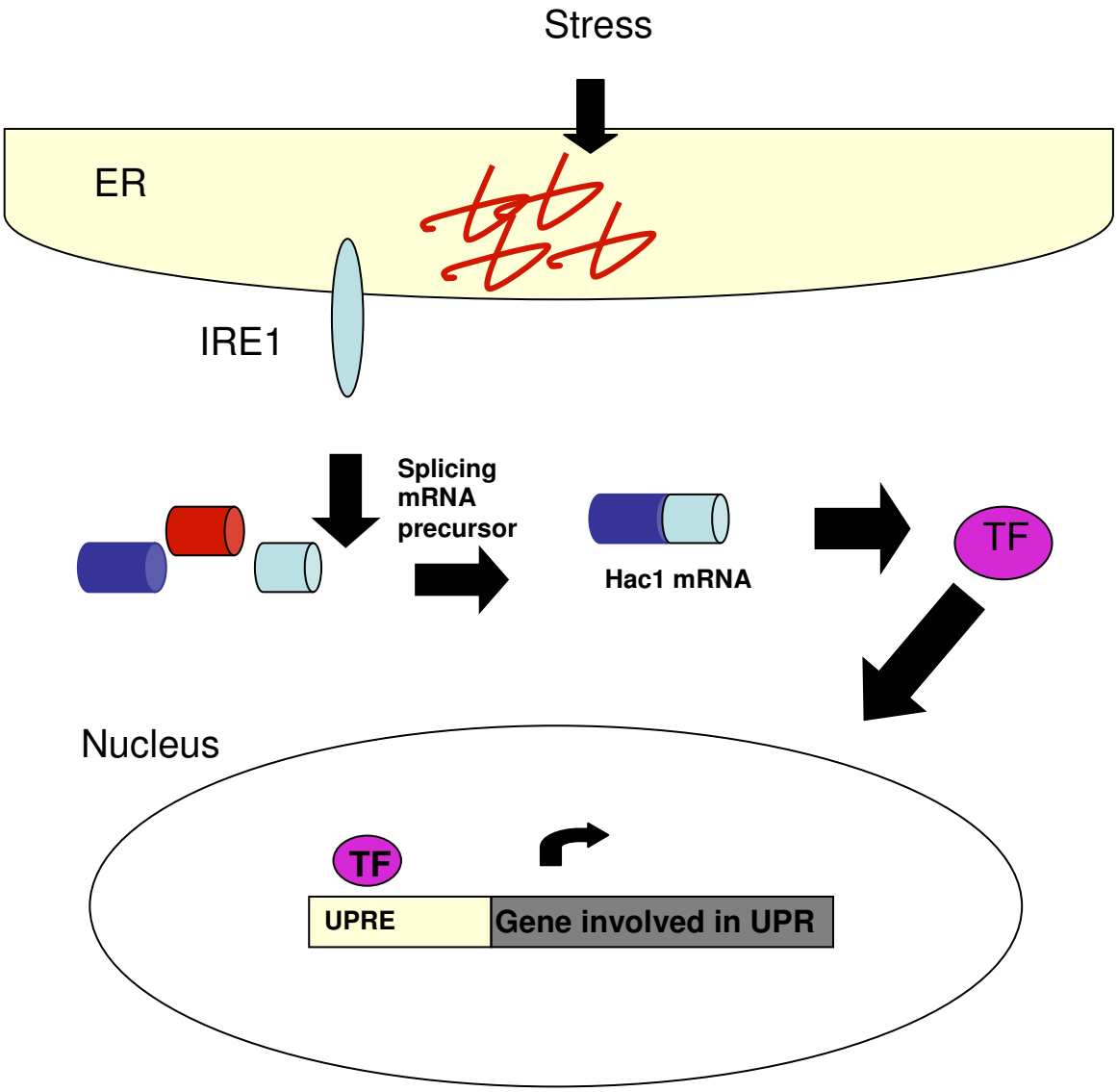


Figure 3.35 The unfolded protein response in yeast. Stress in the ER causes unfolding of proteins. This stress is sensed by the type I transmembrane protein Ire1. The C-terminal of Ire1 exhibits RNase activity which splices to splice *HAC1* precursor mRNA in the cytoplasm. The mature mRNA is translated into a transcription factor (Hac1), which moves to the nucleus. Hac1 binds to promoters of genes that contain UPRE, which encode protein chaperones and protein folding catalysts.

Hsp70 or its activity for the inhibition of Ire1 and the F475S mutation somehow inhibits this process causing the rise in expression of the UPRE.

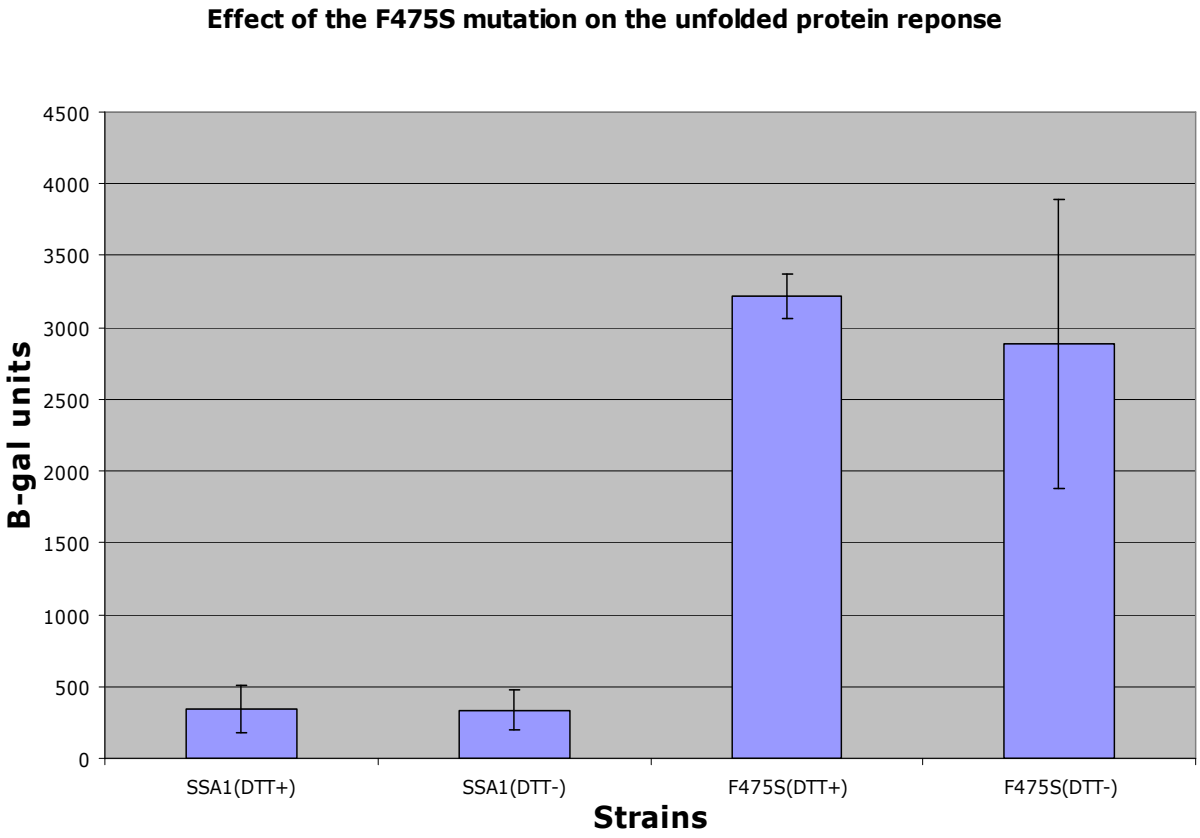


Figure 3.36 The effect of Ssa1^{F475S} on the unfolded protein response. Yeast cells expressing wildtype Ssa1 or Ssa1^{F475S} were transformed with the UPRE promoter ß-galactosidase reporter palsmid (pUPRE)**.** ß-galactosidase assays were carried out described in Figure 3.33, with the exception of adding 5mM DTT into cultures for thirty minutes at 30^{o}C. The X-axis indicates the strains used in the assay; wildtype Ssa1 and Ssa1^{F475S} with and without DTT. The Y axis indicates the ß-gal units.

3.16 Microarray analysis of Ssa1^{F475S}

To gain further insight into the affects of expressing Ssa1^{F475S} as the sole source of Ssa1, we decided to analyze the affects of the mutant on gene expression in comparison to cells expressing wildtype Ssa1. Detailed microarray analysis data is

supplemented as a hard copy (CD). As previously mentioned, the PBD mutant Ssa1^{F475S} impairs prion propagation and has a t.s 37^{o}C phenotype that can be remediated by growth in the presence of 1M sorbitol, which may implicate Ssa1 as playing a role in the CWI signaling pathway. The presence of the F475S mutation also appears to have an affect on the expression of genes involved in diverse cellular functions such as; the pleiotropic drug response, unfolded protein response and expression of Ssa1.Table 3.7 lists genes that are exclusively up-regulated in Ssa1^{F475S} (up-regulation of genes compared to expression of genes in Ssa1, 2, 3 and 4). Twelve genes are up-regulated by more than three-fold in the Ssa1^{F475S} strain. Genes that are up-regulated appear to be involved in glucose transportation (*GAL2* and *SNF3*), Iron homeostasis (*FET4* and *FIT3*) and cell wall integrity (*SSD1*).

As Ssa1 ^{F475S} may implicate Ssa1 as playing a role in the CWI signaling pathway, up-regulation of the *SSD1* gene may provide further insight into this. Ssd1 plays a role in the maintenance of cellular integrity. It has been reported that Ssd1 may regulate a pathway parallel to the Pkc1 pathway to maintain cell wall structure and cell wall integrity (Kaeberlein and Guarente, 2002). Interestingly it has also been reported that Ssd1 is required for Hsp104 mediated protein dissaggregation (Mir *et al*., 2009). Ssd1 influences the ability of Hsp104 to hexamerize, which then permits interaction with the co-chaperone Sti1 and consequently binding of protein aggregates (Mir *et al.,,* 2009).

ORF/Gene	Gene function	$Ssa1^{F475S}$
YLR081W/*GAL2*	Galactose permease, required for utilization of galactose; also able to transport glucose.	6.241
YOR383C/*FIT3*	Mannoprotein that is incorporated into the cell wall via a glycosylphosphatidylinositol (GPI) anchor, involved in the retention of siderophore-iron in the cell wall.	3.922
YMR319C/*FET4*	Low-affinity Fe(II) transporter of the plasma membrane	3.756
YPR101W/*SNT309*	Member of the NineTeen Complex (NTC) that contains Prp19 and stabilizes U6 snRNA in catalytic forms of the spliceosome containing U2, U5, and U6 snRNAs	3.556
YAL055W/*PEX22*	Putative peroxisomal membrane protein required for import of peroxisomal proteins.	3.397
YDR293C/*SSD1*	Protein with a role in maintenance of cellular integrity, interacts with components of the TOR pathway	3.187
YDR019C/*GCV1*	T subunit of the mitochondrial glycine decarboxylase complex, required for the catabolism of glycine to 5, 10-methylene-THF.	3.156
YNR067C/*DSE4*	Daughter cell-specific secreted protein with similarity to glucanases, degrades cell wall from the daughter side causing daughter to separate from mother	3.129
YLD194W/*SNF3*	Plasma membrane low glucose sensor that regulates glucose transport	3.125
YDL078C/*ATG8*	Component of autophagosomes and Cvt vesicles	3.085
YDR406W/*PDR15*	Plasma membrane ATP binding cassette (ABC) transporter, multidrug transporter and general stress response factor implicated in cellular detoxification; regulated by Pdr1, Pdr3 and Pdr8.	3.043
YOR378W/Unknown	Unknown	3.040

Table 3.7 Up-regulation of genes in Strains expressing $Ssa1^{F475S}$. Analysis of microarray data revealed twelve genes that are up-regulated in the $Ssa1^{F475S}$ strain. Expression of genes was compared to expression in Ssa1. Functions of genes were obtained from the *Saccharomyces* genome database (SGD).

The Ssa1^{F475S} strain was the only strain that exhibited up-regulation of the *SSD1* gene. Up-regulation of this gene may be linked to some of the phenotypes caused by the F475S mutation. For example, if the Ssa1^{F475S} mutation somehow affects the Pkc1 CWI signaling pathway, alternative pathway may be up-regulated in order to deal with stress.

Another up-regulated gene in strains expressing Ssa1^{F475S} is *PDR15.* Pdr15 is regulated by the transcription factors Pdr1 and Pdr3, and along with Pdr10 and Pdr5, it encodes ATP- binding cassette (ABC) transporters that operate as drug efflux pump. We have observed that the Ssa1^{F475S} had an affect on the transcription level of the PDR5 promoter (Figure 3.33). As previously stated, Ssa1 was found to co-purify with the transcription factor Pdr3 and further studies revealed that binding of Ssa1to Pdr3 keeps this protein in a low activity state (Shahi *et al*., 2007). The Ssa1^{F475S} may have an affect on the binding of Ssa1 to Pdr3, thereby inhibiting this interaction. To study this further, we observed the expression of *PDR3*, *PDR5* and *PDR10* in the Ssa1 ^{F475S} strain (Table 3.8). Expression of *PDR3*, *PDR5* and *PDR10* were not up-regulated to the same extent as *PDR15* (Table 3.8). In order to confirm this result we chose to carry out RT-PCR to detect expression levels of *PDR5*, *PDR10* and *PDR15, ACT1* was used as a control (Figure 3.37). From RT-PCR analysis, no detecTable change is observed in any of the *PDR* gene's expression in the Ssa1^{F475S} strain.

We also analyzed genes that appeared to be down-regulated in strains expressing Ssa1^{F475S} in comparison to wildtype. Thirteen genes were found to be down-regulated by five-fold or more in cells expressing Ssa1^{F475S} (Table 3.9). Down- regulated genes are involved in a variety of cellular processess and components including protein export

ORF/Gene	Gene Function	Ssa1 ^{F475S}
YBL005W/ *PDR3*	Transcriptional activator of the pleiotropic drug resistance network regulates expression of ATP-binding cassette (ABC) transporters through binding to cis-acting sites known as PDREs (PDR responsive elements).	1.331
YOR152W/ *PDR5*	Plasma membrane ATP-binding cassette (ABC) transporter, multidrug transporter.	1.284
YOR328W/ *PDR10*	ATP-binding cassette (ABC) transporter, multidrug transporter involved in the pleiotropic drug resistance network.	1.012
YDR406W/ *PDR15*	Plasma membrane ATP binding cassette (ABC) transporter, multidrug transporter and general stress response factor implicated in cellular detoxification	3.043

Table 3.8 Up-regulation of *PDR* genes in Ssa1^{F475S}. Analysis of microarray data revealed three *PDR* genes that were very slightly up-regulated in the Ssa1^{F475S} strain. The *PDR15*is the most up-regulated as it is expressed three times higher in Ssa1^{F475S} strain. Expression of *PDR* genes were compared to expression in Ssa1. Functions of genes were obtained from the *Saccharomyces* genome database (SGD).

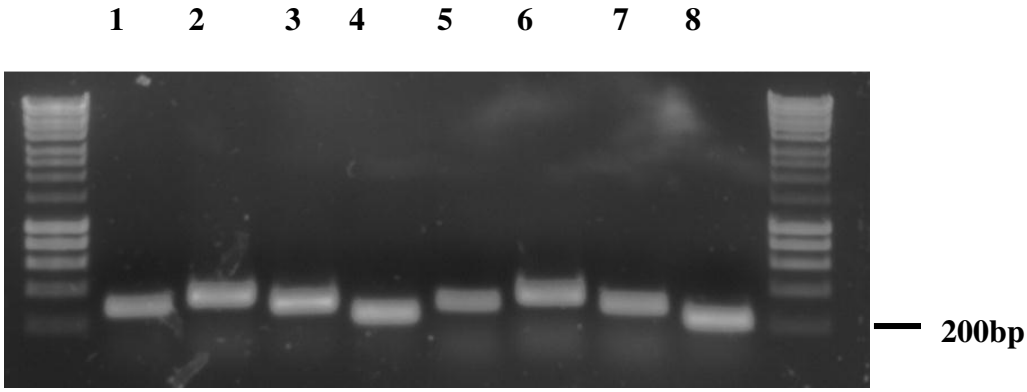


Figure 3.37 RT-PCR analysis of *PDR* genes. RNA was extracted from cells expressing wildtype Ssa1 or Ssa1^{F475S} and cDNA was prepared. Primers were designed to amplify approximately 200bp fragments of *PDR5*, *PDR10* and *PDR15* and the housekeeping gene *ACT1* that was used as a control. Lanes 1-4 contain amplified genes *PDR5*, *PDR10*, *PDR15* and *ACT1* respectively from wildtype strain and lanes 5-8 amplified genes *PDR5*, *PDR10*, *PDR15* and *ACT1* respectively from Ssa1^{F475S} strain.

ORF/Gene	Gene Function	$Ssa1^{F475S}$
YNL036W/*NCE*103	Carbonic anhydrase; involved in non-classical protein export pathway	0.006
YER153C/*PET122*	Mitochondrial translational activator specific for the COX3 mRNA	0.146
YHR174W/*ENO2*	Enolase II, a phosphopyruvate hydratase that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate during glycolysis and the reverse reaction during gluconeogenesis; expression is induced in response to glucose	0.146
YIL076W/*SEC28*	Epsilon-COP subunit of the coatomer; regulates retrograde Golgi-to-ER protein traffic	0.150
YLR061W/ *RPL22A*	Protein component of the large (60S) ribosomal subunit	0.152
YGR159C/ *NSR1*	Nucleolar protein that binds nuclear localization sequences, required for pre-rRNA processing and ribosome biogenesis	0.155
YIL053W/ *RHR2*	Constitutively expressed isoform of DL-glycerol-3-phosphatase; involved in glycerol biosynthesis	0.167
YPL113C/ Unknown	Glyoxylate reductase; acts on glyoxylate and hydroxypyruvate substrates	0.187
YOL124C/ *TRM11*	Catalytic subunit of an adoMet-dependent tRNA methyltransferase complex.	0.195
YJL177W/*RPL17B*	Protein component of the large (60S) ribosomal subunit	0.204
YER052C/*HOM3*	Aspartate kinase (L-aspartate 4-P-transferase); cytoplasmic enzyme that catalyzes the first step in the common pathway for methionine and threonine biosynthesis	0.207
YBL043W/ *ECM13*	Unknown	0.208
YOR302W	CPA1 uORF, Arginine attenuator peptide, regulates translation of the CPA1 mRNA	0.209
YDR341C	Arginyl-tRNA synthetase	0.209
YGR060W/*ERG25*	C-4 methyl sterol oxidase, catalyzes the first of three steps required to remove two C-4 methyl groups from an intermediate in ergosterol biosynthesis	0.212

Table 3.9 Down-regulation of genes in strains expressing $Ssa1^{F475S}$. Analysis of microarray data revealed sixteen genes that were down-regulated strains expressing $Ssa1^{F475S}$ by approximately five-fold or more. Expression of genes were compared to expression in Ssa1. Functions of genes were obtained from the *Saccharomyces* genome database (SGD).

glycolysis, ribosomal biogenesis, methionine, threonine and ergosterol biosynthesis. The presence of the F475S mutation in Ssa1 appears effect the expression levels of numerous genes in comparison to wild-type Ssa1, which may contribute to phenotypes observed in strains expressing this mutant.

3.17 Discussion

We have identified five novel mutations, within the peptide-binding domain of the major cytosolic chaperone Hsp70-Ssa1 of *S. cerevisiae,* that impair [*PSI*$^+$] propagation. Previous Hsp70 genetic screens have mainly isolated mutated residues that affect [*PSI*$^+$] propagation located in the ATPase domain, highlighting the importance of this domain for prion propagation (Jung *et al.,* 2000; Jones and Masison, 2003; Loovers *et al.,* 2007). The majority of PBD mutants did not have a major effect on cell growth.

Aggregated Sup35 in [*PSI*$^+$] cells are composed of individual SDS-insoluble polymers and large aggregates. The large aggregates are composed of complexes formed by SDS-insoluble polymers and other associated proteins (Kryndushkin *et al*. 2003). It has been proposed that Hsp104 generates prion seeds by breaking down polymers and separating polymers from aggregates, increasing the amount of free polymers (Kryndushkin *et al*., 2003). Studies on the PBD mutant, *SSA1-21,* show that there is a ten-fold decrease in the number of transmissible seeds per cell but only a two to three fold reduction in the amount of aggregated Sup35 (Jung *et al.,* 2000). By utilizing a Sup35-GFP fusion protein, it was observed that aggregates present in strains expressing *SSA1-21* are larger as there are more polymers per aggregate (Song *et*

*al.,*2005). Therefore, if there are a large proportion of polymers associated with large aggregates, there will be fewer free polymers. Efficiency of Sup35 recruitment into prion aggregates depends on the number of seeds present. This may explain in *SSA1-21,* how the reduced number of seeds affects Sup35 solubility (Song *et al.,* 2005). Similarly, to *SSA1-21,* F475S is a dominant mutant with regard to prion propagation. The PBD mutant residues isolated in this study are closely located to *SSA1-21*. Both F475S and *SSA1-21* share many phenotypic similarities. Therefore, it may be possible that F475S and the other PBD mutants are having a similar affect on number of prion seeds and size of Sup35 aggregates, causing loss of the prion.

Another explanation as to why mutations of Ssa1, such as *SSA1-21* and F475S may impair prion propagation, is they affect the Ssa1 function of dismantling polymers from Sup35 aggregates. It has recently been shown that Ssa1/2 interacts with SDS resistant polymers (Bagriantsev *et al.,* 2008). Work carried out has shown, the interaction of Ssa1/2 was weaker in non-prion Sup35 than in Sup35 prion form and this process is independent of levels of Ssa1 (Bagriantsev *et al.,* 2008). Binding of Ssa1/2 to the prion form of Sup35 may be due to the refolding of the N-terminal domain of Sup35 during prionization (Bagriantsev. *et al.,* 2008). Previous work suggests the PBD mutant *SSA1-21* has increased substrate binding in comparison to wildtype Ssa1 (Jones and Masison, 2003; Jones *et al*., 2004). Interestingly, *SSA1-21* second site suppressor E540K is homologous to the bovine Hsp70 residue E543K, which has been shown to cause a decrease in affinity for substrate (Ha *et al*., 1997). Although we have not tested the rate of substrate binding of F475S, this mutation shows many similarities to *SSA1-21;* they are closely located, both suppressed by the P636S mutation, both do not affect luciferase recovery and both exhibit an increase in

ATPase activity. Therefore, it is possible that F475S will also show an increase in substrate binding. Ssa1/2 binding more avidly to Sup35 aggregates, may affect the Hsp70 function of dismantling aggregates into polymers directly or by an association with Hsp104 (Song. *et al.,* 2005).

The isolation of second-site suppressors of both *SSA1-21* and F475S, allows us to gain further insight on how these PBD mutations may be effecting prion propagation. The P636S mutation counteracts the [*PSI*$^+$] impairment phenotypes of both *SSA1-21* and F475S. The ATPase domain of Hsp70 regulates the function of the PBD. In an ATP bound state, the PBD is in an open confirmation and this allows for rapid substrate exchange. Hydrolysis of ATP to ADP converts the PBD into a closed confirmation, establishing a tight association with its substrate. Hsp70 ATPase activity is stimulated by the PBD, which suggests inter-domain communication, which is influenced by co-chaperones, and TPR co-chaperones motifs (Shomura *et al.,,* 2005). The P636 residue is located within a conserved octapeptide GPTVEEVD. This motif in humans interacts with the Hop1, suggesting that this mutation may suppress *SSA1-21* and F475S by interfering with their ability to interact with Sti1 (yeast homologue of Hop1). It appears a weak interaction of Hsp70 with Sti1 is favorable for [*PSI*$^+$] propagation, overexpression of Sti1 (promotes substrate binding) impairs [*PSI*$^+$] propagation whereas deletion of Sti1 restores prion propagation (Jones *et al*., 2004). Conversely, the NEF Fes1 facilitates the release of substrates. Overproduction of Fes1inhibits the impairment of [*PSI*$^+$] propagation by *SSA1-21* and depletion of Fes1 (which prolongs substrate binding) impairs [*PSI*$^+$] propagation in both wildtype and *SSA1-21* (Jones *et al*. 2004). This shows that in order for [*PSI*$^+$] propagate, a weak interaction with Fes1 is required.

Previously isolated mutants of Ssa1 that impair [*PSI*$^{+}$] propagation have not had a significant effect on cell growth, leading to the conclusion that Ssa function in essential cellular pathways can be separated from Ssa function in prion propagation (Jung *et al*., 2000; Jones and Masison, 2003; Loovers *et al.,* 2007). However, the F475S mutation appears to be unique as it demonstrates temperature sensitivity at elevated temperatures, suggesting that essential cellular pathways are not separate to the Ssa function in prion propagation. Remediation of the t.s phenotype by osmotic support and recovery by constitutively expressed components of the signaling cascade, leads us to believe that the F475S mutation may implicate Ssa1 as having a role in the CWI signaling pathway. Chaperones and co-chaperones that function in concert with Hsp70 are involved in the CWI signaling pathway. Hsp90 binds Slt2 and is essential for activating the downstream target Rlm1 (Millson *et al*., 2005). Sse1 functionally partners with Hsp90 as a Hsp70 NEF, and it has been shown that Sse1 binds to Slt2 *in vivo,* yet Slt2 stability and phosphorylation is not affected in cells that are ΔSse1yet activation of Rlm1 does not occur *(*Shaner *et al., 2008)*. Cells containing an *Ydj1* deletion also exhibit phenotypes consistent with cell wall defects and overexpression of Pkc1 improves cell growth (Wright *et al.,* 2007). Hsp70 maybe indirectly affects CWI through any of the above components or alternatively it maybe directly affecting the pathway its components such as Pkc1 or Bck1. Functional conservation of the F475S mutation in Ssa2 and Ssa4 suggests that it may be a more of a general involvement of Hsp70 family members in the CWI signaling pathway.

Analysis of the effects of Ssa1^{F475S} on the promoter activity of *PDR5*, UPRE and *SSA1* expression may indicate that this mutant is having an effect on these genes. Alternatively, western blot analysis indicated that there was a lower abundance of

Ssa1 when the F475S mutation was present (Figure 3.6). Lower levels of Ssa1 may explain the effects of the $Ssa1^{F475S}$ mutation on the expression of the various promoters. If Ssa1 is required for binding to Pdr3, causing it to go into a low activity state, reduced amounts of Ssa1 may not be sufficient for this process and an increase in Pdr5 is observed in comparison to the effects of wildtype Ssa1.Similarly reduced amounts of Ssa1 may not be sufficient for negative regulation of its expression. Ssa1 levels may also be a reason for the increase of UPRE promoter activity.

Isolation of novel mutations in the PBD of Hsp70, that effect prion propagation and other cellular functions, has provided us with new insight into the role of Hsp70 in *S. cerevisiae*. These mutations have implicated the PBD as playing a role in prion propagation, which may be caused by alterations to inter-domain communication, binding of Ssa1 to Sup35 or interactions of co-chaperones with Ssa1. The F475S mutation suggests that Ssa function in essential cellular pathways cannot be separated from Ssa function in prion propagation as the mutation appears to implicates Ssa1 in the CWI signaling pathway and has an effect on other diverse cellular functions.

4.0 Introduction

The aim of this chapter is to further characterize and perform biochemical analysis on previously isolated Hsp70 ATPase mutants that affect prion propagation and to assess the effects of C-terminally truncated Hsp70 on prion propagation. Hsp70 is composed of a 44kDa ATPase domain (NBD), an 18kDa peptide-binding domain (PBD) and a 10kDa C-terminal variable domain. The function of Hsp70 requires coordinated action between these three domains. As the effects of mutations of Ssa1 in the PBD has already been assessed, this chapter will assess the role of the ATPase domain and C-terminal (CTD) in [PSI^+] propagation.

The importance of the Hsp70 ATPase domain for [PSI^+] propagation is highlighted by the isolation of numerous mutants located within the domain that impair prion propagation (Jones and Masison, 2003; Jones *et al*., 2004 and Loovers. *et al.,* 2007). A majority of these mutants appear to be located in regions of the NBD that are important for inter-domain communication between the ATPase domain and PBD domain (Loovers. *et al.,* 2007). A number of isolated ATPase mutants are located in the IIB region of the NBD (Loovers. *et al.,* 2007). This region has been identified in mammals for the binding of the nucleotide exchange factor (NEF), HspBP1, which is the mammalian homologue of the yeast Ssa1 NEF, Fes1. The isolation of these ATPase mutants and ATPase mutants that can suppress the PBD mutant *SSA1-21* has implicated inter-domain communication and the function of Hsp70 NEF's and co-chaperones as playing an essential role in prion propagation (Jones and Masison, 2003; Jones *et al*., 2004 and Loovers. *et al.,* 2007).

The CTD forms a lid like structure over the SBD that enables the trapping of substrates in the PBD. Substrate trapping by this lid like structure is dependent on communication between the SBD and the PBD. The Hsp70 co-chaperone Ydj1, which stimulates the activity of Hsp70, interacts with the NBD and the CTD to facilitate the transfer of substrates to the PBD. Deletion of the C-terminal in the Hsp70 *E.coli* homologue DnaK, exhibits a five- fold reduction in peptide binding affinity in comparison to wildtype (Pellecchia. *et al.,* 2000). Furthermore, the addition of ATP to truncated DnaK lowers peptide binding by a magnitude similar to wildtype (Pellecchia. *et al.,* 2000). This evidence suggests that the CTD is not necessary for inter-domain communication. However, it was observed that deletion of the CTD, increased peptide release induced by ATP by almost one hundred fold in comparison to wildtype (Pellecchia. *et al.,* 2000).

To understand the precise role of Hsp70–Ssa in amyloid prevention, formation, and propagation, biochemical analysis is required in addition to genetic analysis. Furthermore, it is necessary to investigate the effects of ATPase mutants on other cellular functions within the yeast cell. It is also necessary to investigate whether there is a role for the CTD in prion propagation by analyzing the affects of various CTD truncations on [*PSI*] propagation.

The main objective of this chapter was to:

- Genetically and biochemically characterize isolated ATPase mutants.
- Analyze the effects of ATPase mutants on other cellular functions.
- Investigate if CTD has a role in [PSI^{+}] propagation independent of co-chaperones.

4.1 Isolation of Hsp70 ATPase mutants that impair [*PSI*$^+$] propagation

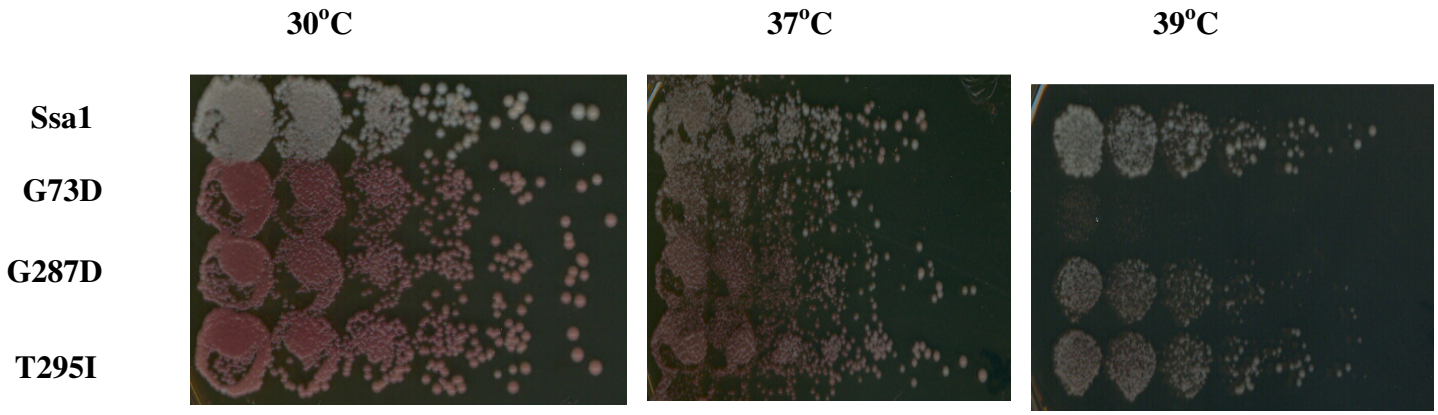
Previous genetic screens have identified the ATPase domain of Hsp70 as playing an essential role in prion propagation (Jones *et al.,* 2003). To further investigate the effects of mutated Hsp70 on prion propagation an extensive genetic screen to identify mutants in both the major cytosolic Ssa proteins was carried out (Loovers *et al*., 2007). All but one of mutations that effected [*PSI*$^+$] propagation are located in the ATPase domain (Table 4.1) (Loovers *et al*., 2007). When ATPase mutants are expressed as the sole source of Ssa1 in yeast cells, only Ssa2^{A176T} exhibited an affect on the growth rate (MGT). A subset of the mutant residues (A53, G73, R74, A146 and T223) are located in a region, which has recently been shown to be important for inter-domain communication in the *E.coli* Hsp70, DnaK (Revington *et al*., 2005). Another subset of the mutant residues (R259, G287, T295 and A297) are located within the IIB region of the ATPase domain. The IIB region of the ATPase domain has been implicated as interacting with the Fes1 homologue, HspBP1, in mammals (Shomura *et al*., 2005). Therefore, mutants may be altering the IIB region in a way that Hsp70 cannot interact with the NEF Fes1 in yeast, which then alters [*PSI*$^+$] propagation. To further assess the role of Hsp70 ATPase mutants on [*PSI*$^+$] propagation, we chose three mutants (G73D, G287D and T295I), which are representative of both subsets. Isolation of G287D and T295I in Ssa2, suggests that Ssa2 is carrying out a similar function as Ssa1 with regard to prion propagation. Furthermore, G73D is suppressed by three previously established second-site suppressors (A519T, E540K and P636S), which suppress other previously isolated ATPase mutants (Jones and Masison, 2003); whereas G287D is unaltered by mutations suggesting G73D and G287D function in different manners.

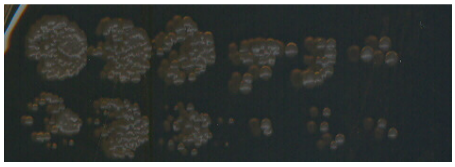
Ssa1 mutants	Ssa2 mutants	Times isolated	MGT % of WT
WT	WT		100
	A4T	1	81 ± 8
	D30N	1	95 ± 1
	G41D	5	118 ± 2
	G50D	1	90 ± 9
A53V		1	95 ± 4
	A53V	2	100 ± 5
	A53T	4	105 ± 3
T64I		3	101 ± 2
	T64I	4	101 ± 4
G73D		3	101 ± 5
R74K		1	94 ± 4
	R74K	1	77 ± 30
S119F		2	96 ± 4
	A146T	2	101 ± 3
A155V		2	113 ± 10
A155T		1	94 ± 4
	A176T	1	131 ± 7
T223I		8	94 ± 2
R259K		2	100 ± 6
G287D		4	102 ± 5
	G287D	5	101 ± 5
T295I		1	95 ± 3
	T295I	1	105 ± 5
A297T		1	107 ± 3
G481D		1	98 ± 5

Table 4.1 Relative effects of Ssa1 and Ssa2 mutants on prion propagation and cell growth. Hsp70 mutants isolated as in chapter 3.1. All but one of mutants are located in the ATPase domain of Ssa1/Ssa2. Mean generation time expressed as a percentage of wildtype. Highlighted mutants (G73D, G287D and T295I), were further characterized in this chapter. This table is an adapted version from Loovers *et al.*,2007.

4.2 Analysis of [*PSI*] propagation and Hsp70 function of ATPase mutants

As the main function of Hsp70 is refolding misfolded or aggregated proteins at elevated temperatures it is necessary to test this function of Hsp70 mutants at elevated temperatures. Previous analysis of ATPase mutants revealed that mutation in the ATPase domain of Hsp70 did not effect Hsp70's heat shock function, suggesting that different regions of Ssa1 caused the same impairment of [PSI^+] without affecting normal Hsp70 function (Jones and Masison, 2003). The isolated mutants G287D and T295I appear not to affect the function of Hsp70, as cells expressing each mutant as the sole source of Ssa1 are viable at 37°C and 39°C**.** However, the G73D mutation causes a 39°C t.s phenotype, which is recovered on 1M sorbitol. This suggests that the G73D mutation may affect the function of Ssa1 at elevated temperatures and could further implicate Ssa1 as playing a role in the CWI signaling pathway.





(B)

Figure 4.1 Effect of elevated temperatures on ATPase mutants. Cells harboring WT Ssa1 and ATPase mutants were incubated at specific temperatures for 48 hours. Cells were diluted by a 1/5 serial dilution. Spots are labeled accordingly. Wildtype and mutants expressed as the sole source of Ssa in yeast. As G73D appeared 39°C t.s we preformed growth assay on 1M Sorbitol at 39 °C (B).

4.3 Hsp70 structural analysis

As stated previously, G73D is located in a region that has recently been shown to be important for inter-domain communication in the *E.coli* Hsp70, DnaK (Revington *et al*., 2005) and G287D and T295I are located in the IIB region of the ATPase domain which has been implicated as interacting with the Fes1 homologue, HspBP1 in mammals (Shomura *et al*., 2005). Previous structural analysis of the ATPase mutants was carried out utilizing the bacterial Hsp70 homologue DnaK has 62.6% similarity to yeast Hsp70. More recently, the bovine Hsp70 (Hsc70) homologue has been crystallized which has 87.3% similarity to yeast Hsp70; therefore mutants were mapped onto the bovine model (Figure 4.2). The crystal structures were downloaded from the protein databank (PDB) and prepared for later homology modeling using the Accelrys Discovery Studio 2.5. Based on the Hsc model. Models were generated by MODELER (Sali and Overington, 1994). From this analysis we can tell, Gly73 residue is located in a turn region of the ATPase domain. A hydrogen bond (HB) interaction is formed with Leu71, while neighboring residues include Ile72, Arg74, Asn75, Glu115, Asn149, Ser151, Gln152, Ala155. The G73D mutation changes a polar uncharged residue to a polar acidic residue. As an Asp the HB to Leu71 is lost and additional neighboring residues include Asp73, Arg153 and Gln154. Gly287 is located in a turn between beta strands. No HBs are present with G287 and neighbors include Leu257, Phe285, Glu286, Ile288, Asp289 and Leu284. With the change from a polar uncharged residues to a polar acidic residue HBs are predicted involving Ile288 and Asp289, additional neighbors include Asp249 and Ile288. The Thr295 is located in beta strand. The polar uncharged Thr295 Hbs to Ile194, Lys268 and Ser278. T295 Neighbors: Gln276 (ADP binding site), Thr277, Ser278, Val279 (neighbors ADP binding site), Ser293, Ile294, Lys268, Arg296, Glu300, Glu280,

Phe299, Ala297 (involved in HSP110 interaction). Although the mutation changes from a polar uncharged to a nonpolar hydrophobic residue both are favored in the middle of Beta strands. When the mutation I295 is present there is a change in the predicted Hydrogen bonding pattern with only the HB with Ser278 being maintained. New neighbors include Ser272.

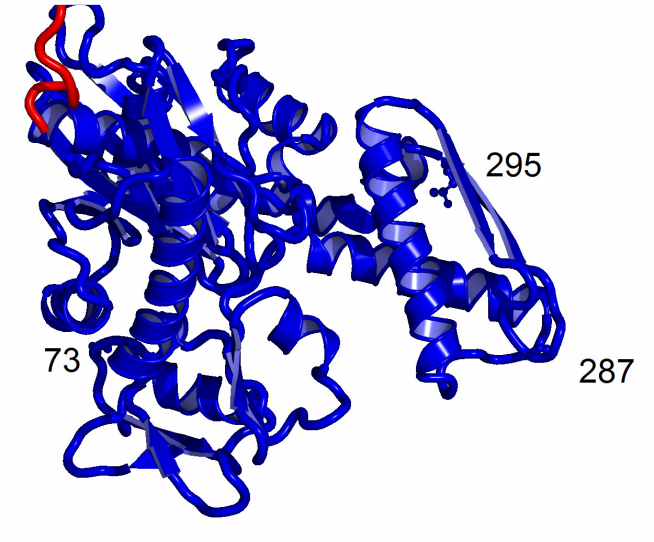


Figure 4.2- Yeast HSP70 ATPase domain model developed from the 37CN crystal structure (chain B). Position of ATPase mutations on Hsp70 crystal structure.

4.3.1 Structural analysis of the binding of the Ssa1 NEF, Sse1 to Hsp70

Binding and release of substrates by Hsp70 is driven by the ATPase cycle. ATP binding to the NBD of Hsp70 induces conformational changes in the C-terminal PBD, which results in rapid substrate exchange. When Hsp70 is in an ADP bound state, the substrate becomes tightly bound. Many factors influence this process, such as Hsp40 and the nucleotide exchange factor (NEF) Hsp110. Two highly homologous members, Sse1 and Sse2, in *S. cerevisiae*, represent Hsp110. Peptide release can be triggered by NEF's, by helping liberate bound ADP and their facilitating ATP rebinding. Recent studies have found Sse1 is required for *de novo* [*URE3*] and [PSI^+] formation and efficient prion propagation (Kryndushkin. *et al.,* 2007; Fan. *et al*., 2007; Sadlish *et al*., 2008). From structural analysis of the interaction between Hsc70 and Sse1 (Figure 4.3) we have defined residues in Hsc70 that are important for Sse1 binding (Table 4.2). The highly conserved Hsp70 residue Ala300H mediates a Van der Walls (vdw) contact to the Sse1 backbone between the Sse1 residues Glu575 and Glu576, which in turn are engaged in hydrogen bond (HB) contacts to Hsp70 residues Thr298 and Lys348. The Sse1 residue Tyr579 makes additional vdw contacts to Ala300 and Glu304. Interestingly, the yeast homologue of the Hsc70 residue Thr298 is Thr 295. A mutation to this residue may affect the ability of Hsp70 to bind to Sse1, this may explain why Ssa1 harboring the mutation T295I cannot maintain [PSI^+]. This further suggests efficient NEF activity is required for [PSI^+] propagation.

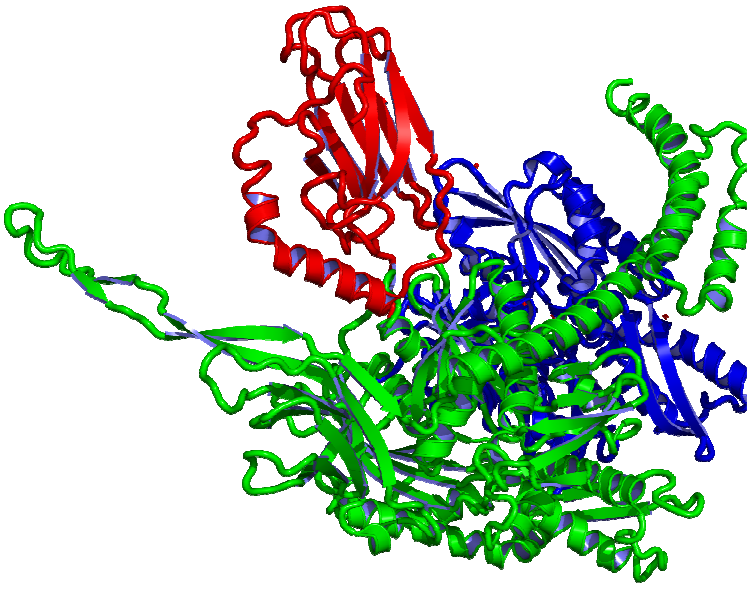


Figure 4.3 Binding of Sse1 to Hsp70. Image is the 37CN crystal structure (Schuermann. *et al*. 2008). In blue is the ATPase domain, in red the peptide-binding domain of Hsp70 and in green is Hsp110. Images were generated using Pymol.

Bovine HSP70	Yeast HSP70
G34	G32
A54	A52
N57	N55
L133	L131
Y134	G132
D285	D282
T298	**T295**
A300	A297
K348	K345

Table 4.2 Bovine HSP70 residues that contact Hsp110 in the 3C7N crystal structure with the corresponding aligned residues in yeast Hsp70. Bovine Hsp70 residues revealed that the T295 Bovine homologue is involved in the binding of Sse1.

4.4 Basal levels of Hsp70 and Hsp104 in yeast expressing ATPase mutants

Analysis of the abundance of Hsp70 and Hsp104 proteins in cells expressing mutants that affect [*PSI*$^+$] propagation is critical as their levels may effect prion propagation. Previous studies indicated that the abundance of Hsp70 or Hsp104 was not affected by mutations in the ATPase domain (Jones and Masison, 2003; Loovers *et al.*, 2007). As previously observed (Loovers *et al*., 2007), levels of Hsp70 or Hsp104 in yeast cells expressing the ATPase mutants were not considerably different in mutant strains in comparison to wildtype (Figure 4.4). This indicates that impairment of [*PSI*$^+$] propagation is not a result of altered amounts of Hsp70.

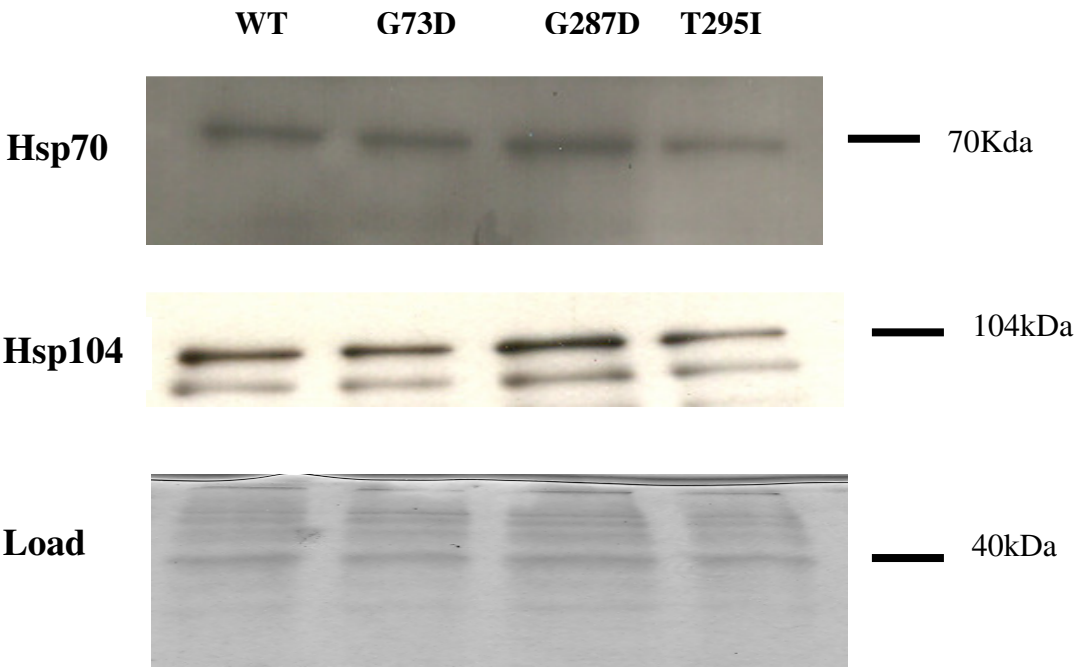


Figure 4.4 Basal levels of Hsp70 and Hsp104 in cells expressing ATPase mutants. Lysates prepared from overnight 30^{o}C cultures. Aliquots of proteins (10µg) were separated on three polyacrylamide gels, gels were either immunoblotted with antibodies either specific for (A) Hsp70 (SPA-822, 1/2000 dilution) and (B) Hsp104 (HSP104, 1/150,000 dilution) or stained (C) Coomassie brilliant blue stained overnight and destained the following day. Mutant alleles as indicated.

4.5 Protein refolding activity of Hsp70 ATPase mutants

As a heat shock protein, one of its major functions of Hsp70 is refolding proteins that have become misfolded due to heat stress. The ability of chaperones to refold misfolded proteins can be measured by performing a luciferase assay. The luciferase enzyme is unstable at high temperatures. In the presence of its substrate decanal, luciferase emits light, which can be measured on a luminometer. Luciferase assays were preformed as in chapter 3.4. As Hsp104 is critical for survival at elevated temperatures and its function is dependent on Hsp70 and Hsp40, a yeast strain with ablated Hsp104 should exhibit a low reading for luciferase refolding whereas a wildtype strain such as G600 should exhibit a high percentage of luciferase refolding as all chaperones are present. Both strains were used as controls to measure the accuracy of assay (Figure 4.5). Yeast strains expressing wildtype Ssa1 and the ATPase mutants G73D, G287D and T295I were transformed with the plasmid (pDCM90), expressing the luciferase protein. Luciferase assays were preformed as in chapter 3.4. As expected, the yeast strain with the *Hsp*104 deletion exhibited low luciferase re-folding (29%) and the wildtype strain G600 exhibited the highest rate of luciferase refolding (99%) (Figure 4.5). There was no significant difference in the rate of luciferase folding between wildtype Ssa1 and ATPase mutants. This result suggests that these specific mutations in the ATPase domain do not effect refolding of aggregated proteins post heat shock.

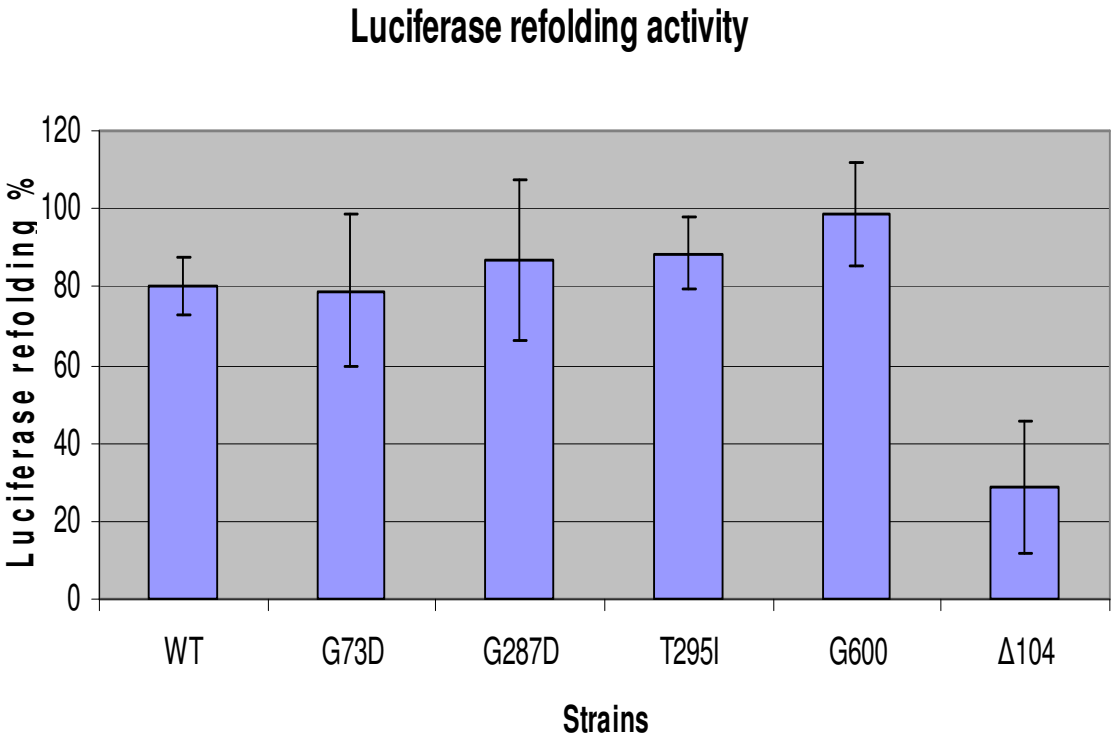


Figure 4.5- Refolding activity of ATPase mutants. Overnight cultures of G600, Δ104 and ATPase assays expressing luciferase were shifted from 30°C to 37°C for 30 minutes and then shifted to 42°C for one hour. Cycloheximide was added 50 minutes after shifting to 42°C to prevent synthesis of luciferase during the recovery period. Luciferase activity, expressed as a percentage of pre-heat shock activity, was measured after allowing cells to recover for 30 minutes at 25°C. Experiment was repeated twice in triplicate. There was no significant difference in the rate of luciferase folding between wildtype Ssa1 and ATPase mutants. The Δ *Hsp104* strain exhibited low luciferase folding and the wildtype strain G600 exhibited the highest rate of luciferase refolding.

4.6 Conservation of ATPase mutations across the Ssa family

There is a high degree of conservation among the Hsp70 family. It is conserved in archaea, eubacteria, eukaryotes and organelles (mitochondria, chloroplasts) (Karlin and Brocchieri, 1998). Ssa1 and Ssa2 are constitutively expressed and have 97% homology; Ssa3 and Ssa4 have 87% homology and are induced when the cell encounters stress. Therefore, conservation of mutations of Hsp70, that impair prion propagation, may be useful targets for therapeutics or designing treatments for prion diseases. To test whether the ATPase mutations were conserved in Ssa2, Ssa3 and Ssa4, mutations were inserted by SDM into plasmids expressing each protein. We were unable to obtain the G73D mutation in *SSA2* and *SSA3.* As previously described the G287D and T295I also impair prion propagation in Ssa2 (Figure 4.6) (Loovers *et al*., 2007). We can now conclude that the G287D and T295I mutations are functionally conserved in Ssa3 and Ssa4. Cells expressing the Ssa3 and Ssa4 mutants appear to be growing at a much slower rate in comparison to Ssa1 and Ssa2 cells harboring the G287D and T295I mutations. The G73D mutation appears to make the Ssa4 protein non-functional as the presence of the mutation appears to be detrimental. Functional conservation of these ATPase mutations and the PBD mutation (F475S) is indicative of a general role of the Hsp70 Ssa family in [*PSI*$^{+}$] propagation.

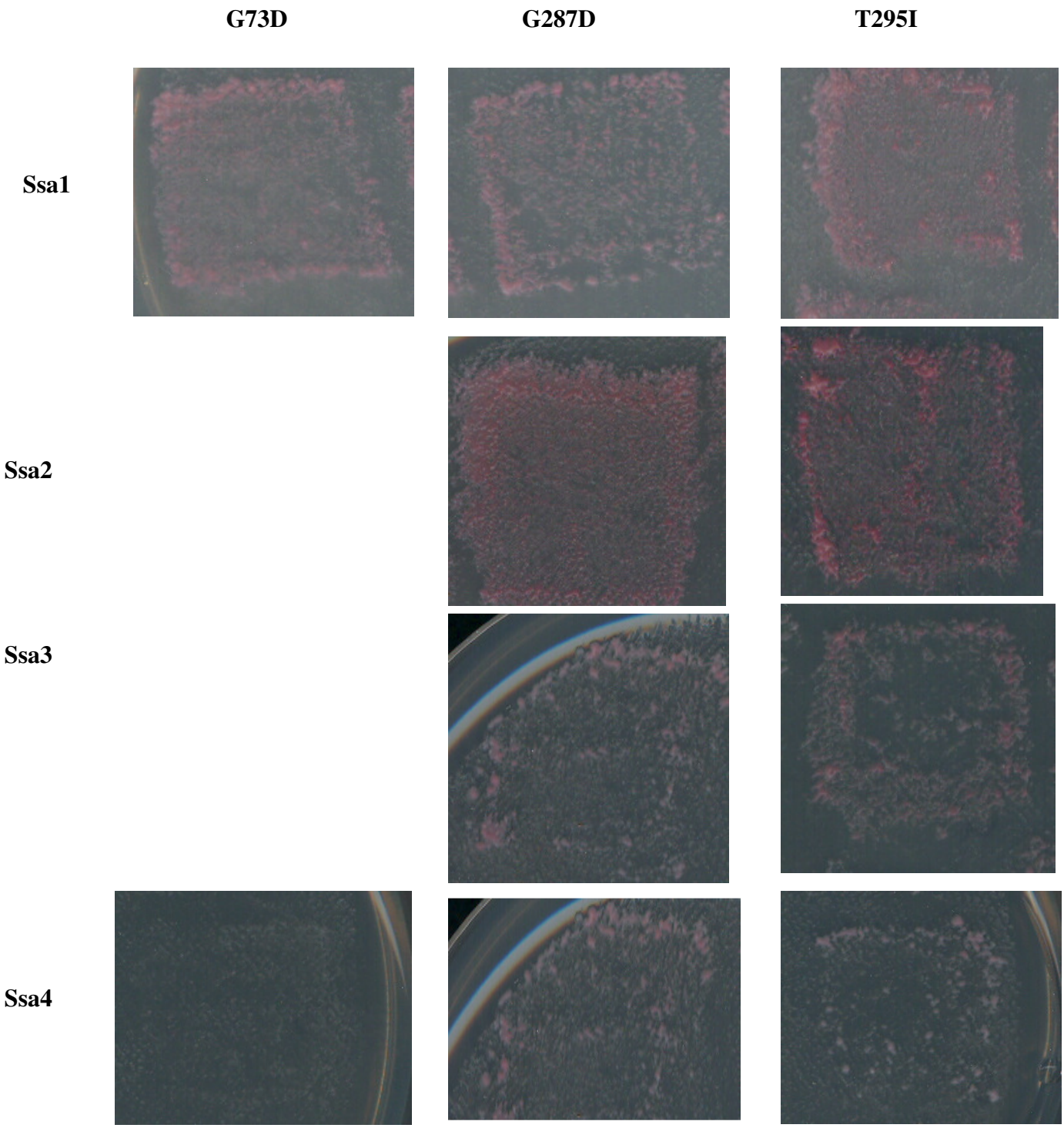


Figure 4.6- Conservation of ATPase mutants across Ssa family. Plasmids expressing Ssa1-4 with ATPase mutations, were transformed into the yeast strain G402 onto SD agar lacking leucine. Cells were grown at 30^{o}C for 48 hours. Colonies from Leucine plates were then restreaked onto SD agar lacking leucine and grown at 30^{o}C for 48 hours. Strains were replicated onto 5-FOA and incubated 30^{o}C for 48 hours.

4.7 Biochemical analysis of ATPase mutants

Mutations in the ATPase domain of Ssa1 (G73D, G287D and T295I) appear to have little effect on the essential cellular functions carried out by this protein chaperone, yet they are able to impair [PSI^+] propagation. To further our knowledge on the ATPase domain mutants, we carried out initial biochemical analysis in addition to genetic studies. As mutants are located in regions important for inter-domain communication (G73D) and binding of NEF's such as Fes1 (G287D and T295I), we decided to carry out ATPase assays to deduce whether mutants affect the rate of ATP turnover. We purified ATPase mutants from *E.coli,* using a well established *E.coli* system. From previous experience it is known that an N-terminally fused His (6X) tag may be lethal to Ssa1 (G. Jones personal correspondence). To test this we expressed Ssa1-(His6X) ATPase mutants in yeast. We were unable to obtain the G287D mutation in the Ssa1-(His6X) construct. The N-terminal His (6X) tag did not affect the viability of ATPase mutants; G73D and T295I *in vivo* (Figure 4.7)

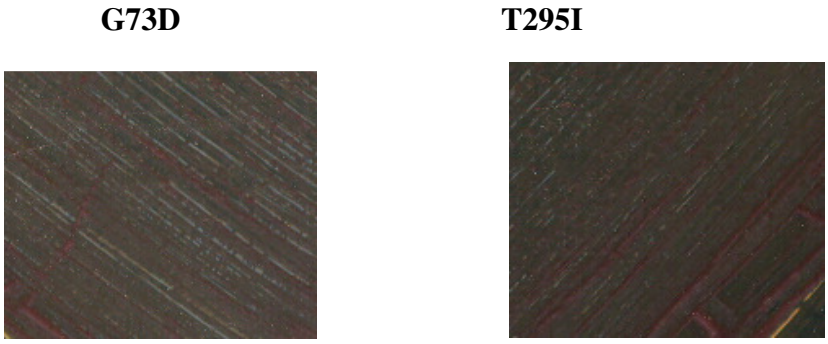
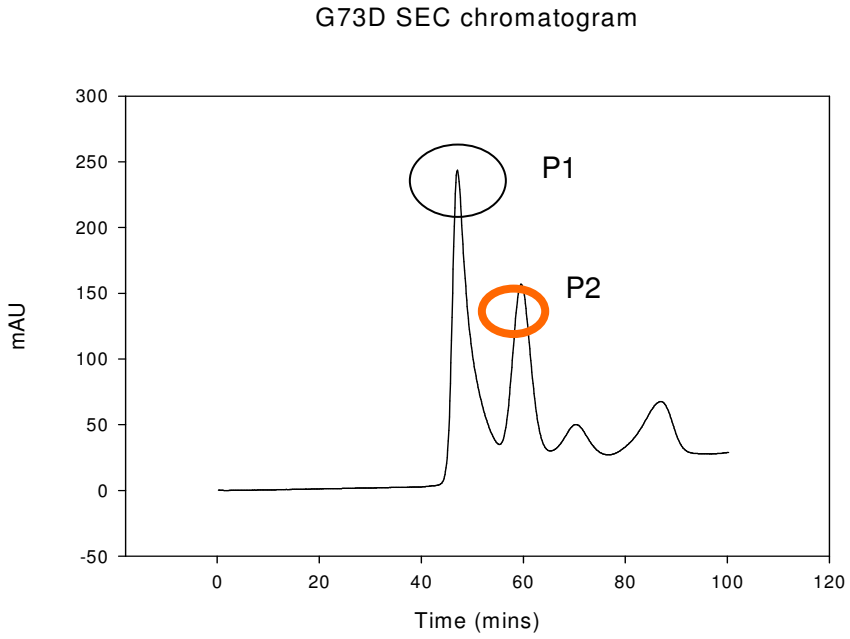


Figure 4.7 -Viability of His(6X) tagged ATPase mutants. ATPase mutations were inserted into pC210 expressing Ssa1 with a N-terminal His(6X) tag by site-directed mutagenesis. Mutant plasmids were then transformed into G402 on SD agar that lacked leucine at 30^{o}C for 48 hours. Colonies from Leucine plates were then restreaked onto SD agar lacking leucine and grown at 30^{o}C for 48 hours. Strains were then replicated onto 5-FOA and incubated 30^{o}C for 48 hours. Strains were then streaked onto YPD and grown at 30^{o}C for 48 hours. Mutants as indicated.

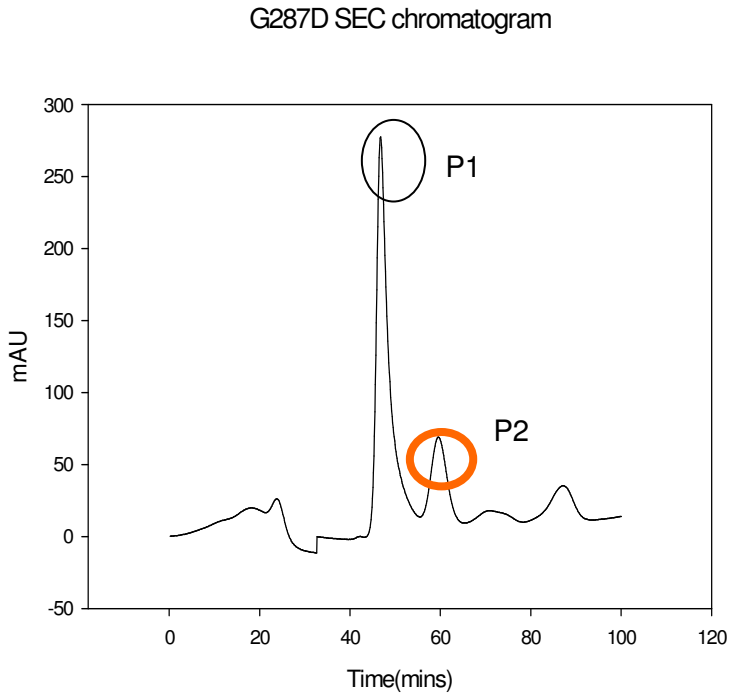
4.7.1 Protein purification of ATPase mutants

We decided to purify ATPase mutants from *E.coli.* This was achieved by inserting the ATPase mutations G73D, G287D and T295I into an expression vector which expressed Ssa1 with an N-terminally fused His (6X) tag. ATPase mutants were purified under native conditions by Ni^{2+} affinity chromatography, dialysis and size exclusion chromatography. To condense large volumes of protein supernatant collected from dialysis and size exclusion chromatography, protein samples were condensed using Millipore condensing tubes. Two protein fractions were collected from size exclusion chromatography; peak one which is aggregated Hsp70 and Peak two which is native Hsp70 (Findings of Perrett group)(Figure 4.8).

(A)



(B)



(C)

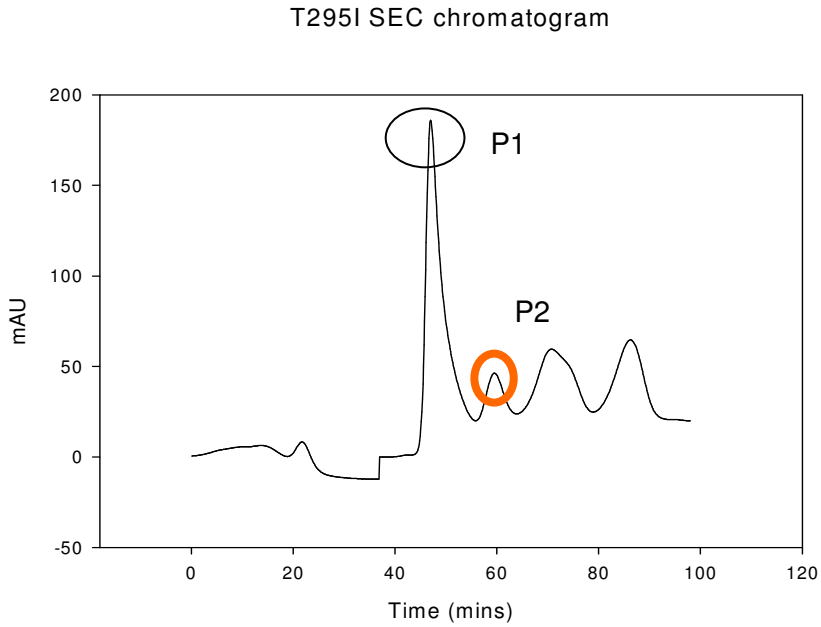
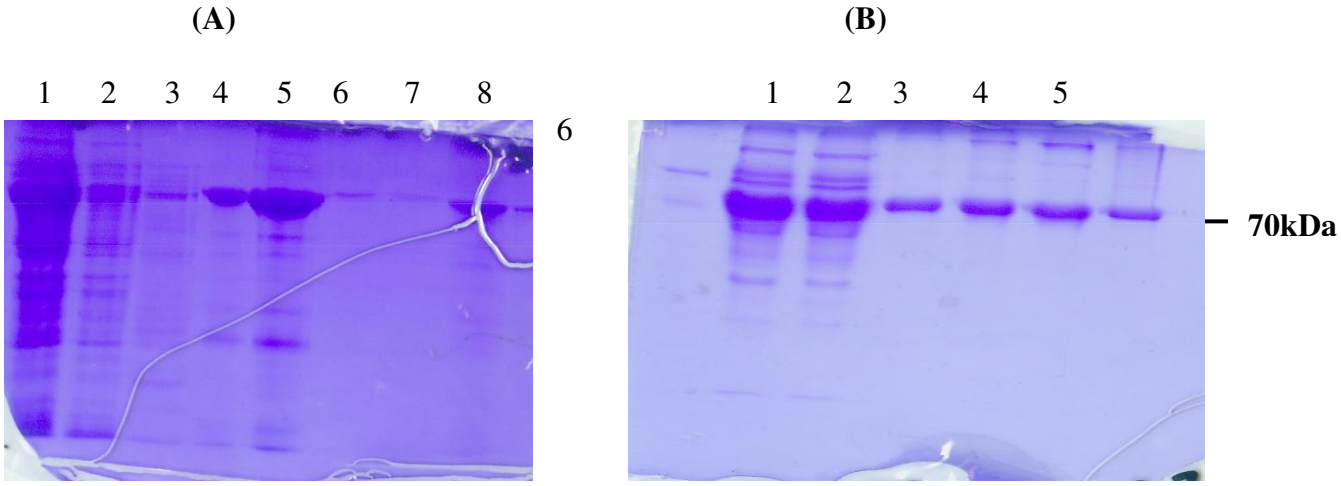


Figure 4.8 Size- exclusion chromatography chromatogram Gel filtration column (HiLoad superDex 200pg, GE Health care) loaded with ATPase mutants supernatant. Peak one represents aggregated Ssa1 and peak two represents native Ssa1. Mutants as indicated. All fractions were collected and stores at -70^{o}C.

4.7.2 Confirmation of purified ATPase mutants

Purity of purified Ssa1 and ATPase mutants was judged by SDS-page and coomaisse staining (Figure 4.9). Pre purified and post purified proteins were run on 12.5% polyacrylamide gels. To further confirm the protein purified was Hsp70, P1 protein samples collected from size-exclusion chromatography were analyzed by western blot. Samples were probed with antibodies specific for Hsp70 and His6X tag (Figure 4.10). Western blot confirms presence of both Hsp70 and His (6X) tag. SDS-page and coomaisse staining confirmed purity of proteins. Immunoblotting of proteins with antibodies specific for Hsp70 and His (6X) tag further clarified that correct protein was purified (Figure 4.10).



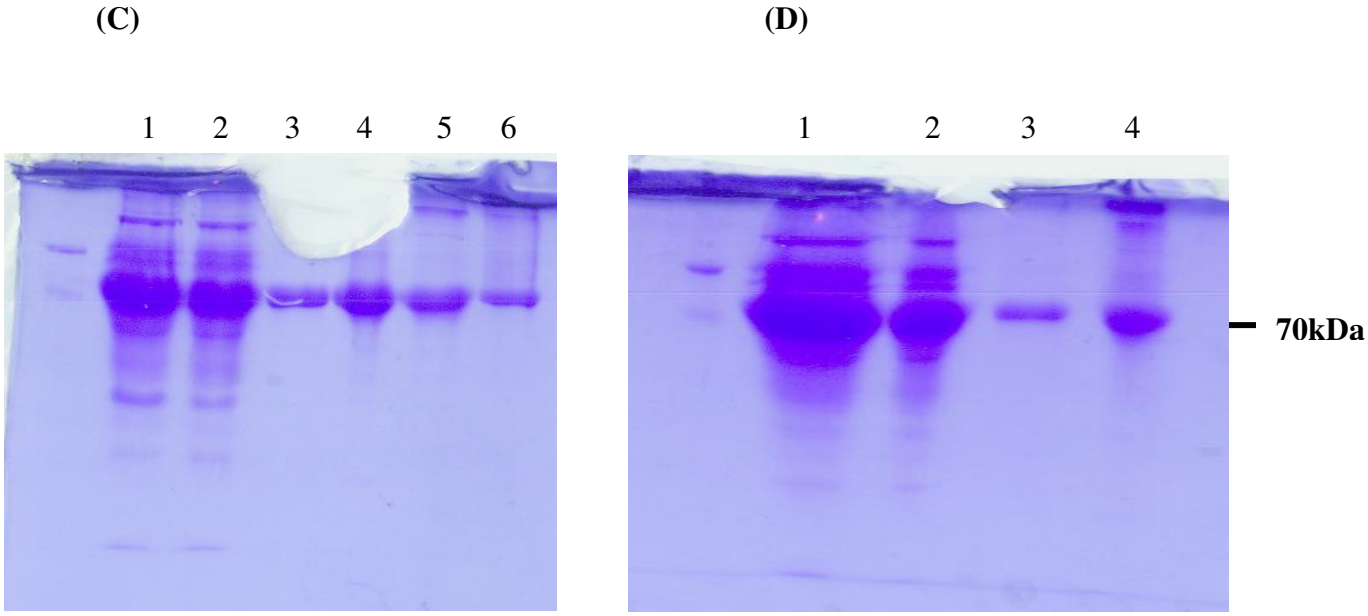


Figure 4.9 Determination of purity of Ssa1p and ATPase mutants by SDS-page.
Protein fractions run on 12.5% polyacrylamide gel at 200V for one hour. Gels stained in coomassie brilliant blue for one hour and destained overnight. Gel (A) represents purification of Ssa1; Lanes 1 represents supernatant pre purification, lanes 2-4 represents fractions collected from Ni column eluted with MCAC-10, MCAC-100 and MCAC-350 respectively, lanes 5 represent post condensing of proteins post dialysis, lanes 6 and 7 represent Peak one (P1) and peak two (P2) post size exclusion chromatography and lane 8 represents P1 post condensing. Gel (B) represents purification of $Ssa1^{G73D,}$ lane 1 loaded with supernatant pre purification, lane 2 loaded with run-through, Lane 3 loaded with fraction post MCAC-350, lane 4 loaded with condensed 350 fraction and lanes 5 and six are peak one and peak two post size exclusion chromatography post condensing. Gel (C) represents $Ssa1^{G287D}$ and is loaded as gel (B). Gel (D) represents purification of $Ssa1^{T295I}$, lane 1 represents supernatant pre purification, lane 2 loaded with run-through, lane 3 loaded with fraction post MCAC-350 and lane 4 loaded with condensed 350 fractions.

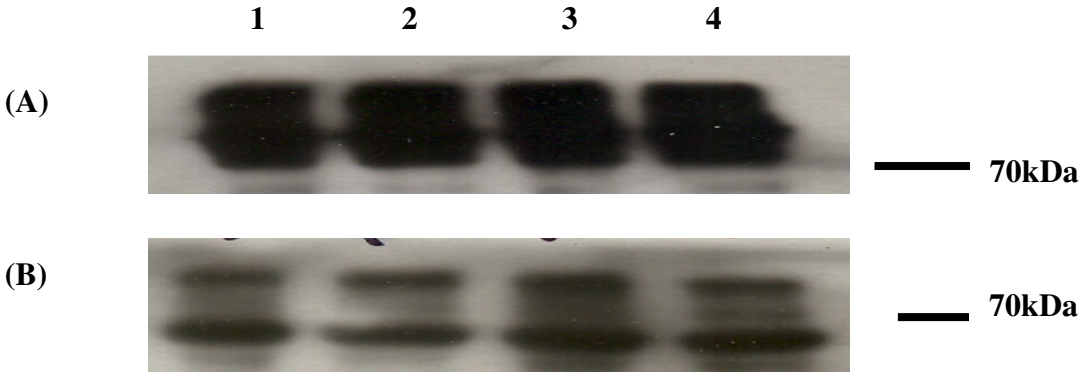


Figure 4.10 Detection of Hsp70 and His6X tag by western blotting. Peak 1 purified Ssa1 and ATPase mutants separated by SDS-page. Gels were immunoblotted with antibodies specific for Hsp70 (SPA-822, 1/2000 dilution) and His6x tag (anti His-Santa Cruz 1/200 dilution). Gel **(A)** shows (1) Ssa1, (2) $Ssa1^{G73D}$, (3) $Ssa1^{G287D}$and (4) $Ssa1^{T295I}$ probed with SPA-822 and gel **(B)** samples loaded in the same order as gel (A) probed with an anti-His tag antibody.

4.7.3 Investigating intrinsic ATPase activity of ATPase mutants

Hsp70 plays many roles in protein quality control such as; synthesis, protection during aggregation caused by stress and recovery of aggregates. Hsp70 functions by binding hydrophobic stretches of incompletely folded proteins, this process depend on conformational changes of Hsp70, which is regulated by ATP hydrolysis and ADP/ATP exchange. When Hsp70 is in an ATP bound state rapid substrate exchange occurs, hydrolysis of ATP to ADP results in an ADP bound state in which the substrate becomes tightly bound. Co-chaperones are also involved in this process, Hsp40 (Ydj1) presents substrates to Hsp70 and stimulates Hsp70 ATP hydrolysis. Nucleotide exchange factors such as Fes1 and Sse1 are also required to accelerate the release of ADP and the rebinding of ATP causing Hsp70 to release its substrate. As the ATPase mutants are located in regions of the ATPase domain involved with inter-domain communication (G73D) and binging of NEF's (G287D and T295I), investigating alterations to the ATPase cycle is critical. ATPase assays were carried out as in chapter 3.10. The ATPase activity of G73D is similar to that of wildtype suggesting that this mutation may not be affecting communication between the ATPase domain and PBD (Figure 4.11). The ATPase activity of G287D and T295I was increased by approximately two fold in comparison to wildtype (Figure 4.11). These results indicate that the ATPase mutants are not having a major effect on the ATPase cycle of Hsp70.

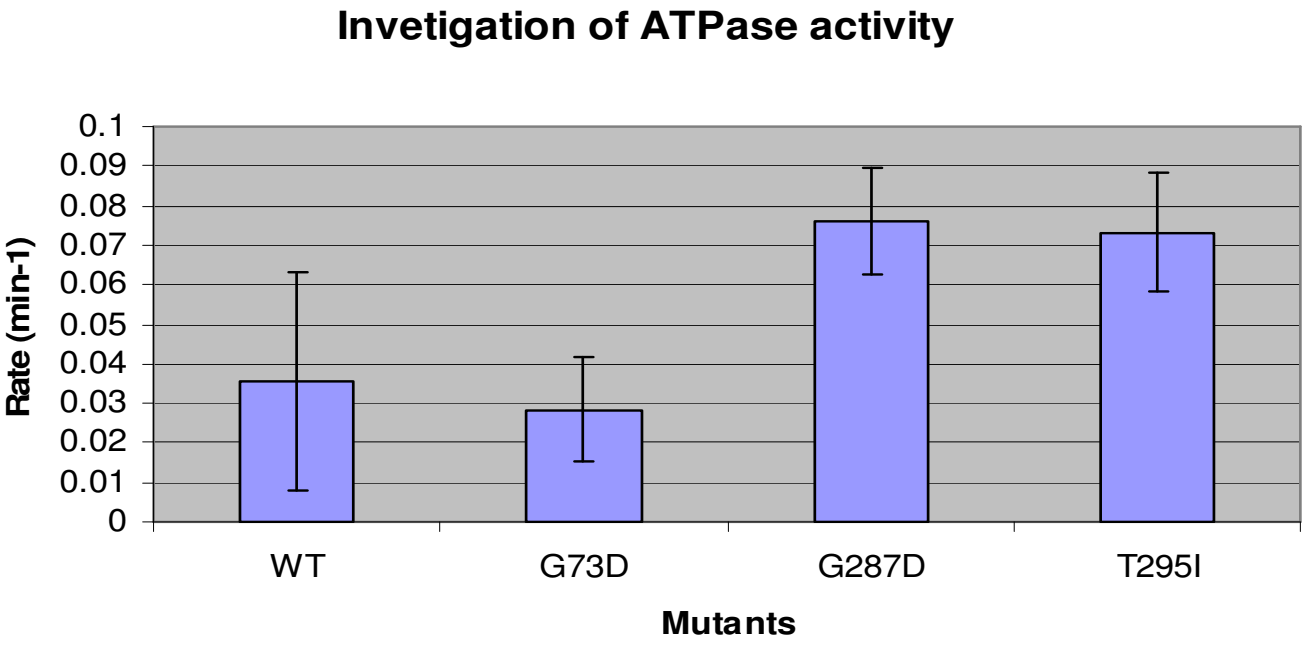


Figure 4.11 Investigation of ATPase activity. Comparison of ATPase activity between wildtype and ATPase mutants was measured. ATPase assay carried out using 2µM purified wildtype and ATPase mutants. The x axis represents proteins being assayed and y axis represents rate of ATP turnover per minute. Assays repeated three times in triplicate.

4.7.4 Stimulation of ATPase activity by Hsp40 (Ydj1)

The hydrolysis of ATP to ADP in the Hsp70 ATP cycle is a critical event in the ATP cycle of Hsp70. A variety of co-chaperones are involved in this event, with Hsp40 being the most prevalent. Hsp40 presents substrate to Hsp70 and coordinates Hsp70 ATPase hydrolysis with substrate binding. In yeast the major Hsp40 is Ydj1, increasing wildtype ATPase activity by seven fold (Needham and Masison. 2008).To test whether Ydj1 stimulates ATPase activity of wildtype Ssa1 and ATPase mutants, we tested the rate of ATPase activity with and without Ydj1. Ydj1 was purified as in chapter 3.10.2. Wildtype ATPase activity increases by four fold in presence of Hsp40. ATPase mutants do not appear to be stimulated by the presence of Ydj1 to the same degree as wildtype.

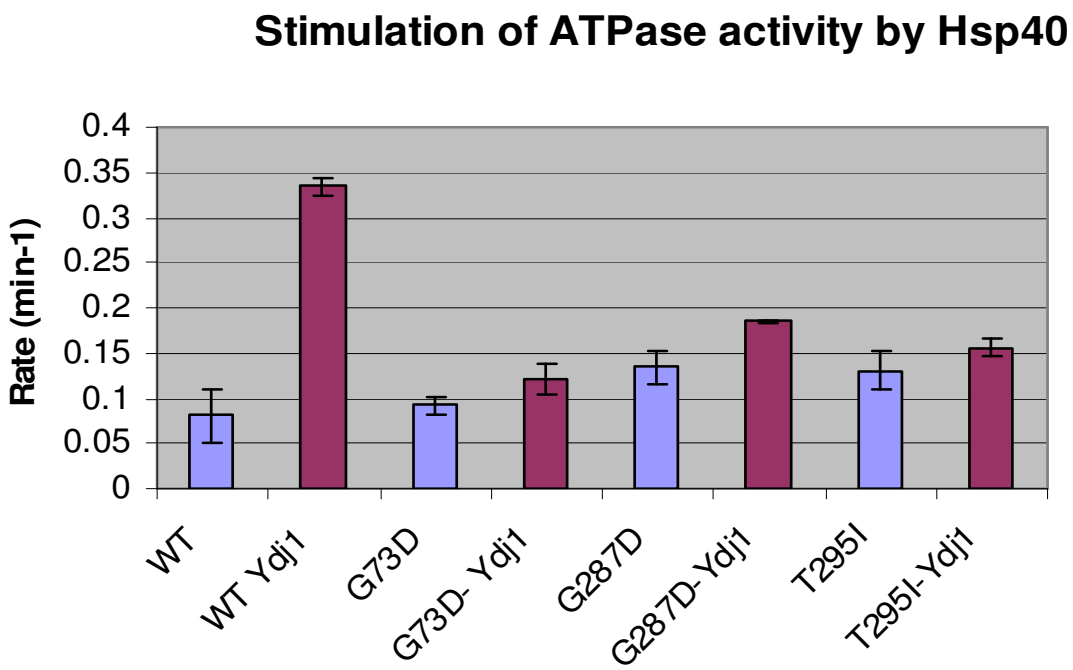
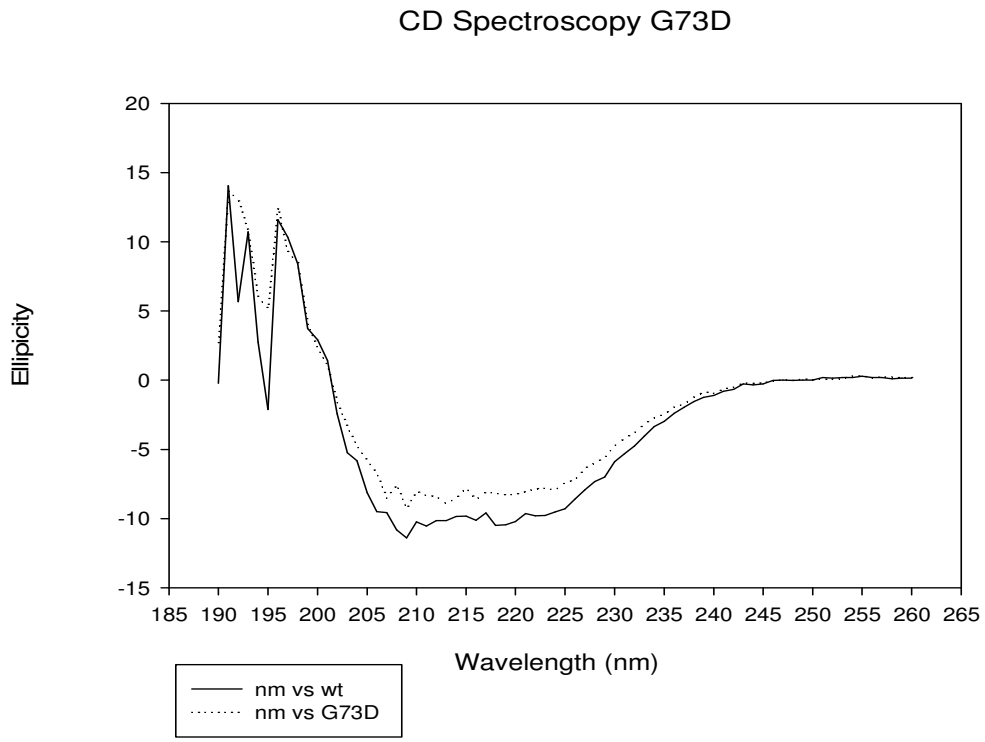
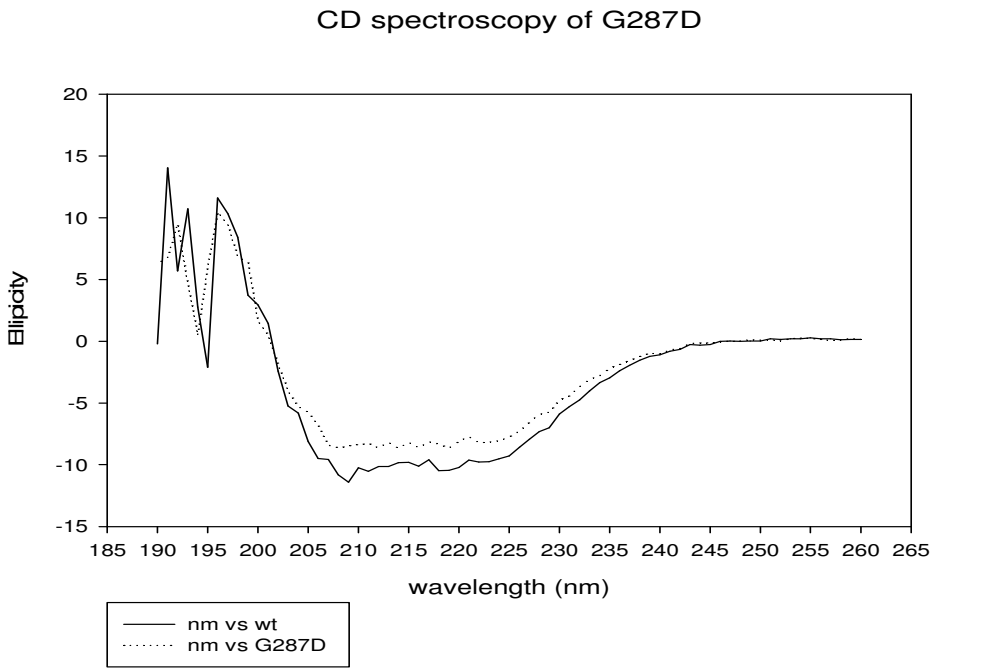


Figure 4.12. Stimulation of Ssa1 ATPase by Hsp40. This graph represents the stimulation of ATPase activity of ATPase mutants by Hsp40. ATPase assay carried out using 2µM of purified proteins. The x axis represents proteins being assayed and y axis represents rate of ATP turnover per minute. Assay carried out once in triplicate.

4.7.5 Analysis of protein structure of ATPase mutants

The ATPase mutants do not appear to significantly alter the ATPase activity of Hsp70; however, a decrease in the rate of ATPase activity by Ydj1 stimulation is observed. This decrease may be due to a structural change in Hsp70 caused by ATPase mutations. To investigate this we analyzed the secondary structures of the ATPase mutants in comparison to wildtype using Circular Dichroism spectroscopy. CD spectra carried out as in chapter 3.11. CD spectra reveals there is no major difference in the secondary structure of ATPase mutants in comparison to wildtype (Figure 4.13).





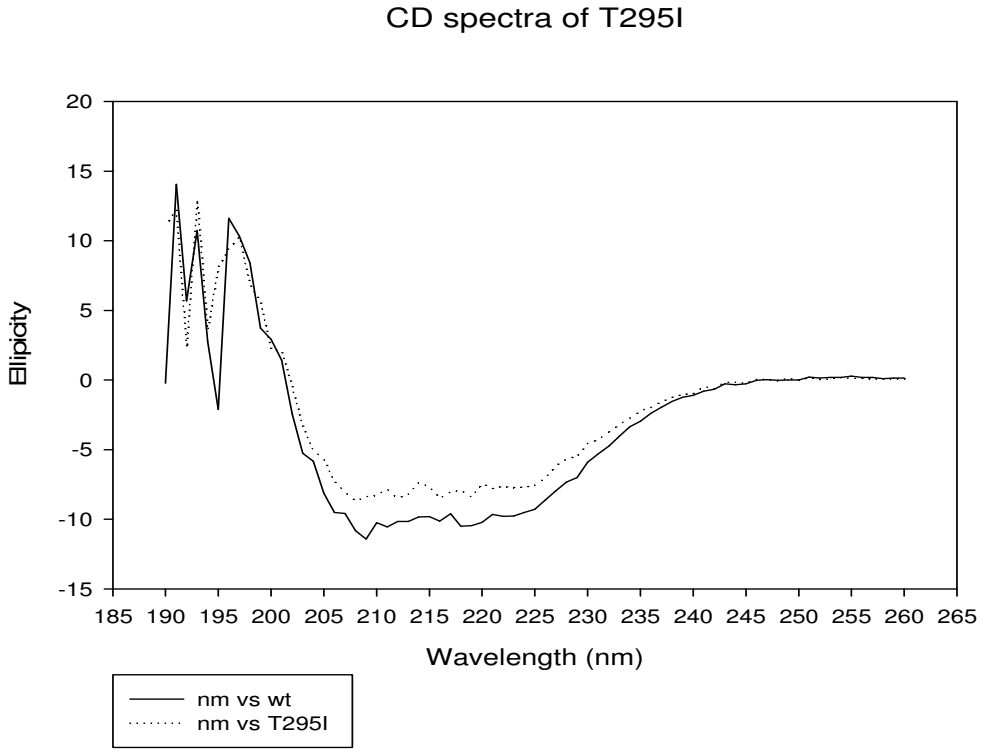


Figure 4.13- CD spectroscopy analysis of ATPase mutants. CD spectroscopy was preformed measuring the absorbance of 4µM of purified ATPase mutants in a total volume of 350µl of dialysis buffer on the Pistar CD stopped flow (Applied biophysics limited)

4.8 Affect of ATPase mutants on cellular functions

Hsp70 is involved in many cellular processes. Therapies based on affecting Hsp70 machinery may have side effects if Hsp70 is involved in a variety of cellular functions. The ATPase mutants have so far only exhibited an effect on prion propagation, no significant differences with respect to cellular functions were observed. This may be advantageous, as if these sites only affect prion propagation they may be used for potential targets for future treatments. To test this hypothesis we decided to investigate the affects of ATPase mutants on other cellular functions in *S.cerevisiae* such as; pleiotropic drug resistance (Pdr), Hsp70 self- regulation and the unfolded protein response.

4.8.1 Affect of ATPase mutants on expression of *PDR5*

Pleiotropic drug resistance (Pdr) enables *S. cerevisiae* to become resistant to numerous toxic compounds. In yeast Pdr1 and Pdr3 are two major transcription factors which control the Pdr response by activating genes such as *PDR5*, *PDR10* and *PDR15* which encode ATP- binding cassette (ABC) transporters which operate as drug efflux pump. ß-galactosidase assays were employed to measure the ATPase mutants effects on the expression of the transcription factor Pdr5. Methodology of *PDR5* ß-galactosidase assays described in section 3.14.1. The ATPase mutants appear to have no significant effect on expression of *PDR5* (Figure 4.14)

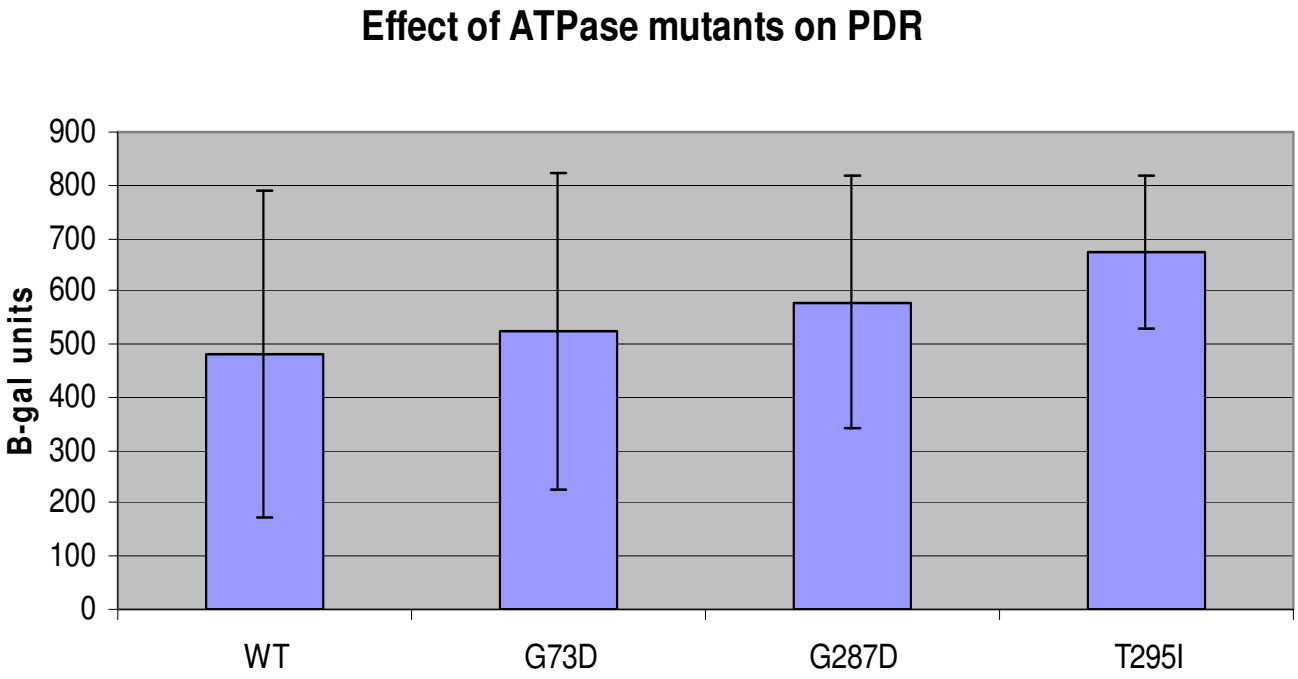


Figure 4.14- Effect of ATPase mutants on *PDR5* expression. Yeast cells expressing wildtype Ssa1 or ATPase mutants were transformed with the Pdr5 ß-galactosidase reporter palsmid. Transformed strains were grown overnight in 5ml of YPD at 30°C to a concentration of $5x10^6$ (cells/ml). Cells were then grown to a concentration of $1x10^7$-$2x10^7$ (cells/ml). Cells were chilled on ice and then harvested at 4°C. Protein was extracted and reaction was initiated by adding ONPG, once a pale yellow color was observed the reaction was terminated by adding 1M Na_2CO_3. *o*-nitrophenol was quantified by measuring absorbance at OD_{420}nm. Units of ß-galactosidase activity (Y axis) were defined as (OD_{420}) X (1.7)/ (.0045) X (volume) X (time) X (protein concentration) (section 2.9)

4.8.2 Effect of ATPase mutants on Ssa1 expression

The induction of heat shock proteins by stress depends on heat shock factors (HSF), which bind to heat shock elements (HSE) in the promoters of heat shock genes. The Ssa1 promoter is known to contain HSE's and Ssa1 is also thought to negatively regulate its own expression (Stone and Craig., 1990). Although protein levels of ATPase mutants are similar to wildtype it was still necessary to investigate whether the ATPase mutants could cause changes at the transcriptional level for the Ssa1 promoter. To analyze the effect of ATPase mutants on transcriptional levels of the Ssa1 promoter we preformed a ß-galactosidase assay as described in section 3.12.2. The mutant G73D was the only ATPase mutant to show increased Ssa1 expression with it being expressed almost one and a half times higher than wildtype (Figure 4.15). A decrease of more than one and a half fold, in Ssa1 expression was observed with the mutants G287D and T295I in comparison to wildtype. Although abundance of Hsp70 appears similar for ATPase mutants, transcriptional levels appear to vary, indicating mutants may some how be affecting this.

4.8.3 Effect of ATPase mutants on unfolded protein response

As one of the primary functions of Hsp70 is the refolding of proteins that have become unfolded due to cellular stress, we decided to investigate the effect of the Hsp70 ATPase mutants on the unfolded protein response (UPR) in yeast. The unfolded protein response is described in chapter 3.14.3. To assess whether the ATPase mutants have an affect on the UPR, we measured the activity of an unfolded protein response element.

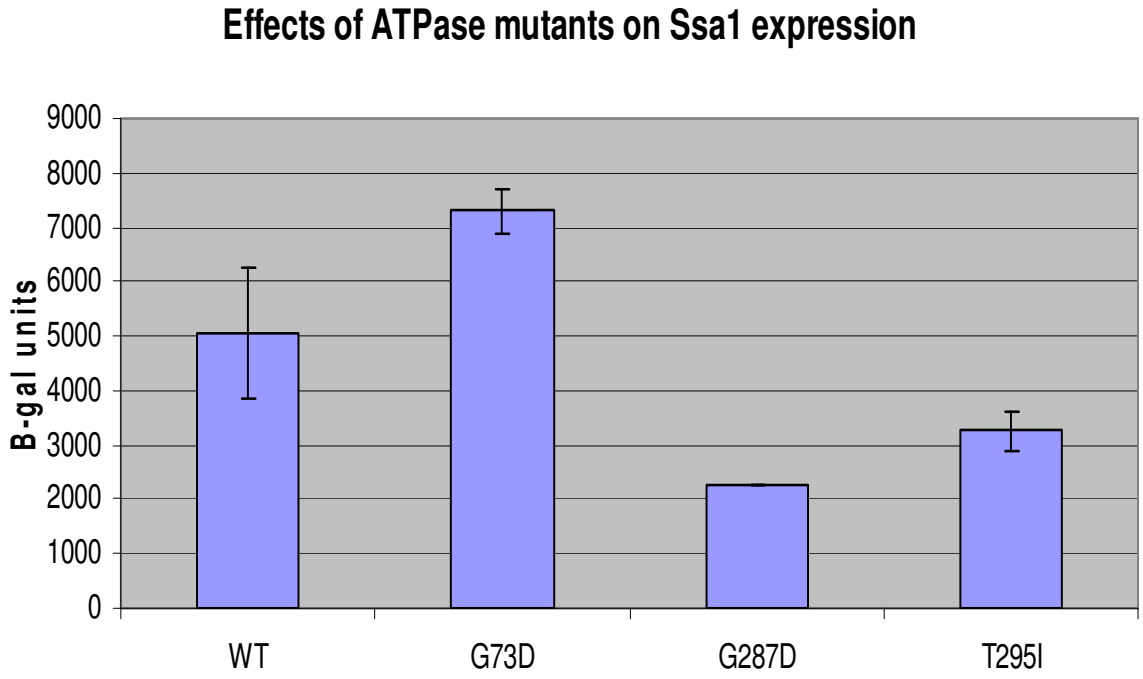


Figure 4.15 Effect of ATPase mutants on Ssa1 expression. Yeast cells expressing wildtype Ssa1 or ATPase mutants were transformed with the *SSA1* promoter ß-galactosidase reporter palsmid (pZFO) . ß-galactosidase assays were carried out as described in Figure 4.14.

This was achieved by utilizing a ß-galactosidase reporter plasmid which expressed the promotor conyaing UPRE elements fused to the *lac*Z gene (chapter 3.14.3). None of the ATPase mutants appear to have a significant effect on the unfolded protein response.

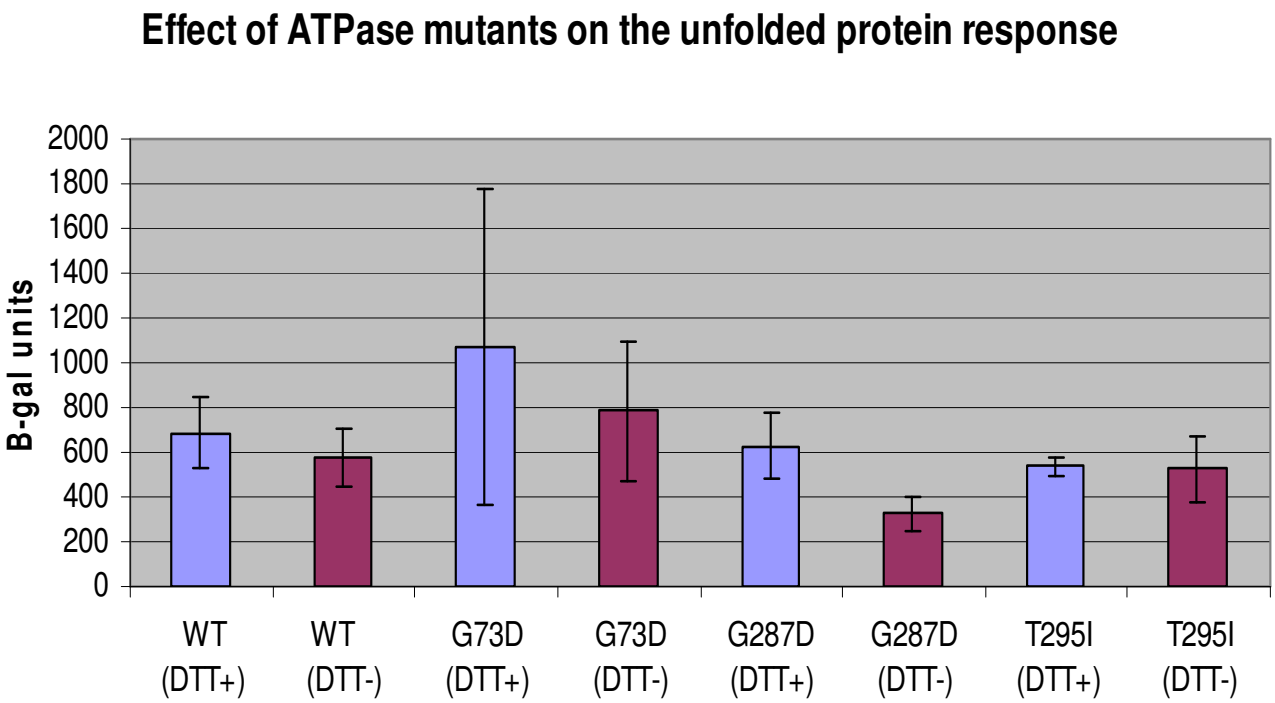


Figure 4.16 -The effect of ATPase on the unfolded protein response. Yeast cells expressing wildtype ATPase mutants were transformed with the UPRE promoter ß-galactosidase reporter palsmid (pUPRE)**.** ß-galactosidase assays were carried out described in section 4.14, with the exception of adding 5mM DTT into cultures for thirty minutes at 30^{o}C. The X-axis indicates the strains used in the assay. The Y axis indicates the ß-gal units.

4.9 Effects of CTD truncations on prion propagation and Hsp70 function

Hsp70 is composed of three domains; PBD, the ATPase domain and the C-Terminal domain (CTD). We have identified mutants located in the PBD and ATPase domain of Hsp70 that affect prion propagation. The construct used to identify PBD mutants contained part of the CTD, yet no mutations were isolated in this region that affected prion propagation. To determine whether the CTD plays a role in prion propagation and other Hsp70 functions, we decided to investigate the affect of various CTD truncations in *S. cerevisiae.* To assess the possible influence of CTD in prion propagation we created three different amino acid truncations of the CTD; Δ20 (1-622), Δ40 (1-602) and Δ60 (1-582) (Figure 4.17). Primers designed to amplify truncated versions of Ssa1 were utilized and truncated Ssa1 PCR products were ligated into the pJ120 vector (Figure 4.18), resulting plasmids were named pJ120-Δ20, Δ40 and Δ60.

```
  1  MSKAVGIDLG TTYSCVAHFA NDRVDIIAND QGNRTTPSFV AFTDTERLIG
 51  DAAKNQAAMN PSNTVFDAKR LIGRNFNDPE VQADMKHFPF KLIDVDGKPQ
101  IQVEFKGETK NFTPEQISSM VLGKMKETAE SYLGAKVNDA VVTVPAYFND
151  SQRQATKDAG TIAGLNVLRI INEPTAAAIA YGLDKKGKEE HVLIFDLGGG
201  TFDVSLLSIE DGIFEVKATA GDTHLGGEDF DNRLVNHFIQ EFKRKNKKDL
251  STNQRALRRL RTACERAKRT LSSSAQTSVE IDSLFEGIDF YTSITRARFE
301  ELCADLFRST LDPVEKVLRD AKLDKSQVDE IVLVGGSTRI PKVQKLVTDY
351  FNGKEPNRSI NPDEAVAYGA AVQAAILTGD ESSKTQDLLL LDVAPLSLGI
401  ETAGGVMTKL IPRNSTIPTK KSEIFSTYAD NQPGVLIQVF EGERAKTKDN
451  NLLGKFELSG IPPAPRGVPQ IEVTFDVDSN GILNVSAVEK GTGKSNKITI
501  TNDKGRLSKE DIEKMVAEAE KFKEEDEKES QRIASKNQLE SIAYSLKNTI
551  SEAGDKLEQA DKDTVTKKAE ETISWLDSNT TASKEEFDDK LKELQDIANP
601  IMSKLYQAGG APGGAAGGAP GGFPGGAPPA PEAEGPTVEE VD*
```

Figure 4.17- Ssa1 protein sequence exhibiting CTD truncations. Ssa1 protein sequence was retrieved from *Saccharomyces* genome datatbase (SGD). Δ20, Δ40 and Δ60 truncations represented by color code; Δ20 truncation highlighted in blue, Δ40 truncation highlighted in blue and pink and Δ60 truncation highlighted in blue, pink and red.

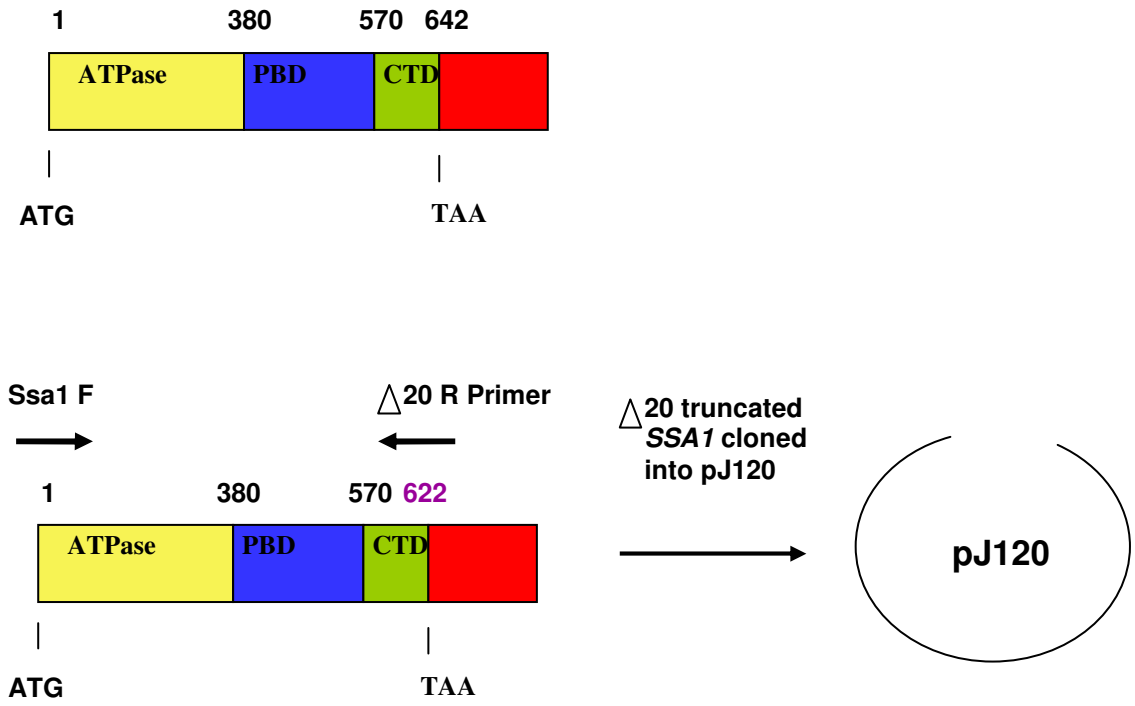


Figure 4.18 Strategy employed for creating CTD truncations of Ssa1- This schematic diagram exhibits the method employed for creating the Δ20 CTD Ssa1 truncation, the Δ40 and Δ60 truncations were made utilizing the same strategy. The Δ20 reverse primer was designed to exclude the last twenty amino acids of Ssa1. Ssa1 was amplified by a PCR reaction utilizing a Ssa1 forward primer and the Δ20 reverse primer. The resulting truncated Ssa1 product was cloned into the pJ120 plasmid by a *Sph*1 and *Nde*1 ligation.

4.9.1 Assessment of viability of Ssa1 CTD truncations

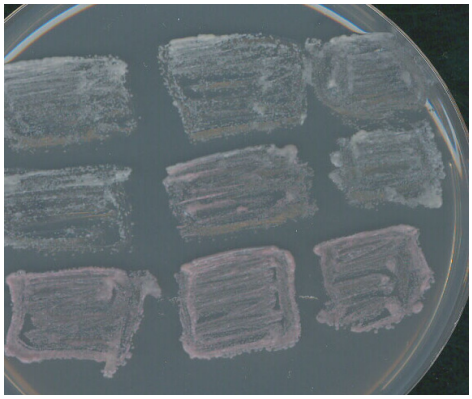
To analyze the effects of CTD truncations on prion propagation and other essential Hsp70 functions, it was necessary to test the viability of these truncations *in vivo.* In order for this to be achieved we transformed [*PSI*$^+$] and [*psi*$^-$] versions of G402 with CTD truncated plasmids, pJ120 (positive control) and pRS315 (negative control). Growth of cells was observed for roughly one week on 5-FOA agar at room temperature. Initially, no cells appeared to grow for the G402 [*PSI*$^+$] cells transformed with truncated CTD plasmid, colonies then began to appear on day four, with most growth appearing on the Δ40 patches, all cells remained [*PSI*$^+$] (Figure 4.19). The [*psi*$^-$] cells expressing the Δ20 and Δ40 CTD truncations exhibited growth after three days. Both strains remained [*psi*$^-$] and growth appeared to be slower than wildtype (Figure 4.20). The Δ60 CTD truncation appears to be unable to support cells growth in both [*PSI*$^+$] and [*psi*$^-$] strains. In both cases controls acted correctly; cells transformed with pRS315 were unable to grow once placed on 5-FOA as cells had no members of the Ssa family being expressed and this is a lethal deletion and cells transformed with pJ120, which expresses Ssa1 grew as normal on 5-FOA. These results suggest that Δ20 and Δ40 CTD truncations can maintain cell viability and Δ60 CTD truncation cannot. This is indicative that a region between the Δ40 and Δ60 is important for Hsp70 function.

PRE-5-FOA

Δ20

Δ40

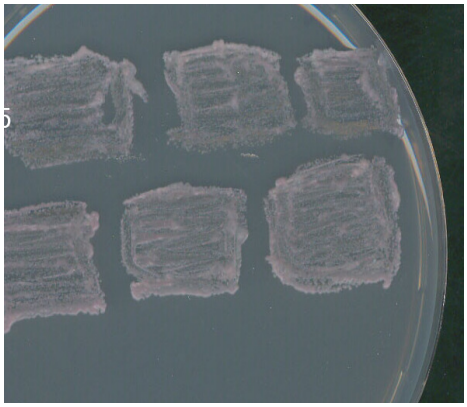
Δ60



PRE-5-FOA

315

pJ120



POST-5-FOA

Δ20

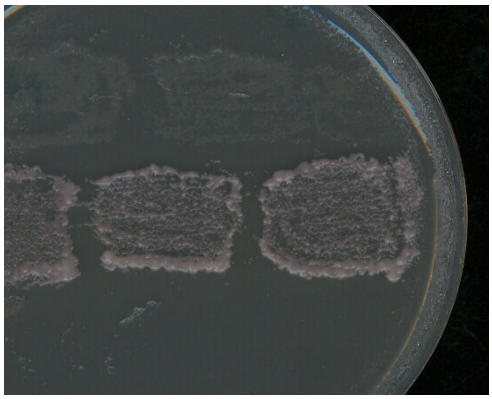
Δ40

Δ60

POST-5-FOA

315

pJ120

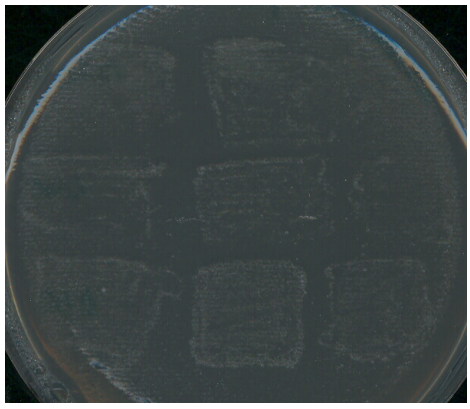


DAY 2

Δ20

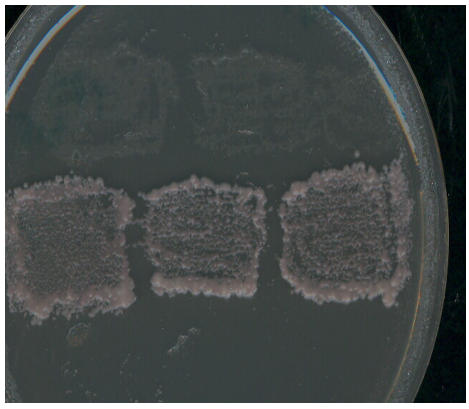
Δ40

Δ60



315

pJ120



DAY 3

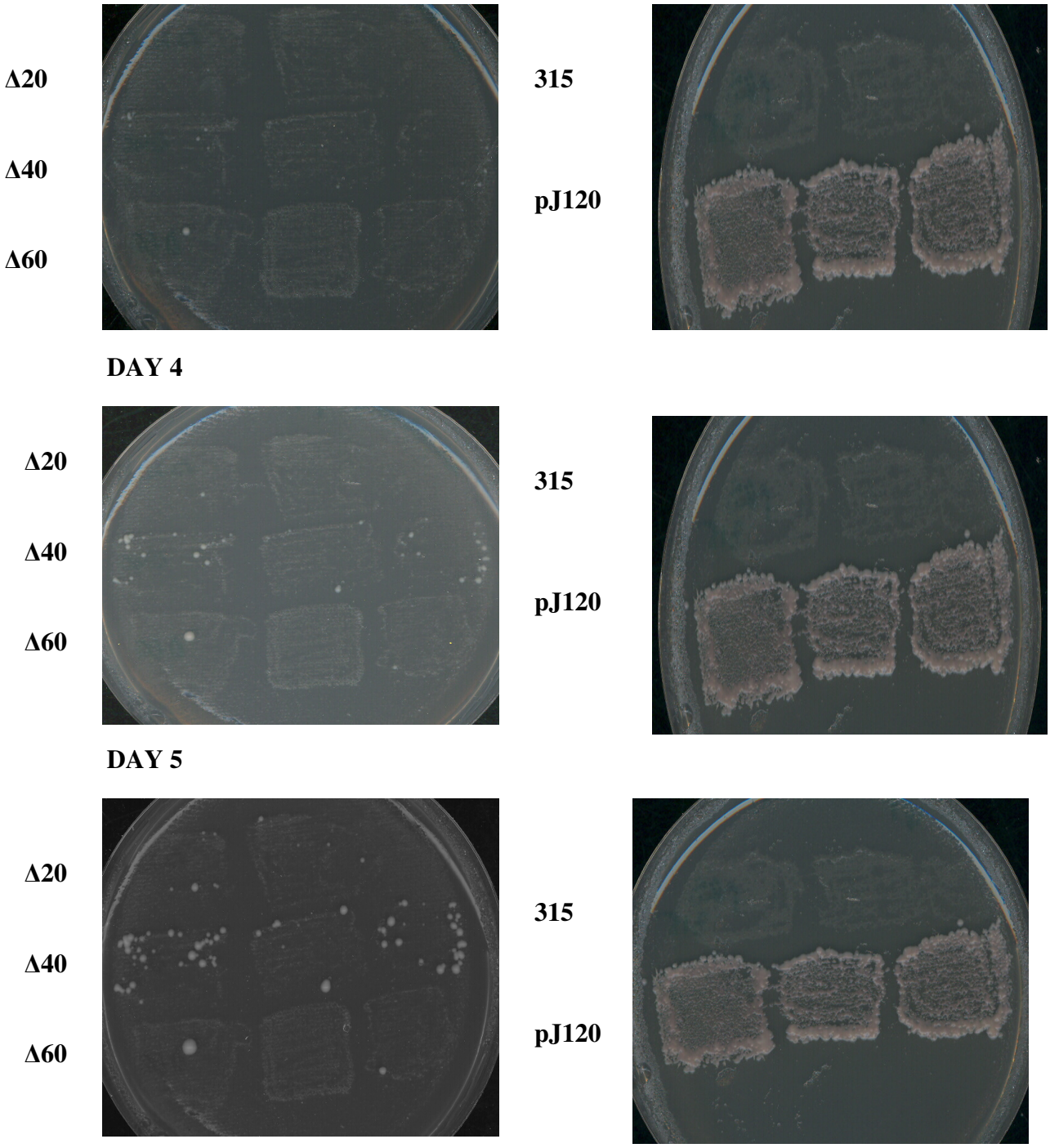


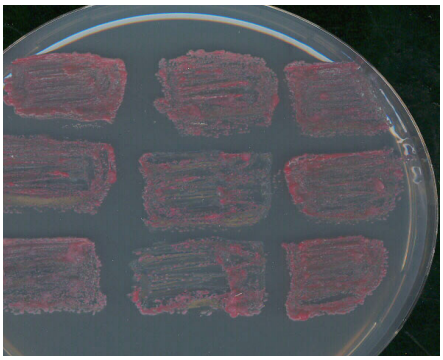
Figure 4.19 Viability of truncated CTD mutants in [*PSI*$^+$] G402. Truncated CTD mutants, pRS315 and pJ120 were transformed into [*PSI*$^+$] G402 and were grown at 30^o for 48 hours on SD agar lacking Leucine. Transformants were patched onto SD agar lacking Leucine (Pre 5-FOA) and grown at 30^o for 48 hours. Patches were replicated onto 5-FOA and growth was observed over six

PRE-5-FOA

Δ20

Δ40

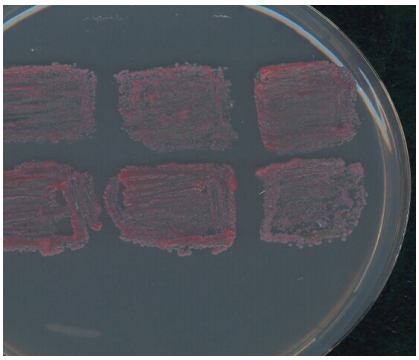
Δ60



PRE-5-FOA

315

pJ120

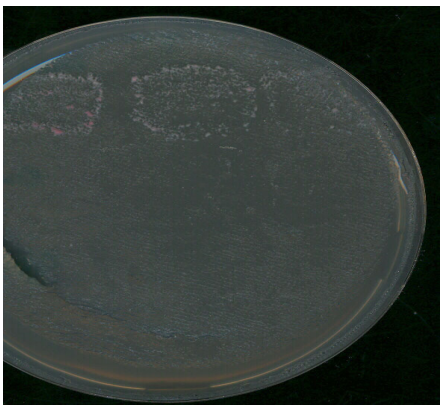


POST-5-FOA

Δ20

Δ40

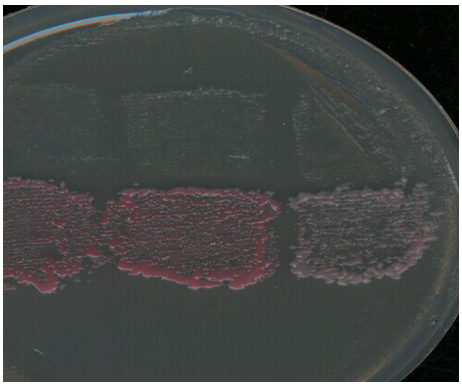
Δ60



POST-5-FOA

315

pJ120

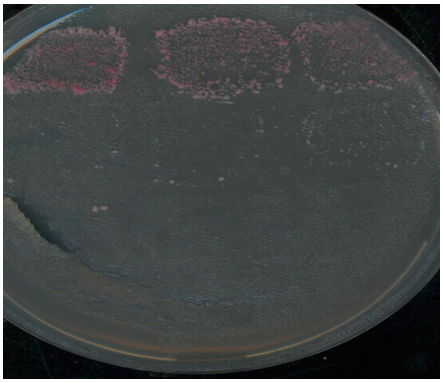


DAY 2

Δ20

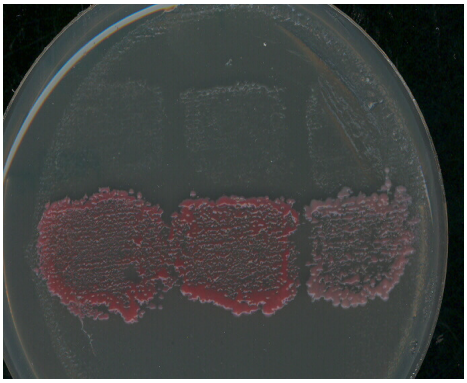
Δ40

Δ60

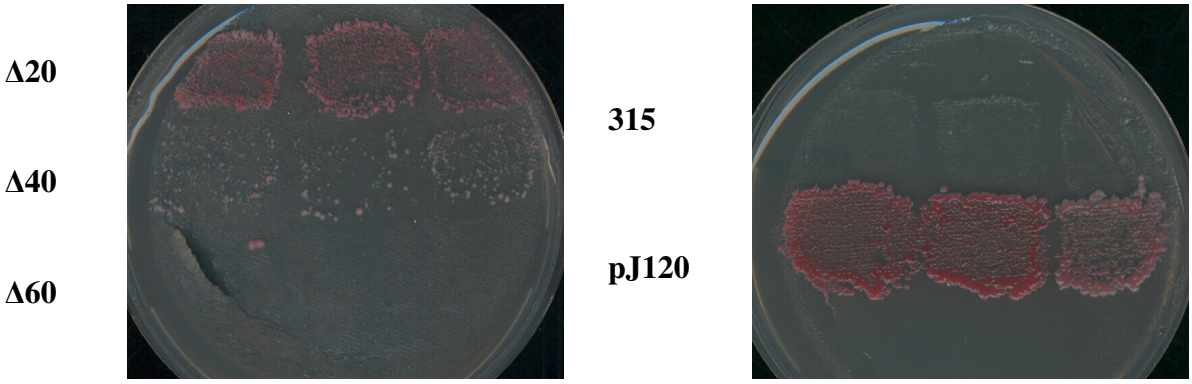


315

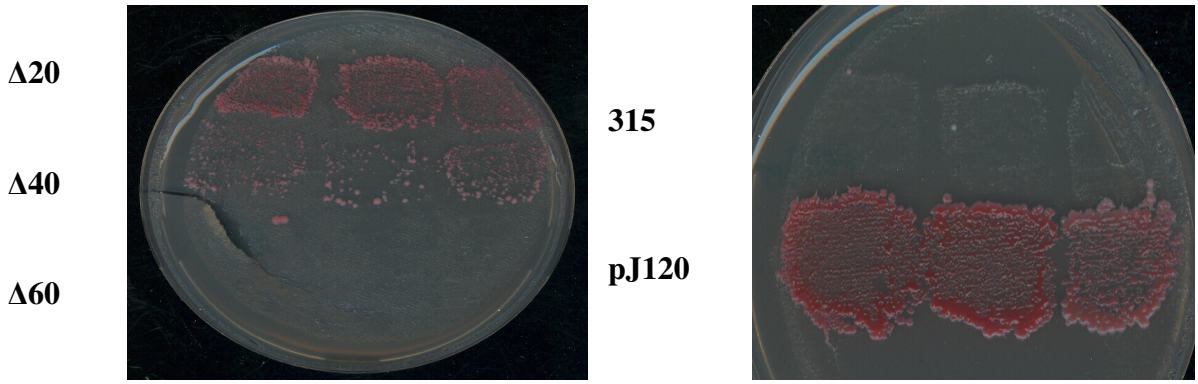
pJ120



DAY 3



DAY 4



DAY 5

4.20 Viability of truncated CTD mutants in [*psi*$^-$] G402. Truncated CTD mutants, pRS315 and pJ120 were transformed into [*psi*$^-$] G402 and were grown at 30^o for 48 hours on SD agar lacking Leucine. Transformants were patched onto SD agar lacking Leucine (Pre 5-FOA) and grown at 30^o for 48 hours. Patches were replicated onto 5-FOA and growth was observed over five days.

4.9.2 Confirmation of CTD truncations

To confirm the strains are expressing truncated versions of Ssa1, we hypothesized that a slight shift in protein size should be visible by Western blot analysis. To test this we extracted total protein from [*psi*$^-$] strains pre 5-FOA and post 5-FOA and immunoblotted with an antibody specific for Hsp70. From Figure 4.20, it is clear that there is a shift in protein size for Δ20 and Δ40 post 5-FOA treatment confirming that truncated proteins are being expressed in the yeast cells and can sustain growth as sole source of Ssa family in strain.

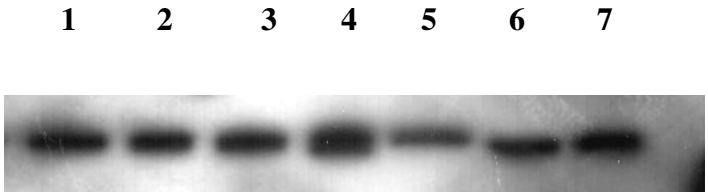


Figure 4.21- Confirmation of expression of CTD truncated proteins. Lysates prepared from overnight 30^{o}C cultures. Aliquots of proteins (10µg) were separated on a polyacrylamide gel. Gels was then immunoblotted with an antibody specific Hsp70 (SPA-822, 1/2000 dilution). Lanes 1-4 represent the samples pJ120, Δ20, Δ40 and Δ60 pre 5-FOA and lanes 5-7 represent samples pJ120, Δ20 and Δ40 post 5-FOA treatment. A shift in protein size is observed Δ20 and Δ40 post 5-FOA treatment, which confirms truncated proteins are being expressed.

4.9.3 Analysis of growth rates of CTD truncation in Yeast

Previous analysis of the effects Ssa1 ATPase and PBD mutation on growth rates of yeast cells has shown that there are no significant alterations to growth rates. As we observed slower growth rates of Δ20 and Δ40 on 5-FOA and no growth for Δ60 (Figure 4.19 and 4.20), we decided to measure the mean generation time of both [*PSI*$^+$] and [*psi*$^-$] strains. As we observed on the plates, Δ40 [*PSI*$^+$] grows slightly quicker than Δ20 [*PSI*$^+$] (Figure 4.19). The [*psi*$^-$] strains also appear to correspond to growth rates on plates with Δ20 growing faster than Δ40 (4.2). These results indicate that truncations of the CTD of Hsp70 have an effect on the growth rate of *S. cerevisiae.* This indicates that the CTD of Ssa1 is essential for normal cellular growth.

CTD Truncations	MGT (Mins)	MGT % of WT
SSA1	110.33	100%
Δ20 [*PSI*$^+$]	142	129% ± 13%
Δ40 [*PSI*$^+$]	135	122% ± 24%
Δ20 [*psi*$^-$]	164	149% ±25%
Δ40 [*psi*-]	201	182% ±18%

Table 4.3 Effects of Hsp70 CTD truncations on the growth rate of yeast cells. Mean generation time of CTD truncations was measured by diluting overnight 30^{o}C cultures to an OD_{600} 0.1. Cultures grown exponentially at 30^{o}C, OD_{600} measured every two hours for approximately ten hours.

4.9.4 Analysis of Hsp70 function in strains expressing CTD truncations

Analysis of the ability of Hsp70 containing mutations in the ATPase and the PBD to recover misfolded protein during heat shock, lead us to believe that mutations in ATPase domain did not affect this function but the PBD mutation Ssa1^{F475S} caused a t.s phenotype at 37^{o}C. As the CTD works in concert with the PBD (CTD forms a lid over PBD trapping substrate), we therefore decided to evaluate the affect of the Δ20 and Δ40 CTD truncations on protein recovery by Hsp70 during heat shock. We analyzed growth of Δ20 and Δ40 at 30^{o}C and 37^{o}C. At 30^{o}C, Δ20 and Δ40 appear to be growing at a slower rate in comparison to wildtype (Figure 4.21), which was previously observed (Table 4.2). Interestingly, Δ20 and Δ40 appear t.s at 37^{o}C (Figure 4.21). This suggests that truncations of the Hsp70 CTD may be inhibiting recovery of misfolded proteins during heat shock or it may further implicate Ssa1 as playing a role in the CWI signaling pathway.

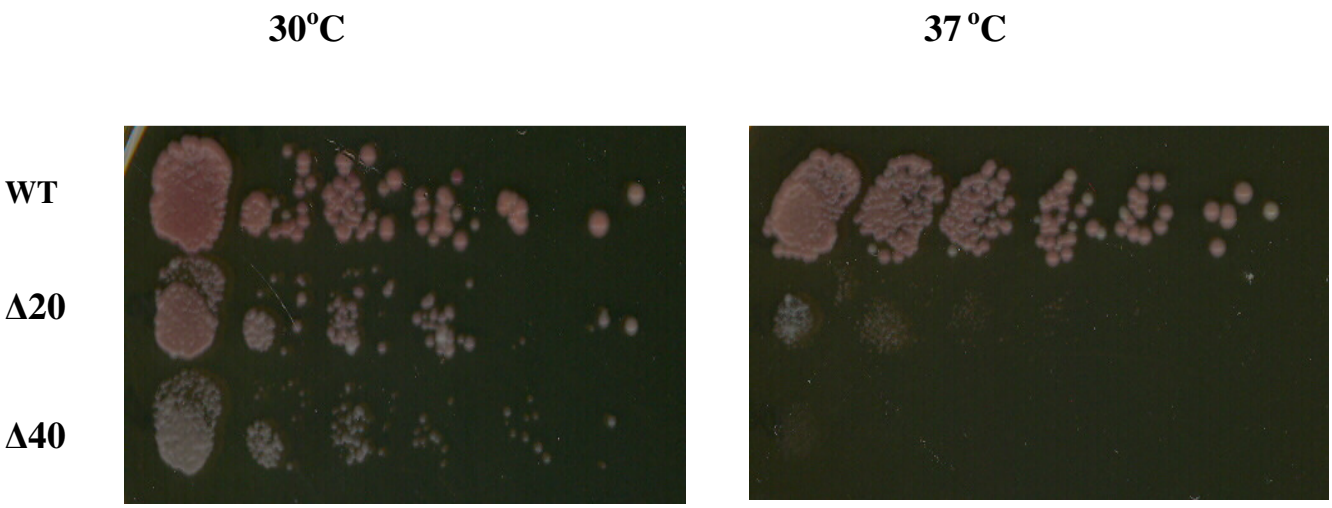


Figure 4.22- Hsp70 function in cells expressing CTD truncations. Cells incubated at specific temperatures for 72 hours on YPD. Cells were diluted by a 1/5 serial dilution. Spots labeled accordingly. Wildtype [*psi*$^{-}$] and mutants expressed in G402 therefore are sole source of Ssa in strain.

4.9.5 Implicating Ssa1 as playing a role in the CWI signaling pathway

The cell wall integrity signaling pathway is a MAP kinase pathway, which contains many components, and results in the phosphorylation of the transcription factor Slt2 that induces the expression of genes involved in cell wall repair and synthesis (Figure 3.28). A variety of chaperones such as Hsp90 and the NEF Sse1 have been implicated in this pathway. Introducing mutations to components of the cell wall integrity (CWI) signaling pathway results in common phenotypes such as temperature sensitivity at 37^{o}C and sensitivity to drugs that damage the cell wall. We have observed that the Ssa1^{F475S} was 37^{o}C t.s and its recovery on 1M sorbitol indicated that Ssa1 might be involved in this pathway. Therefore, we decided to test for recovery of the Δ20 and Δ40 37^{o}C t.s phenotype on 1M sorbitol. Both Δ20 and Δ40 exhibited recovery of the t.s phenotype when gown at 37^{o}C on YPD supplemented with 1M sorbitol (Figure 4.22). This result further indicates that Ssa1 may play a role in the CWI signaling pathway.

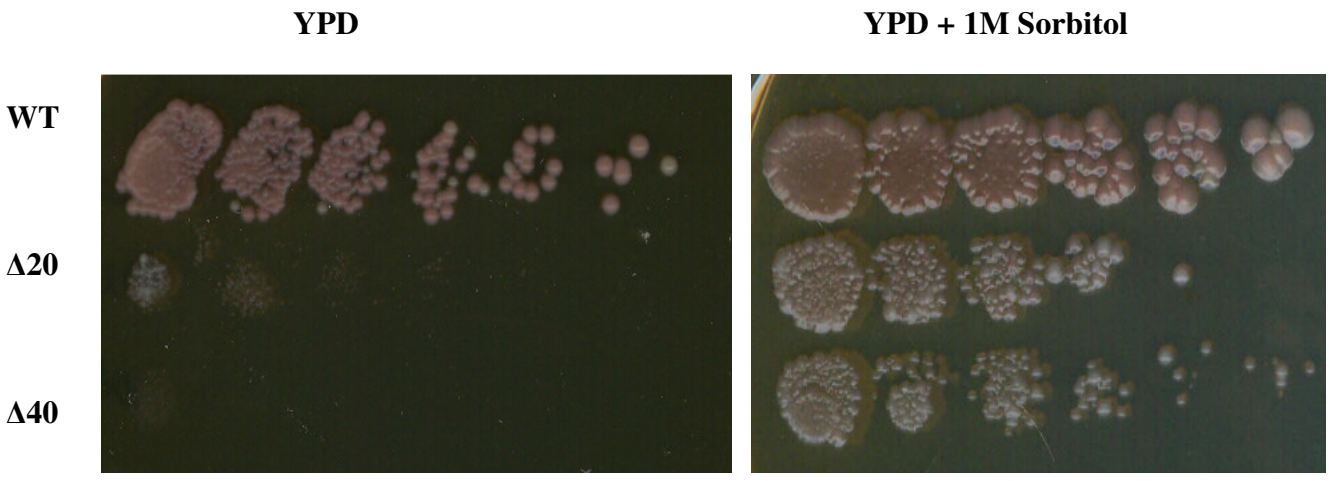


Figure 4.23- Recovery of CTD truncations at 37^{o}C. Cells incubated at 37^{o}C for 72 hours on YPD and YPD+ 1M sorbitol. Cells were diluted by a 1/5 serial dilution. Spots labeled accordingly. Wildtype [*psi*$^{-}$] and mutants expressed in G402 therefore are sole source of Ssa in strain.

4.9.6 Further evaluation of [*PSI*$^+$] strain expressing CTD truncations

Assessment of the viability of CTD truncations in the G402 strain, lead us to observe single white colonies appearing on the Δ20 and Δ40 patches after four days. To further test these colonies we decided to assess their curing ability. Millimolar concentrations of the chaotropic salt guanidine hydrochloride (GdnHCL) eliminates [*PSI*$^+$] from cells, resulting in a change from [*PSI*$^+$] (white) to [*psi*$^-$] (red) (Tuite *et al.*, 1981). To test the Δ20 and Δ40 ability to cure, colonies were picked from patches and restreaked onto YPD plate's containing 3mM GdnHCL. We observed that Δ20 and Δ40 cured when placed on 3mM GdnHCL.

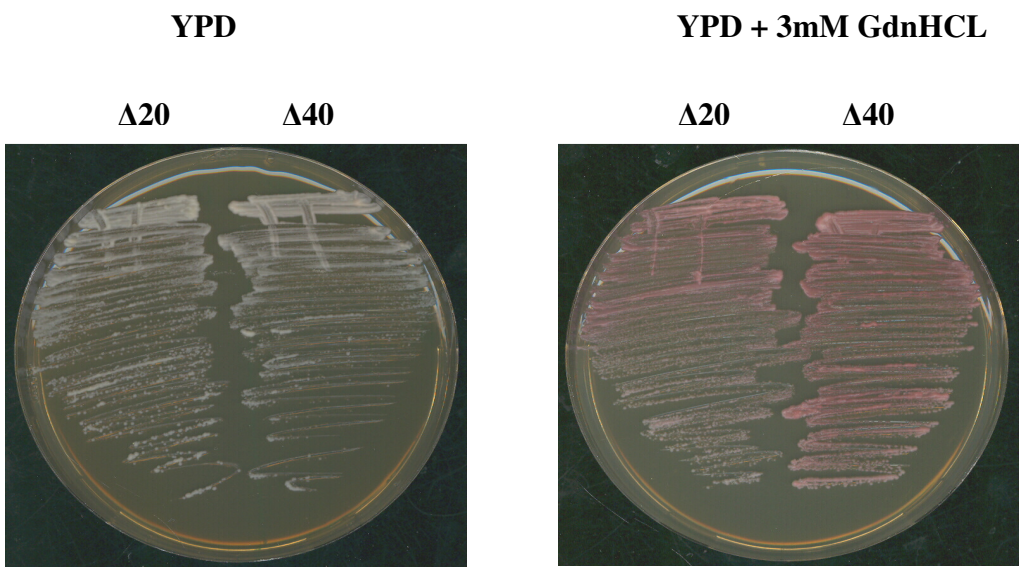


Figure 4.24 Curing of [*PSI*$^+$] Δ20 and Δ40 strains by 3mM GdnHCL. The [*PSI*$^+$] strains harboring the CTD truncation were streaked onto YPD and YPD + 3mM GdnHCL and incubated at 30^{o}C for 48 hours.

4.9.7 Growth rates of cured Δ20 and Δ40 strains

Initially when we assessed the growth rates of the [*PSI*$^+$] and [*psi*$^-$] strains expressing CTD truncations, Δ40 appeared to grow faster in [*PSI*$^+$] strain and slower than Δ20 in [*psi*$^-$] strain. To assess whether curing of Δ20 and Δ40 [*PSI*$^+$] strains made cells act similarly to [*psi*$^-$] strains expressing Δ20 and Δ40 we analyzed the growth rates of [*PSI*$^+$], cured [*PSI*$^+$] and [*psi*$^-$] cells expressing the CTD truncations. Cured versions of [*PSI*$^+$] appeared to grow more similarly to [*psi*$^-$] strains with Δ40 growing slower in both cases (Table 4.4) This suggest the presence of the [*PSI*$^+$] prion has an affect on the functioning of truncated Hsp70.

CTD truncations	**MGT (mins)**	**MGT % of WT**
Ssa1	105	100%
Δ20 [*PSI*$^+$]	142	135% ±14%
Δ40 [*PSI*$^+$]	135	129% ±25%
Δ20 Cured	**137**	**130% ±18%**
Δ40 Cured	**151**	**144% ±31%**
Δ20 [*psi*$^-$]	138	131%±14%
Δ40 [*psi*$^-$]	172	163% ±38%

Table 4.4 Growth rate of cured [*PSI*$^+$] cells expressing CTD truncations. Mean generation time of CTD truncations was measured by diluting overnight 30^{o}C cultures to an OD_{600} 0.1. Cultures grown exponentially at 30^{o}C, OD_{600} measured every two hours for approximately ten hours (chapter 2.7.1).

4.10 Microarray analysis of Δ20

To further analyze the affects of Ssa1 CTD truncations, we investigated the effects of expressing Δ20 as the sole source of Ssa1 on gene expression in G402. We found a considerable amount of genes that were regulated differently in strains expressing Δ20 in comparison to wild-type. We found eight genes in the Δ20 strain that were up-regulated by three-fold or more in comparison to the wild-type strain. The *DAN1* and *TIR1* genes are both up-regulated in cells expressing Δ20 (Table 4.5). Both these genes are part of the *DAN/TIR* family, which is comprised of eight genes and are up-regulated during adaptation to anaerobic growth conditions (Sertil *et al*., 2003, Tai *et al*., 2005). Other genes up-regulated include genes involved in methionine biosynthesis (*MET2*) (Forlani *et al*., 1991) and *de novo* lipid synthesis (*PAH1*) (Han *et al.*,2006).

From the microarray analysis we also observed that a number of genes were down-regulated in cells expressing Δ20 in comparison to wild-type (Table 4.6). Nine genes were down-regulated by five-fold or more in Δ20 cells, including genes involved in a diverse range of cellular structures and functions, including ribosomal structure (*RPS8A*), cytokenesis (*EGT2*) and transcriptional activation (*MSN1*) (Table 4.6). The down-regulation of the *EGT2,* which encodes a GPI cell wall protein required for correct cytokenesis after cell separation, may result in the reduced growth rate observed in cells harboring the Δ20 mutation. By analyzing gene expression in strains harboring Δ20 mutant, it was observed that the presence of an Ssa1 CTD truncation has an affect on the regulation of a number of genes. Detailed microarray analysis data is supplemented as a hard copy (CD).

ORF/Gene	Gene function	Ssa1-Δ20
YNR034W/*SOL1*	Protein with a possible role in tRNA export.	5.506
YHL021C/*AIM17*	Unknown	4.752
YGR204C-A/Unknown	Unknown	3.586
YHR179W/*CYE*	Conserved NADPH oxidoreductase containing flavin mononucleotide may be involved in sterol metabolism, oxidative stress response, and programmed cell death	3.509
YNL277W/*MET2*	L-homoserine-O-acetyltransferase, catalyzes the conversion of homoserine to O-acetyl homoserine which is the first step of the methionine biosynthetic pathway	3.328
YMR165C/*PAH1*	Mg^{2+}-dependent phosphatidate (PA) phosphatase, catalyzes the dephosphorylation of PA to yield diacylglycerol and P_i, responsible for *de novo* lipid synthesis	3.301
YJR150C/*DAN1*	Cell wall mannoprotein with similarity to Tir1, Tir2, Tir3, and Tir4.	3.062
YER011W/*TIR1*	Cell wall mannoprotein of the Srp1/Tip1 family of serine-alanine-rich proteins	3.014

Table 4.5 Up-regulation of genes in cells expressing Δ20. Analysis of microarray data revealed eight genes that were up-regulated in strains expressing the Ssa1 CTD truncation Δ20 by approximately three-fold or greater. Expression of genes was compared to expression in Ssa1. Functions of genes were obtained from the *Saccharomyces* genome database (SGD).

ORF/Gene	Gene function	Ssa1-Δ20
YLR431C/*ATG23*	Peripheral membrane protein required for the cytoplasm-to-vacuole targeting (Cvt) pathway and efficient macroautophagy	0.087
YKR022C/*NTR2*	Essential protein that forms a dimer with Ntr1; also forms a trimer, with Ntr2 and the DExD/H-box RNA helicase Prp43, that is involved in spliceosome disassembly	0.089
YNL186W/ *UBP10*	Ubiquitin-specific protease that deubiquitinates ubiquitin-protein moieties; may regulate silencing by acting on Sir4; involved in posttranscriptionally regulating Gap1 and possibly other transporters; primarily located in the nucleus	0.102
YoL116W/*MSN1*	Transcriptional activator involved in regulation of invertase and glucoamylase expression, invasive growth and pseudohyphal differentiation, iron uptake, chromium accumulation, and response to osmotic stress; localizes to the nucleus	0.109
YNL004W/*HRB1*	Poly (A+) RNA-binding protein, involved in the export of mRNAs from the nucleus to the cytoplasm.	0.166
YJR155W/*AAD10*	Putative aryl-alcohol dehydrogenase with similarity to P. chrysosporium aryl-alcohol dehydrogenase	0.180
YFR008W/*FAR7*	Protein involved in recovery from cell cycle arrest in response to pheromone	0.186
YNL327W/*EGT2*	Glycosylphosphatidylinositol (GPI)-anchored cell wall endoglucanase required for proper cell separation after cytokinesis	0.196
YBL072C/*RPS8A*	Protein component of the small (40S) ribosomal subunit	0.205

Table 4.6 Down-regulation of genes expressing Δ20. Analysis of microarray data revealed nine genes that were down-regulated strains expressing the Ssa1 CTD truncation Δ20 by approximately five-fold or more. Expression of genes was compared to expression in Ssa1. Functions of genes were obtained from the *Saccharomyces* genome database (SGD).

4.11 Discussion

We have identified twenty-five mutants within the Ssa family that impair [*PSI*$^+$] propagation in *S. cerevisiae* (Loovers. *et al.,* 2007). Five of theses mutants were identified in both Ssa1 and Ssa2, further highlighting the importance of the Ssa family in prion propagation. Similarly to previous studies (Jones and Masison, 2003), a majority of the mutations (with the exception of G481D) were located in the ATPase domain. This demonstrates the importance of ATPase regulation of Hsp70 as being an important function for [*PSI*$^+$] propagation.

Hsp70 chaperones are two domain proteins that assist in protein folding. The functioning of Hsp70 is highly dependent on inter-domain communication between the ATPase domain and the PBD. The protein folding activity of the PBD is regulated by the ATPase domain, studies have indicated that inter-domain communication is mediated by a twelve amino acid PBD helix that rests in a grove between lobes IA and IIA of the ATPase domain (Jiang *et al*., 2005). Also when Hsp70 is in a peptide bound state, conformational changes are observed in the ATPase domain (Revington *et al.,* 2005). A subset of the Ssa1 ATPase mutants (A53, G73, R74, A146 and T223) are located in a region that has recently been shown to be important for inter-domain communication in the *E.coli* Hsp70, DnaK (Revington *et al*., 2005). Structural analysis of the G73D mutation reveals that presence of the mutation changes this amino acid from a polar uncharged residue to a polar acidic residue and also there are additional neighbors when mutation is present, suggesting that the mutation may cause a slight conformational change which in turn may affect the inter-domain communication of Hsp70.

Structural analysis of another subset of mutant residues (R259, G287, T295 and A297) revealed that these mutants are located within the IIB region of the ATPase domain. The IIB region of the ATPase domain has been implicated as interacting with the Fes1 homologue, HspBP1, in mammals (Shomura *et al*., 2005). The NEF Fes1 facilitates the release of Ssa1 substrates. Previous studies on the Ssa1 PBD mutant *SSA1-21* showed that overproduction of Fes1inhibits the impairment of [*PSI*$^+$] propagation by *SSA1-21* and depletion of Fes1 (which prolongs substrate binding) impairs [*PSI*$^+$] propagation in both wildtype and *SSA1-21* (Jones *et al*., 2004). This indicates that in order for [*PSI*$^+$] to propagate, a weak interaction with Fes1 is required. Mutations to this region may have an affect on Fes1 interaction with Ssa1, which then may affect [*PSI*$^+$] propagation. Structural analysis of the Hsp70 bovine homologue, Hsc70, revealed that the residue T298 (homologue of T295) forms a hydrogen bond with residues in the Nucleotide exchange factor Sse1. Recent studies have found Sse1 is required for *de novo* [*PSI*$^+$] formation and efficient prion propagation (Kryndushkin *et al.,* 2007; Fan *et al*., 2007). A mutation at the T295 residue may effect binding of Sse1 to Hsp70, as both Sse1 and Hsp70 are crucial for prion propagation, altering this interaction may lead to impaired prion propagation. These results indicate that alterations to the ATPase cycle of Hsp70 in favor towards an ADP bound state appear to impair [*PSI*$^+$] propagation.

Enhanced substrate binding may impair [*PSI*$^+$] propagation in numerous ways. Firstly, prolonged substrate binding may affect the Hsp104 disaggregase function. Generation of yeast prion seeds requires the disaggregating function of Hsp104. It is believed that Hsp104 breaks down Sup35 polymers into prion seed which are then passed on to daughter cells during cell division (Shorter and Lindquist, 2004). If mutations are

making Ssa1 bind more avidly to Sup35 aggregates, this may restrict access of Hsp104 to disaggregate Sup35 polymers. If this was the case, this indicates that Hsp104s disaggregase function for prions differs from its disaggregase function for thermally denatured substrates as ATPase as the mutations did not affect the ability of Hsp104 to refold luciferase post heat shock. Another way that enhanced substrate binding by Hsp70 might impair [*PSI*$^+$] propagation is that it may affect the role of Ssa1 in dismantling polymers from Sup35 aggregates. Ssa1/2 have a stronger interaction with the prion form of Sup35 in comparison to the non-prion form (Bagriantsev *et al.,* 2008). If Ssa1 has prolonged substrate binding, this may affect the Hsp70 function of dismantling aggregates into polymers directly or by an association through Hsp104.

Unlike the PBD mutant Ssa1^{F475S} the ATPase mutants do not appear to have a significant affect on the expression of factors involved in a variety of cellular functions, with the exception of Ssa1 self-regulation. Although abundance of Hsp70 appears similar for ATPase mutants, transcriptional levels of the Ssa1 promoter appear to vary; indicating mutants may increase or decrease the transcription of Ssa1. The G73D mutation was the only ATPase mutation to show a 39^{o}C t.s phenotype, indicating that this mutation may alter Hsp70 function. Overall, mutations in the ATPase domain of Hsp70, that impair [*PSI*$^+$] propagation, do not appear to have a significant affect on a majority of the functions of Hsp70. Although Ssa1^{T295I} may effect the interaction between Ssa1 and the NEF Sse1, there appears to be no significant difference in ATPase activity of Ssa1^{T295I} in comparison to wildtype. The fact that these mutations do not appear to have an effect on Hsp70 function but impair

prion propagation and Hsp70 is a highly conserved protein, may suggest that these residues are potential targets for future treatments.

As we had analyzed the effects of alterations to the ATPase domain and PBD of Hsp70 with respect to prion propagation and Hsp70 function, we decided to investigate the effects of alterations to the CTD. A sixty amino acid truncation of the CTD proved to be detrimental when expressed in both [*PSI*$^+$] and [*psi*$^-$] versions of G402. Expression of twenty and forty amino acid truncations of the Hsp70 CTD exhibited viability in both [*PSI*$^+$] and [*psi*$^-$] strains, indicating that that a region between the 582 and 602 residues is important for Hsp70 function. Expression of Δ20 and Δ40 CTD truncations did not appear to have an effect on [*PSI*$^+$] propagation. Growth rates were severely affected especially in [*psi*$^-$] strain at normal and elevated temperatures. The 37^{o}C t.s phenotype of Δ20 and Δ40 were remediated by growth on 1M sorbitol, further indicating that Ssa1 could potentially be involved in the CWI signaling pathway.

The CTD truncations further highlight the importance of co-chaperones in [*PSI*$^+$] propagation. The regulatory motif EEVD is a highly conserved sequence at the extreme end of the C-terminus (Freeman *et al*., 1995). This motif is involved in the recognition of co-chaperones that contain tetratricopeptide repeats (TPR). In humans this motif interacts with the Hsp90 co-chaperone Hop1 (Scheufler *et al*., 2000) which is the homologue of Hsp90 co-chaperone Sti1 in yeast. Sti1 interacts with Ssa and activates ATPase activity. Cells harboring Δ20 and Δ40 CTD truncations can maintain [*PSI*$^+$] propagation indicating that Sti and Hsp90 are not required for prion maintenance. This is in agreement with previous results that depletion of Fes1

improved [*PSI*$^+$] propagation whereas overexpression weakened it (Jones *et al*., 2004). Although prion propagation and maintenance does not appear to be affected by the CTD truncations, cell growth and Hsp70 function appears to be severely affected. Deletion or mutation of the EEVD motif has an affect on ATPase activity and ability of Hsp70 to interact with substrates (Freeman *et al*., 1995). This may explain why Δ20 and Δ40 CTD truncations are non-functional at 37^{o}C.

The isolation of Ssa1 ATPase mutations and CTD truncations has provided a new insight into the role of Hsp70 and co-chaperones in prion propagation. The ATPase mutations may have implicated alterations to inter-domain communication; Ssa1 NEF's and increased Ssa1 substrate binding as affecting [*PSI*$^+$] propagation. With the exception of G73D, the mutations do not appear to affect other cellular functions, which may make theses regions potential therapeutic targets. The Δ20 and Δ40 CTD truncations confirm that Hsp90 and its co-chaperone, Sti1 are not necessary for [*PSI*$^+$] propagation and the truncations have severe affects on Ssa1 function.

5.0 Introduction

The aim of this chapter is to gain insight into the influence of individually expressing members of the Ssa family, and analyzing their effects on prion propagation and Hsp70 functions. In addition we also propose to analyze the effects of N-terminally fused His (6X) tag on Ssa1 viability, growth rates and affect on prion propagation. We also propose to analyze trends in gene expression in cells solely expressing Ssa1-4, the PBD mutation $Ssa1^{F475S}$ and the CTD truncation Δ20.

Hsp70 constitutes as one of the most conserved protein families, members of this family may be heat inducible or constitutively expressed (Daugaard *et al*., 2007). Hsp70 from different species share functional redundancy for e.g. *Drosophila* Hsp70 can protect mammalian cells from heat stress (Pelham 1984). Yeast contain nine cytosolic Hsp70's, which include the Ssa family, Sse family and three ribosome associated Hsp70's (Ssb1-2 and Ssz). The Ssa sub-family, of cytosolic Hsp70, consists of four members Ssa1-4, which are functionally redundant and abundance of at least one is necessary for growth (Werner-Washburne *et al*., 1987). The constitutively expressed Ssa1/2 are 98% identical, the heat-inducible Ssa3/Ssa4 are 88% identical to one another and are 80% identical to Ssa1/2 (Sharma and Masison, 2008).

Due to the high degree of conservation among Hsp70 family and the fact that the presence of one family member is essential for growth, it was hypothesized that some of these family members may be functionally redundant (Kabani and Martineau, 2008). However, recent evidence concerning prion propagation and biofilm formation, suggests that this is not the case and there is functional difference among Hsp70 orthologs. Altered abundance of Hsp70 appears to affect prion propagation in

different ways. Overexpression of Ssa1 appears not to weaken the [*PSI*$^{+}$] prion but cures the [*URE3*] prion, conversely depleting Ssa1 weakens [*PSI*$^{+}$] but not [*URE3*] (Schwimmer and Masison, 2002; Roberts *et al*., 2004). Interestingly, the Ssa2 appears to have the opposite effect, depletion weakens [*URE3*] but not [*PSI*$^{+}$] (Sharma and Masison, 2008). Hsp70 has been reported to play a role in biofilm formation in yeast (Martineau *et al*., 2007). Deletion of Ssa1 had a more severe affect on biofilm formation than an Ssa2 deletion. The additional deletions of Ssa3 and Ssa4 appeared to enhance the defects caused by the Ssa1/Ssa2 deletions, suggesting that there is cooperation between constitutive and inducible Hsp70's (Martineau *et al*., 2007).

As mentioned previously Ssa1 is involved in prion maintenance, propagation, and many cellular functions such as translation, translocation and protecting cells from stress by preventing aggregation. In order to extensively study the affects of mutations of Ssa1 on these functions, it is necessary to purify the Ssa1 protein to carry out biochemical assays. Fusing a His(6X) to the N-terminal of Ssa1 allows purification of Ssa1 by Metal-Chelate Affinity Chromatography (MCAC), in which metal ions (Nickel) are bound to solid matrices that bind to Histidine residues therefore capturing the protein of interest. As previously observed, fusing a His (6X) tag to Ssa1^{F475S} caused Ssa1 to become non-functional (Figure 3.16). Therefore, we decided to analyze the affects of a N-terminally fused His (6X) tag on wildtype Ssa1.

The main objective of this chapter was to:

- To gain insight into the differences and similarities between yeast strains expressing individual members of the Ssa family
- To decipher the affects of a N-terminally fused His(6x) tag on Ssa1 functions and prion propagation

- To analyze common trends in gene expression in yeast cells expressing Ssa1-4.

5.1 Strategy for Ssa1-4 comparison

In order for us to carry out comparative analysis on the Ssa1-4 proteins, we first had to obtain plasmids expressing each gene under a constitutively active promoter. To achieve this each member of the Ssa family was cloned into a plasmid under the control of the Ssa2 promoter. After failed attempts of cloning Ssa2 and Ssa4, we received plasmids expressing Ssa2 (pDCM64) and Ssa4 (PA4) from the Masison group. Ssa1 was previously cloned into the pRS315 plasmid under the control of the Ssa2 promoter (pC210) by a former member of our lab. The *SSA3* gene (2KB) was amplified from the genomic DNA of the wildtype yeast strain G600, utilizing primers which added thirty base pair overhangs at the 5' and 3' ends of *SSA3* (Figure 5.1(A)) . The overhangs were homologous to sequence at gene incision site of pC210. The pC210 (6KB) plasmid was digested by an *Nde*1 and *Sph*1 restriction digest to remove the *SSA1* (2KB) insert (Figure 5.1(B)) and digested plasmid was isolated by gel extraction (Figure 5.1(c)). The *SSA3* PCR construct and digested pC210 were simultaneously transformed into G600 to allow homologous recombination to occur, potential colonies harboring *SSA3* clones were selected for on SD media lacking Leucine. To confirm homologous recombination was successful, diagnostic *Nde*1 and *Sph*1 restriction digests were preformed on plasmids isolated from colonies that exhibited growth on SD media lacking Leucine. Diagnostic digest (Figure 5.1 (D)) and DNA sequencing confirmed *SSA3* was cloned into pC210.

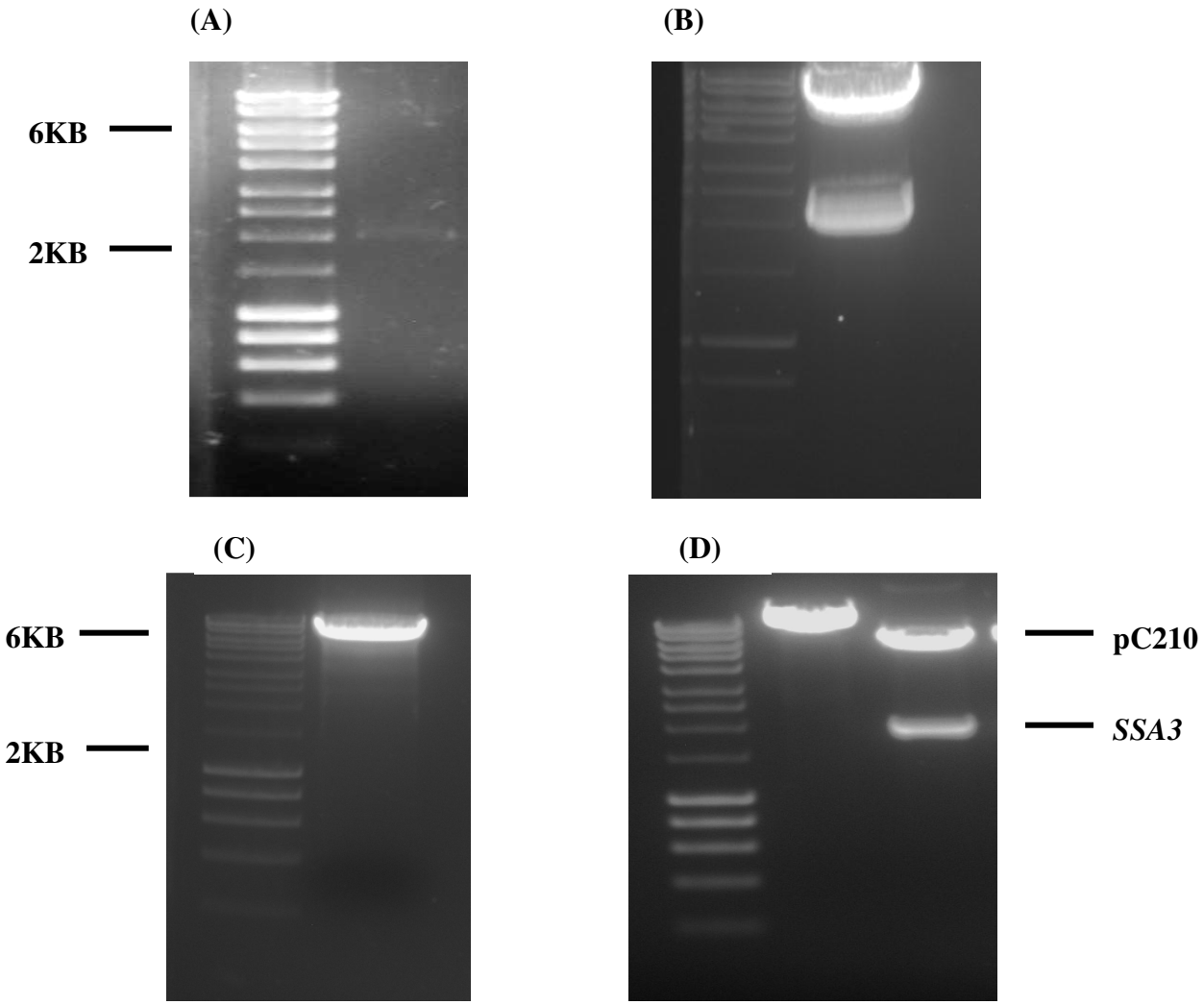


Figure 5.1 Cloning Ssa3 into pC210. All agarose gels made from 0.8% agarose and 1µl of Ethidium Bromide, dissolved in 50ml of 1X TAE buffer. Agarose gels were run at 90V for approximately thirty minutes **(A)** A faint band is observed at 2KB which represents *SSA3* with pC210 overhangs, amplified from G600 genomic DNA. **(B)** *Sph*1 and *Nde*1 restriction digest of pC210, the top band is located around 6KB and represents the digested pC210 vector and the lower band represents *SSA1* which was liberated by restriction digest and is located at 2KB. **(C)** Digested pC210 vector post gel extraction. **(D)** Potential *SSA3* clones were isolated and a *Sph*1 and *Nde*1 restriction digest was carried out. As a control undigested pC210 was run alongside digestion. Diagnostic digest reveals that *SSA3* is present.

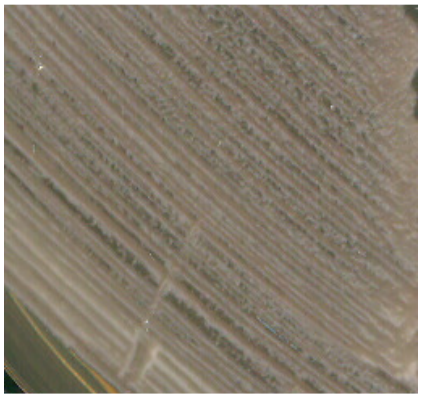
5.1.1 Expression of Ssa family in G402

In order to compare the functions of individual Ssa members, each member must be expressed as the sole source of Ssa in a strain. To achieve this, each plasmid was transformed into a [*PSI*$^+$] version of G402 and potential transformants were selected on SD agar lacking Leucine. Colonies from SD lacking Leucine were replicated onto 5-FOA to eliminate the pRDW10 plasmid and were restreaked onto YPD (Figure 5.2 A-D). All members of the Ssa family individually support growth of G402. In cells expressing Ssa3 the prion was maintained (Figure 5.2C), [*PSI*$^+$] was also maintained in cells expressing Ssa1 and Ssa2 but not to the same degree as Ssa3 (Figure 5.2A-B). All strains were curable by 3mM GdnHCL (Figure 5.2E-F). Cells expressing Ssa4 were unable to propagate the prion (Figure 5.2 D). These results are indicative that prions are affected differently by different Hsp70's, which indicates members of the Ssa family are distinct from one another with respect to prion propagation.

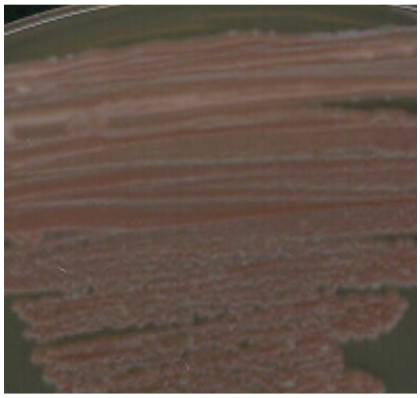
5.1.2 Growth rates of cells expressing Ssa1-4

The Ssa subfamily of Hsp70 is essential for growth in *S.cerevisiae*; we have shown that each member of the Ssa family individually supports growth of *S.cerevisiae* that lacks all four chromosomal *SSA* genes (Figure 5.2 A-D). To assess whether individual Ssa members affected the growth rate of *S.cerevisiae* differently, we analyzed the growth rates of cells expressing Ssa members. Cells expressing the constitutively expressed Ssa1 and Ssa2 grow quicker than the heat inducible Ssa3 and Ssa4 (Table 5.1). Ssa3 appears to have the greatest affect, with the growth rate being reduced by approximately 50% in

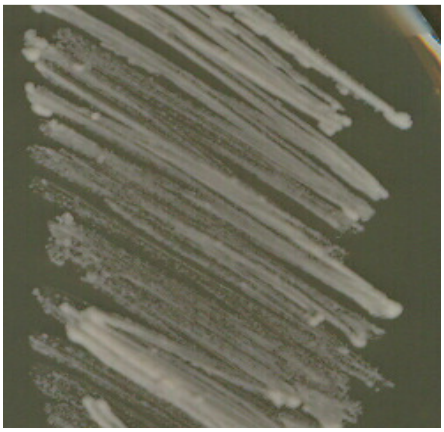
(A)



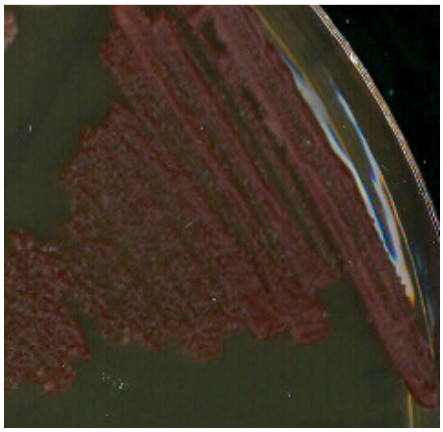
(B)



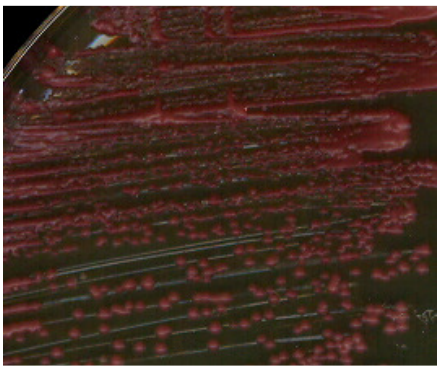
(C)



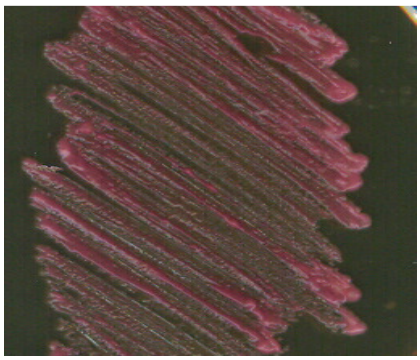
(D)



(E)



(F)



5.2 Expression of Ssa1-4 in G402. Ssa1-4 were transformed into G402 and each was expressed as the sole source of the Ssa family by the plasmid shuffle technique. Cells were streaked from 5-FOA onto YPD and incubated at 30^{o}C for forty-eight hours. Figures A-D show Ssa1-4 respectively. Figures E and F represent cured versions of Ssa1 and Ssa3 respectively. Strains cured by incubating cells on 3mM GdnHCL at 30^{o}C for forty-eight hours.

comparison to Ssa1 (Table 5.1). Previous studies indicate that Ssa4 had the greatest affect on cell growth (Sharma *et al*., 2009). Although our results vary from the previous study, it is clear that the heat inducible members of the Ssa family do not support growth rates of *S.cerevisiae* as efficiently as the constitutively expressed Ssa1 and Ssa2.

Strain	MGT (Mins)	MGT %WT
Ssa1	103	100%
Ssa2	113	109% ±5.6%
Ssa3	156	151% ±9%
Ssa4	128	124% ±4.2%

Table 5.1 MGT of cells expressing individual members of Hsp70 subfamily Ssa. Overnight cultures of cells expressing individual Ssa's were diluted from overnight 30^{o}C cultures to an OD_{600} 0.1. The OD_{600} of cultures was measured every two hours for approximately ten hours (chapter 2.7.1)

5.1.3 Investigation of Ssa1-4 temperature sensitivity

Members of the Hsp70 subfamily Ssa function to protect cells from stress injuries by preventing aggregation of partially unfolded proteins. In order to gain insight on whether individual Ssa's can protect cells at elevated temperatures, we tested cells expressing individual Ssa's at various temperatures. There was no affect on growth of cells expressing individual Ssa members up to 37 ^{o}C. Cells individually expressing Ssa3 and Ssa4 were t.s at 39^{o}C (Figure 5.3). This shows that Ssa3 and Ssa4 are not efficient for protection against stress injuries, when solely expressed in yeast.

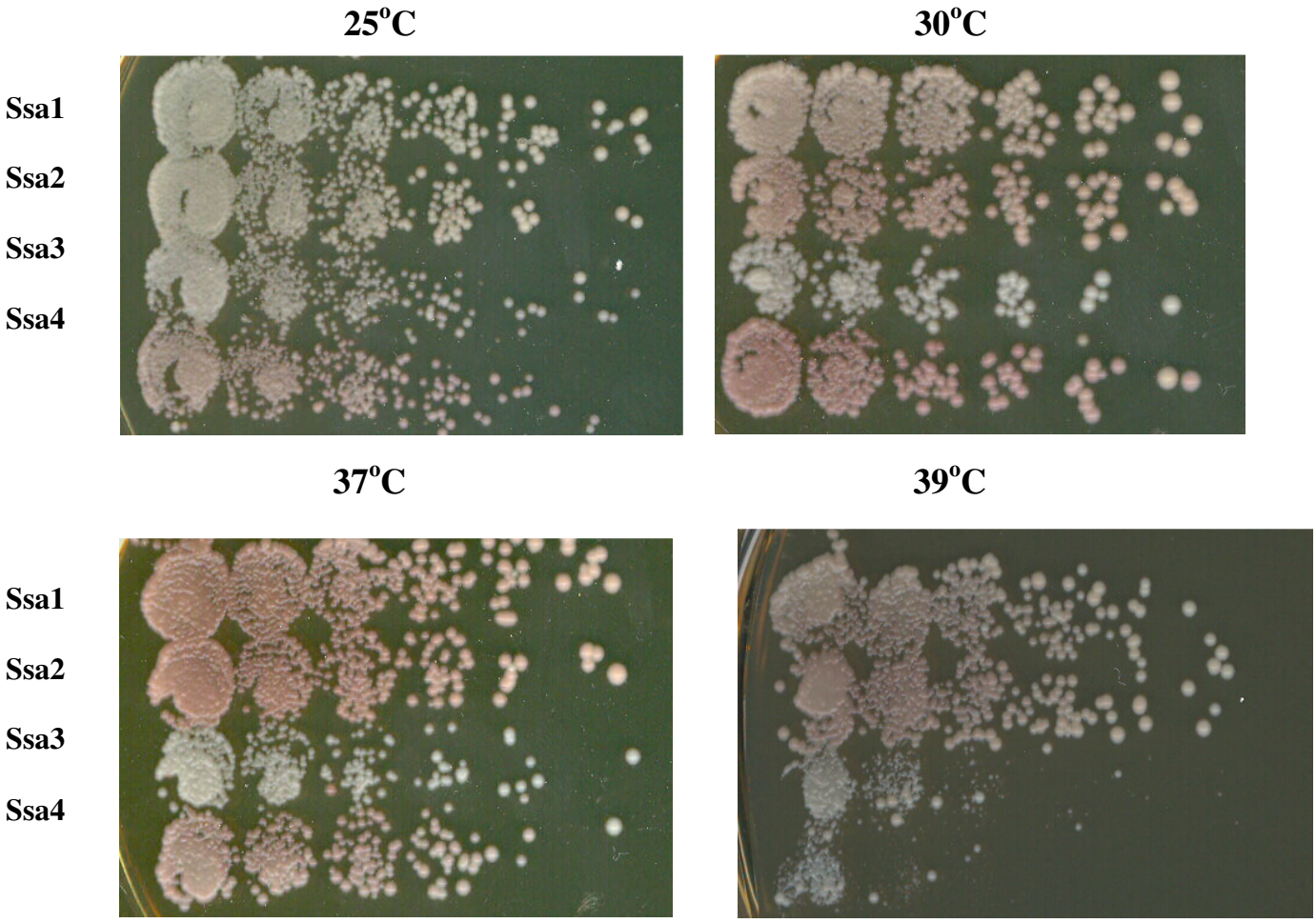


Figure 5.3 Temperature sensitivity of cells expressing Ssa1-4. Cells incubated at specific temperatures for 48 hours on YPD. Cells were diluted by a 1/5 serial dilution. Spots labeled accordingly. Ssa1-4 expressed in G402 as sole source of Ssa in strain.

5.1.4 Recovery of t.s 39°C phenotype by 1M sorbitol

As previously described, the F475S mutation in the PBD of Ssa1, 2 and 4 causes a t.s 37°C phenotype, which is recovered by growth on 1M sorbitol (Figure 3.29). This may implicate the Hsp70 Ssa subfamily in the CWI signaling pathway. As Ssa3 and Ssa4 are both t.s at 39°C we decided to analyze the effects of 1M sorbitol on their growth at 39°C. Growth of both Ssa3 and Ssa4 is rescued by growth on 1M Sorbitol. This is a common phenotype we have observed with Ssa1 t.s mutants ($Ssa1^{F475S}$, $Ssa1^{G73D}$, Δ20 and Δ40). This could quite possibly suggest that Ssa1 is playing either a direct or indirect role in the CWI signaling pathway. It may also be the case that these mutations are causing the Ssa1 protein to become unstable and the sorbitol is somehow making the Ssa1 protein unstable.

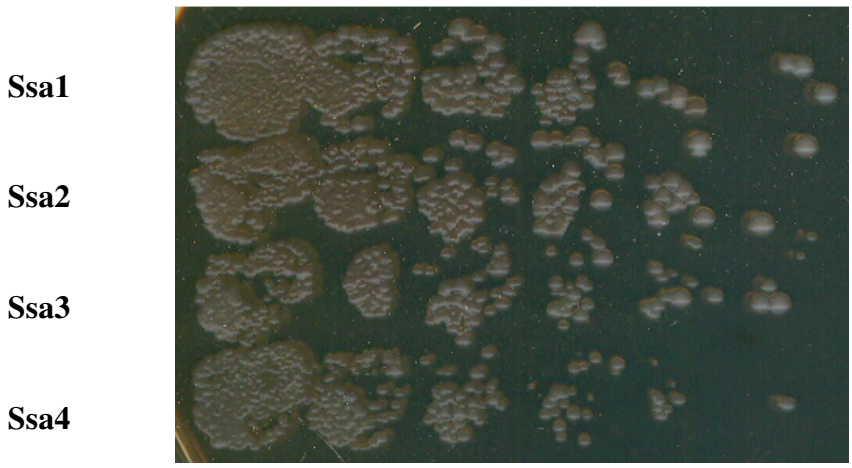


Figure 5.4 Recovery of Ssa3 and Ssa4 by 1M Sorbitol at 39°C. Cells were incubated at 39°C for 48 hours on YPD supplemented with 1M sorbitol . Cells were diluted by a 1/5 serial dilution. Spots labeled accordingly. Ssa1-4 expressed in G402 as sole source of Ssa in strain.

5.1.5 Effect of Ssa1-4 on thermotolerance

In order for Hsp70 to prevent aggregation of partially unfolded proteins, it must cooperate with the molecular chaperones Hsp40 and Hsp104. As Ssa3 and Ssa4 are t.s at 39^{o}C, we decided to analyze the effects of a short lethal heat shock (52^{o}C), on cells individually expressing Ssa1-4. Survival rates of yeast post lethal shocks are improved by pre-treating cells with a non-lethal shock, which stimulates elevated heat shock proteins. To assess the affects of a lethal heat shock on cells individually expressing Ssa1-4, cells were grown at 30^{o}C until a final concentration of 5x10^{6} cells/ml was reached. Cells were then shifted to 37^{o}C for one hour to induce elevated expression of heat shock proteins; 1ml of the culture was then removed and stored on ice. Culture was then shifted to 52^{o}C for thirty minutes, with samples being removed at fifteen and thirty minutes. Cells were then replicated onto YPD and grown at 30^{o}C for forty-eight hours. Apart from Ssa3 colonies appearing slightly smaller, there appears to be no significant difference between members of the Ssa family with regards to thermotolerance, indicating that individual members of Ssa family can cope with lethal heat shock in the presence of elevated levels of chaperones such as Hsp40 and Hsp104.

5.1.6 Protein refolding activity of Ssa family

A major function of Hsp70 is refolding proteins that have become misfolded due to heat stress. The ability of chaperones to refold misfolded proteins can be measured by performing a luciferase assay. The luciferase enzyme is unstable at high temperatures and omits light in presence of its substrate decanal, which can be measured on a luminometer. Cells individually expressing Ssa1-4 were transformed with the pDCM90 plasmid

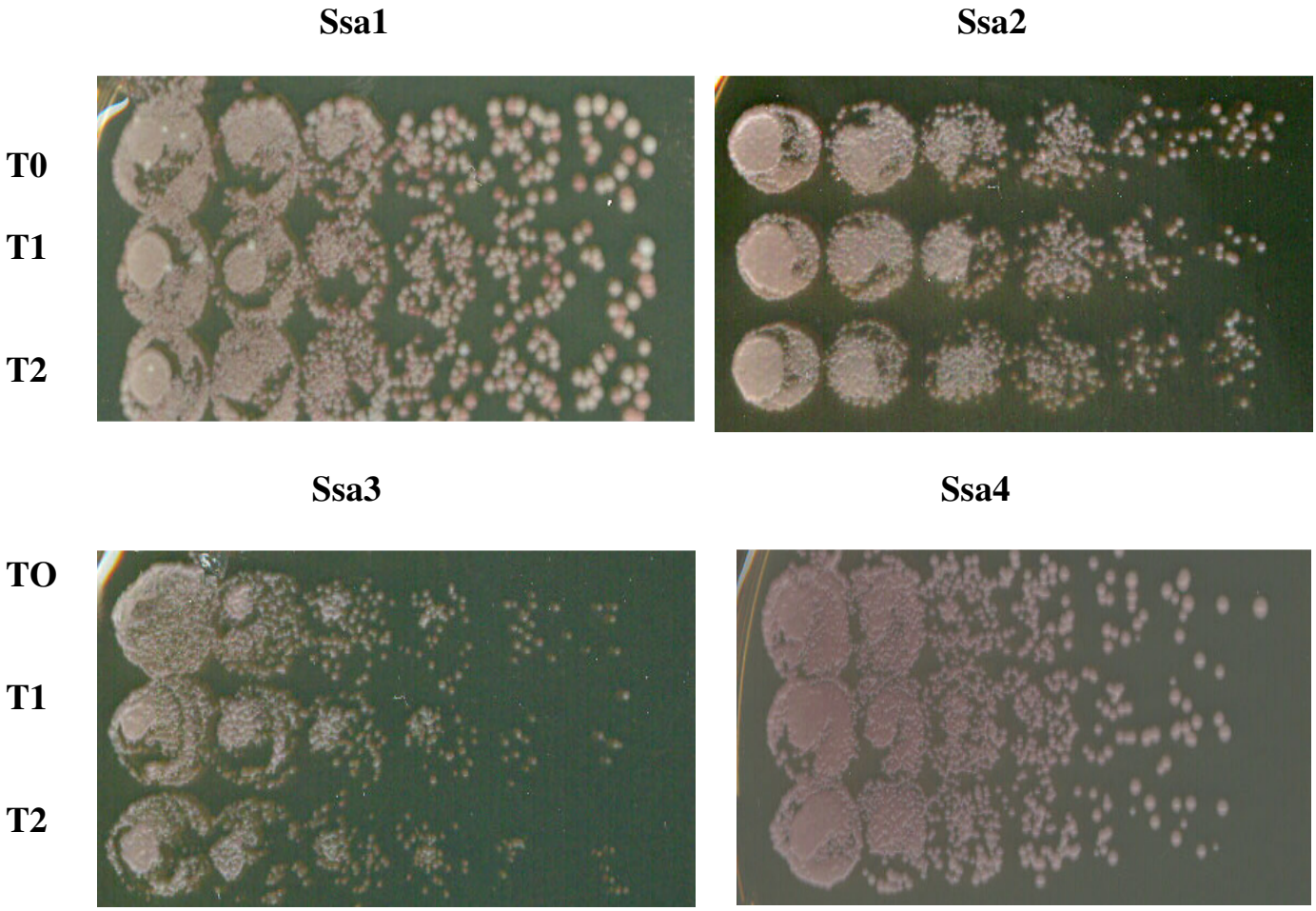


Figure 5.5 Effects of solely expressing Ssa1-4 on thermotolerance in yeast. Cells individually expressing Ssa1-4 were grown overnight at 30°C in YPD. Cells were diluted to an OD_{600} of 0.15 and were grown at 30°C in YPD, until a final concentration of $5x10^6$ cells/ml was reached. Cells were then shifted to 37°C for one hour. Following this one hour incubation, 1ml of cells from each culture was transferred into a sterile 1.5ml tube and stored on ice (T0). The remainder of the culture was shifted to 52°C for thirty minutes, 1ml samples were removed at fifteen minutes (T1) and another 1ml was removed at thirty minutes (T2). Cells were diluted by a 1/5 serial dilution and replicated onto YPD and incubated at 30°C for forty-eight hours. Spots labeled accordingly.

and luciferase assays were carried out as in chapter 3.4. In Ssa1 86% of luciferase activity was restored which agrees with our previous results (Figure 3.7 and 4.5). Ssa2 and Ssa4 have similar luciferase recovery rates at 76% and 70% respectively. Ssa3 has the lowest recovery rate with only 60% of luciferase being recovered; this may explain why colonies appeared smaller for Ssa3 thermotolerance as recovery of unfolded proteins is slower.

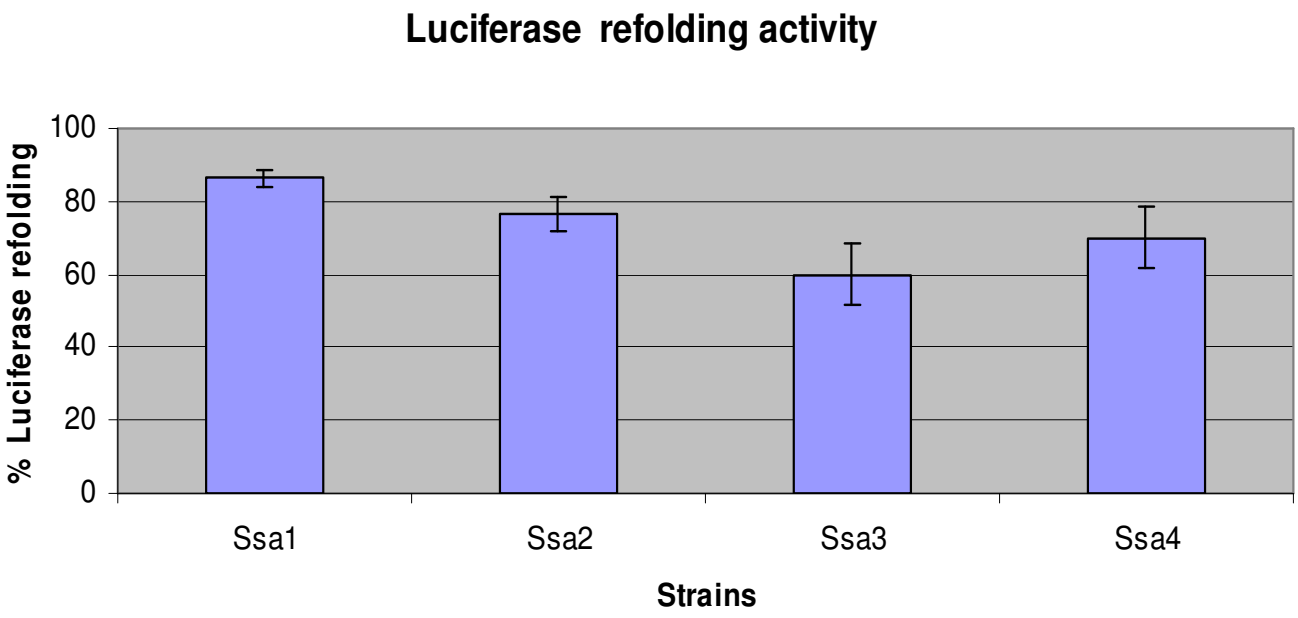


Figure 5.6 Refolding activity of Ssa1-4. Cultures of strains expressing luciferase and Ssa1-Ssa4 were shifted from 30°C to 37°C for 30 minutes and then shifted to 42°C for one hour. Cycloheximide was added 50 minutes after shifting to 42°C to prevent synthesis of luciferase during the recovery period. Luciferase activity, expressed as a percentage of pre-heat shock activity, was measured after allowing cells to recover for 30 minutes at 25°C. Experiment was repeated twice in triplicate.

5.1.7 Basal levels of Hsp70 and Hsp104 in cells expressing individual Ssa's

Abundance of Hsp70 and Hsp104 in a yeast cell may be a contributing factor for impairment of [*PSI*$^+$] propagation. Protein levels for Ssa1-4 were observed by Western blotting. Total protein was extracted from cells and probed with a general Hsp70 (SPA-822), Ssa1/2 (Santa Cruz), Ssa3/4 (60591) and Hsp104 specific antibodies. In addition to analyzing abundance of Hsp70 and Hsp104 in cell, western blot should also confirm each strain is expressing individual members of Ssa family. Abundance of Ssa3 and Ssa4 appears slightly lower in comparison to Ssa1 and Ssa2 when probed with the general Hsp70 antibody. This difference in abundance may be due to Ssa3/4 being only 80% identical to Ssa1/2, therefore the affinity of a general Hsp70 antibody may be weaker in Ssa3/4 if binding epitope is slightly different than Ssa1/2. The Ssa1/2 and Ssa3/4 antibodies show that strains are expressing individual members of the Ssa family and the there appears to be no variation in the amount of Hsp104 being expressed between strains. Strains expressing Ssa3 and Ssa4 appear to have a similar level of Hsp70 yet exhibit opposite phenotypes with respect to [*PSI*$^+$] propagation; cells solely expressing Ssa3 are [*PSI*$^+$] whereas cells solely expressing Ssa4 cannot maintain the prion and are [*psi*$^-$]. This may indicate that Ssa3 and Ssa4 are functionally different with respect to [*PSI*$^+$] propagation.

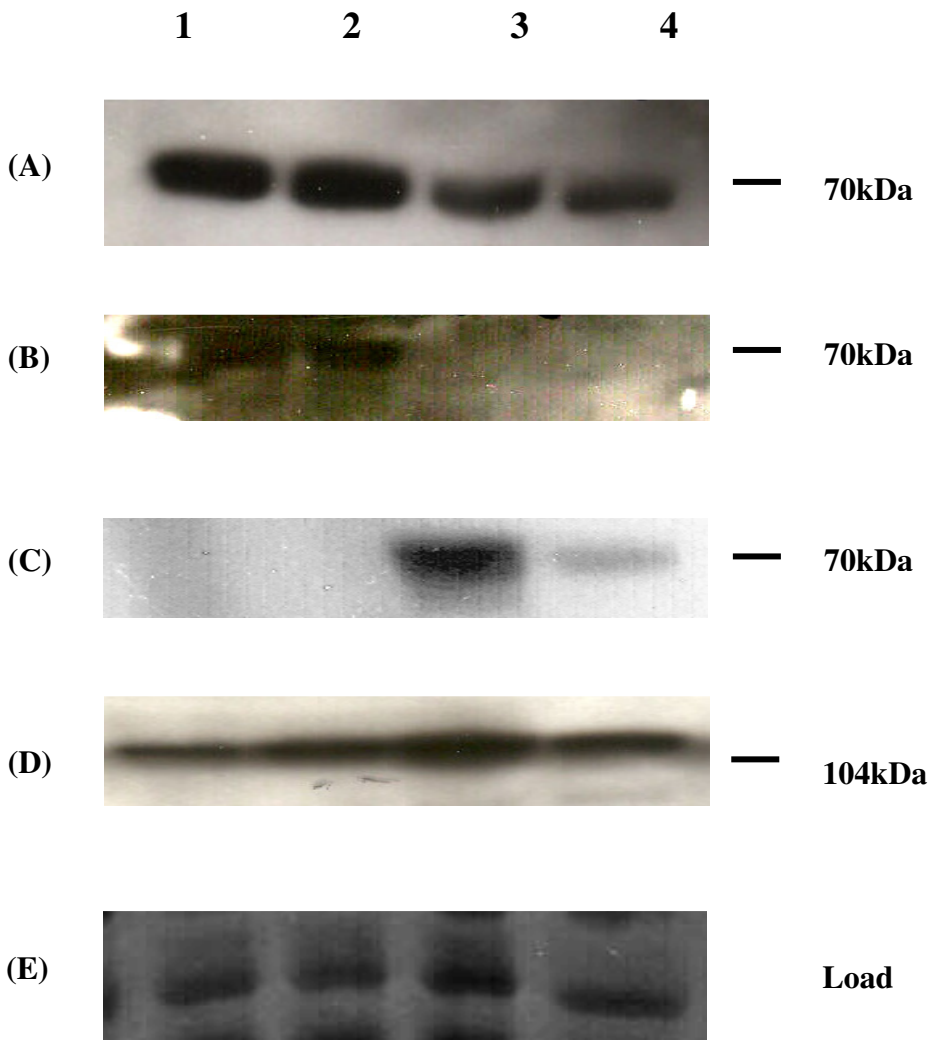


Figure 5.7 Basal levels of Hsp70 and Hsp104. Lysates prepared from overnight 30^{o}C cultures. Aliquots of proteins (10µg) were separated on five polyacrylamide gels, gels were either immunoblotted with antibodies either specific for (A) Hsp70 (SPA-822, 1/2000 dilution) and (B) Ssa1/2 (1/200, Santa Cruz)(C) Ssa3/4 (1/5000, 60591) (D) Hsp104 and (E) Coomassie brilliant blue stained overnight and destained the following day.

5.2 Affect of fusing an N-terminal HIS (6X) tag on Ssa1 *in vivo*

Fusing a His (6X) tag to the N-terminal of a protein of interest proves as a useful tool for protein purification. Our chosen system for purifying Ssa1 was *E.coli.* Assays preformed on purified Ssa1, such as ATPase assays and CD spectra, were preformed *in vitro*. Therefore it was necessary to analyze the affects of fusing a His (6X) tag to the N terminal of Ssa1 *in vivo,* as a His (6X) tag may alter the structure or function of a protein. In order to achieve this we first had to clone the His (6X) Ssa1 construct into a yeast expression vector (pC210). We chose to clone the *SSA1*-His (6X) construct into the pC210 vector by homologous recombination. The *SSA1*- His(6X) gene (2KB) was amplified from the pROEX-HTb plasmid, utilizing primers that added thirty base pair overhangs at the 5' and 3' ends of the gene (Figure 5.8 (A)). The overhangs were homologous to sequence at gene incision site of pC210. The 2kB band which represents the *SSA1*-His(X6) construct was isolated by gel extraction (Figure 5.8 (B)). The pC210 (6KB) plasmid was digested by an *Nde*1 and *Sph*1 restriction digest (Figure 5.8 (C)) to remove the *SSA1* (2KB) insert and digested plasmid was isolated by gel extraction. The *SSA1*-His(X6) *PCR* construct and digested pC210 were simultaneously transformed into G600 to allow homologous recombination to occur, potential colonies harboring *SSA1*-His(X6) clones were selected for on SD media lacking leucine. To confirm homologous recombination was successful, diagnostic *Nde*1 and *Sph*1 restriction digests were preformed on plasmids isolated from colonies that exhibited growth on SD media lacking leucine (Figure 5.8 (D)). Diagnostic digest and DNA sequencing confirmed *SSA1*-His(X6) was cloned into pC210.

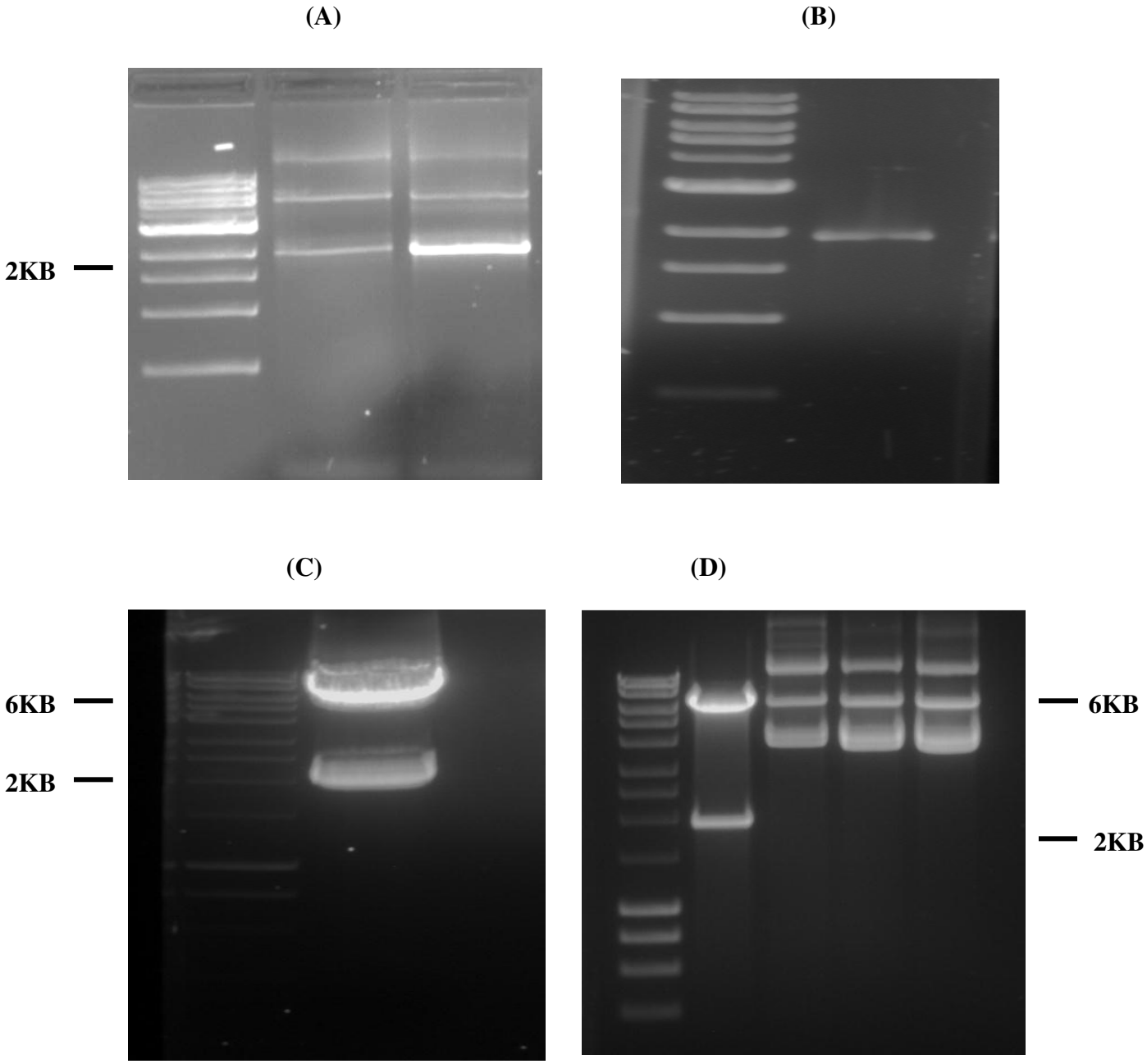


Figure 5.8 Cloning *SSA1*-His(6X) into pC210.All agarose gels made from 0.8% agarose and 1µl of Ethidium Bromide, dissolved in 50ml of 1X TAE buffer. Agarose gels were run at 90V for approximately thirty minutes **(A)** Band at 2KB represents *SSA1*-His(6X) with pC210 overhangs, amplified from the pPROEX-HTb plasmid. **(B)** *SSA1*-His(6X) with pC210 overhangs post gel extraction **(C)** *Sph*1 and *Nde*1 restriction digest of pC210, the top band is located around 6KB and represents the digested empty pC210 vector and the lower band represents *SSA1* which was liberated by restriction digest and is located at 2KB. **(D)** Potential *SSA1*-His(6X) clones were isolated and a *Sph*1 and *Nde*1 restriction digests were carried out. Out of a potential four clones, one was successful. Plasmid was verified by sequencing.

5.2.1 Expression of Ssa1-His (6X) *in vivo*

To assess the affects of a His (6X) tag on the function of Ssa1, the Ssa1-His (6X) plasmid must be expressed as the sole source of the Ssa family in yeast. To achieve this we transformed the plasmid into [*PSI*$^+$] and [*psi*$^-$] versions of G402 and selected for colonies on SD media lacking Leucine. Potential transformants were restreaked onto SD media lacking Leucine and replicated onto 5-FOA to eliminate the pRDW10 plasmid. Ssa1-His (6X) is functional in both [*PSI*$^+$] and [*psi*$^-$] strains (Figure 5.9-A). When Ssa1-His(6X) is expressed as the sole source of Ssa, the [*PSI*$^+$] prion appears to be maintained more efficiently than when wildtype Ssa is expressed.

We previously observed that His (6X) tags in combination with mutations in the PBD of Ssa1 such as F475S (Figure 5.9-B) and L483W (G. Jones personal communication), result in a non-functional Ssa1. Furthermore fusing a His (6X) tag to the N-terminal of Ssa1 containing Δ20, Δ40 and Δ60 deletions also results in non-functional Ssa1 (Figure 5.9-C). Interestingly, mutations in the ATPase domain such as G73D and T295I in combination with a His (6X) tag appear not to affect the function of Ssa1 (Figure 5.9-B). This may indicate that the N-terminal region of Ssa1 is important for the functioning of the PBD and CTD of Ssa1, whereas alterations to the N-terminus may not affect the functioning of the ATPase domain.

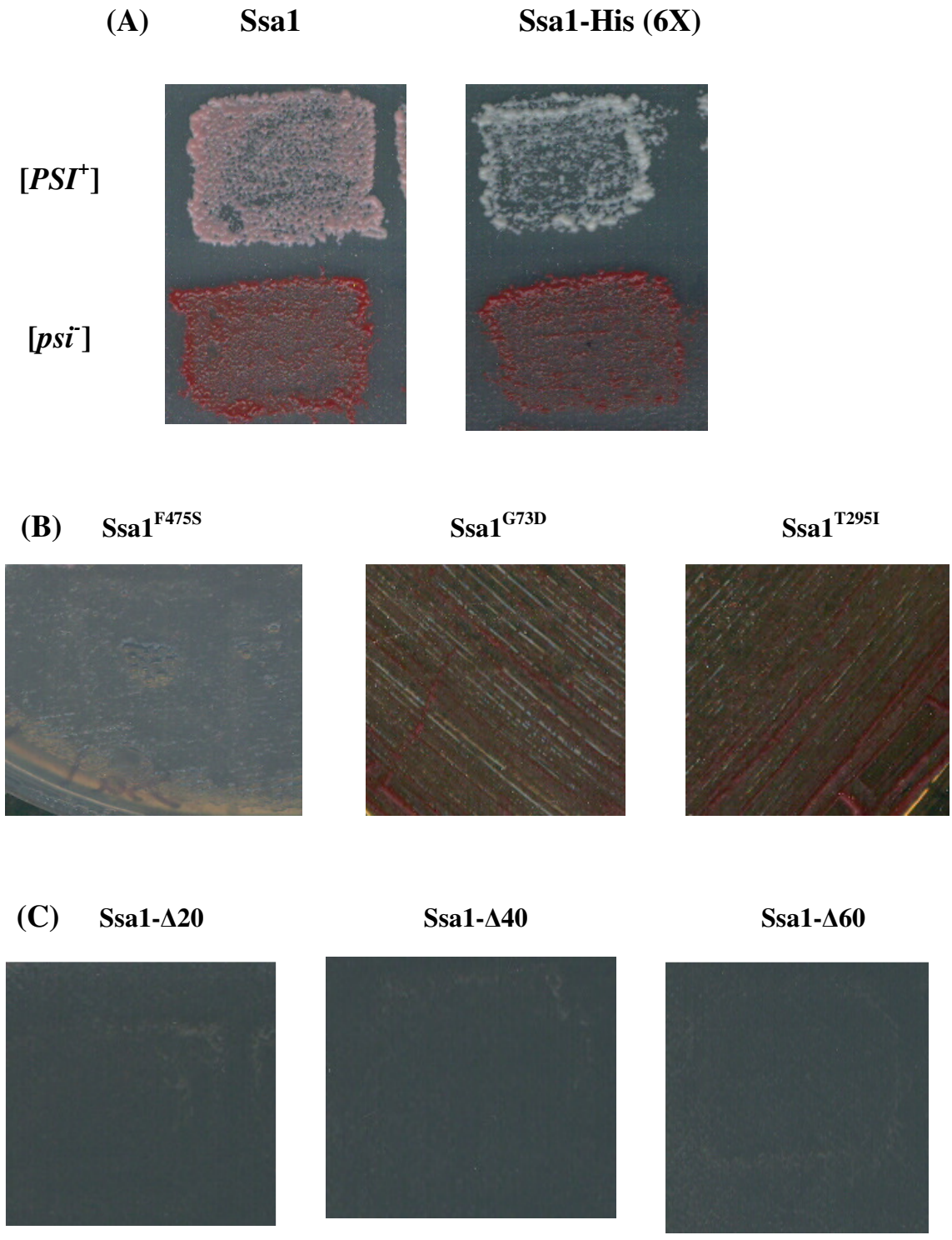
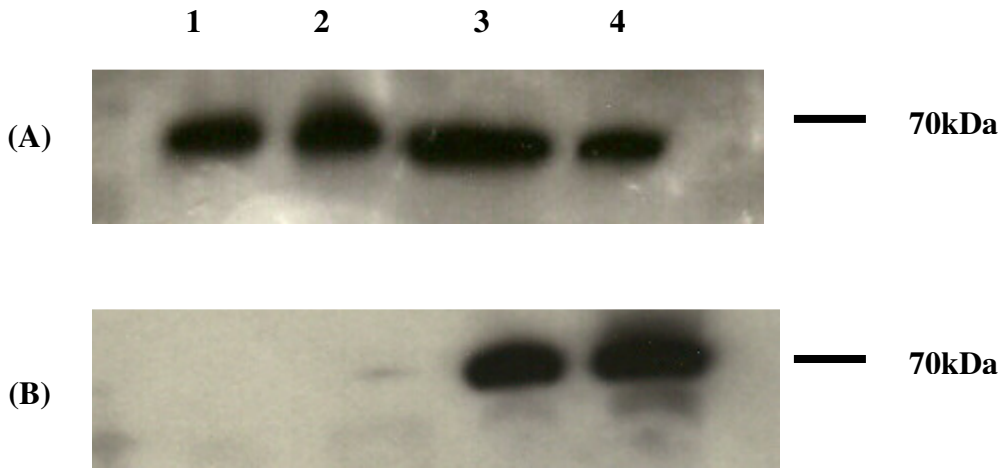


Figure 5.9 Effects of Ssa1-His(6X) tag on yeast viability. (A) [*PSI*$^+$] and [*psi*$^-$] versions of G402 were transformed with Ssa1and Ssa1-His(6X). Potential transformants were selected on SD lacking Leucine and the replicated onto 5-FOA and grown at 30^{o}C for 48 hours. **(B)** PBD and ATPase mutations inserted into Ssa1-His(6X) by SDM **(C)** His(6X) fused Δ20, 40 and 60 CTD truncations expressed as sole source of Ssa in G402.

5.2.2 Confirmation of Ssa1-His (6X) expression

To further confirm the Ssa1 being expressed has a fused His (6X) tag, we analyzed total protein from cells expressing Ssa1 and Ssa1-His (6X) by Western blot analysis. Total protein was extracted from cells and probed with antibodies specific for either Hsp70 or His (6X). Western blot analysis shows a signal for cells expressing the Ssa1-His (6X) construct but no signal was detected for wildtype when probed with anti-His (6X) antibody. This confirms that the His (6X) is fused to the N-terminal of Ssa1.



5.10 Confirmation of His(6X) tag. Total protein from Ssa1 and Ssa1-His(6X) [*PSI*$^+$] and [*psi*$^-$] strains was separated by SDS-page. Gels were immunoblotted with antibodies specific for **(A)** Hsp70 (SPA-822, 1/2000 dilution) and **(B)** His6x tag (anti His-santa cruz 1/200 dilution). Lanes 1-4 represent Ssa1 [*PSI*$^+$], Ssa1 [*psi*$^-$], Ssa1-His(6X) [*PSI*$^+$] and Ssa1-His(6X) [*psi*$^-$] respectively.

5.2.3 Investigation of Ssa1-His (6X) growth rates

The Hsp70 Ssa subfamily is essential for cell growth; we have already observed that expressing individual Ssa proteins under the control of the *SSA2* promoter in the yeast strain G402 can support cell growth (Figure 5.2). We have observed that Ssa1-His (6x) can also support cell growth when being expressed as the sole source of Ssa. To gain insight on whether the His (6X) tag is affecting the growth of yeast cells we analyzed the growth rates of cells expressing Ssa1-his (6X) in [*PSI*$^+$] and [*psi*$^-$] cells. In [*PSI*$^+$] and [*psi*$^-$] cells expressing Ssa1-His (6X) the growth rate appears to be reduced by 22% and 39% respectively. This indicates that the addition of a His-(6X) tag to the N-terminus of Ssa1 has an affect on the growth rate of G402.

Strain	MGT (mins)	MGT %WT
Ssa1 Ψ^+	104	100%
Ssa1 Ψ^-	117	112% ±2.12%
Ssa1-His(6X) Ψ^+	133	128% ±11.31%
Ssa1-His(6X) Ψ^-	145	139% ± 12%

Table 5.2 Growth rates of cells expressing Ssa1 and Ssa1-His(6X). Overnight cultures of cells expressing Ssa1 and Ssa-His(6X) were diluted from overnight 30^{o}C cultures to an OD_{600} 0.1. The OD_{600} of cultures was, measured every two hours for approximately ten hours (chapter 2.7.1). MGT expressed as a percentage of wildtype.

5.2.4 Affect of His (6X) tag on Ssa1 function

Hsp70 functions as a heat shock protein, refolding misfolded or aggregated proteins at elevated temperatures. As previously observed fusing of a His (6X) tag to the N-terminus of Ssa1 appears to have a larger affect on the PBD of Ssa1 in the presence of mutations (Figure 5.9). This may indicate the N-terminally fused His (6X) tag of Ssa1 is having a specific affect on the PBD and may affect the ability of Ssa1 to rescue proteins at elevated temperatures. To analyze this we observed growth of cells solely expressing Ssa1 and Ssa1 His (6X) at 37°C. Both [*PSI*$^+$] and [*psi*$^-$] cells expressing Ssa1-His (6X) were capable of growth at 37 °C, indicating that Ssa1 is still functional despite the presence of an N-terminal His (6X) tag.

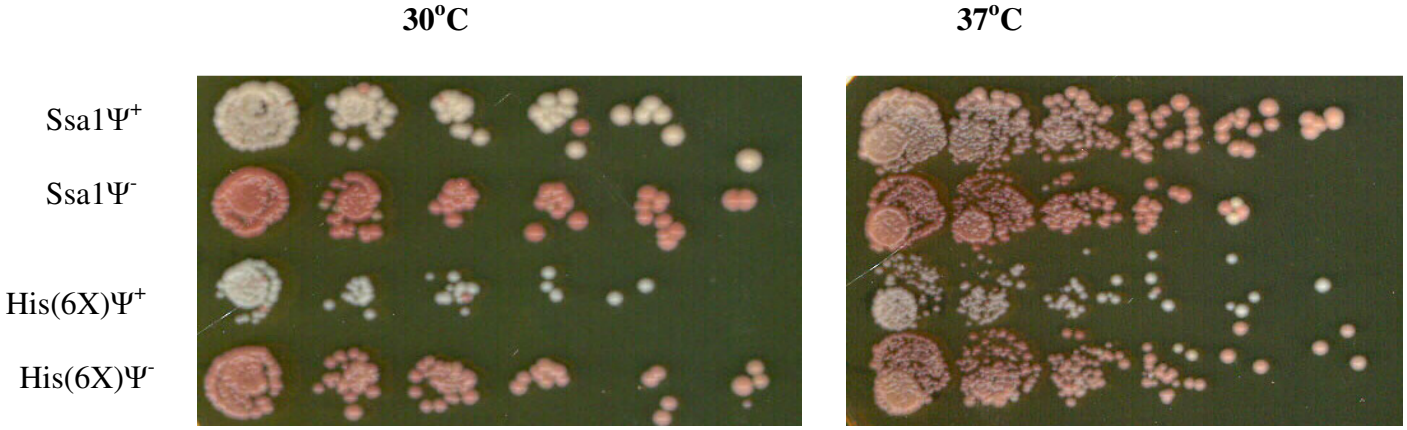


Figure 5.11 Temperature sensitivity of cells expressing Ssa1 and Ssa1-His(6X). Cells incubated at specific temperatures for 48 hours on YPD. Cells were diluted by a 1/5 serial dilution. Spots labeled accordingly. Ssa1 and Ssa1-His(6X) expressed in [*PSI*$^+$] and [*psi*$^-$] G402 as sole source of Ssa in strain.

5.3 Microarray analysis Ssa1-4

We have observed that individually expressing the Hsp70 subfamily Ssa1-4 in *S. cerevisiae,* can lead to different phenotypes with regards to prion propagation (Figure 5.2) and cell growth (Figure 5.3) at elevated temperatures. It appears that each member of the Ssa family may functionally differ. To obtain a more complete view of functional relationships between the individual Ssa members we evaluated gene expression in cells expressing Ssa1-4, each as the sole source of Ssa in the cell by microarray analysis. Previous studies saw microarray analysis carried out on a Δ*ssa1/2* strain to investigate the genomic response at an mRNA to heat shock (Matsumoto *et al*., 2005). Analysis revealed that genes involved in stress response, including chaperones, were up-regulated similarly in Δ*ssa1/2* and wildtype strains (Matsumoto *et al*., 2005). Up-regulation of genes involved in protein synthesis was observed in Δ*ssa1/2* but were suppressed in wildtype, also up-regulated in Δ*ssa1/2* were genes involved in ubiquitin-proteosome protein degradation, whereas genes encoding elements of the unfolded protein response were highly expressed in heat shocked wild-type (Matsumoto *et al*. 2005). These results suggest that recovery of denatured proteins in Δ*ssa1/2* differs from wild-type.

To gain further insight into the functional relationships between Ssa1-4, we compared the up and down-regulation of genes in [*psi*$^{-}$] strains expressing Ssa2, 3 and 4. Strains expressing Ssa1 was used as a control to compare gene expression levels. RNA was extracted from individual strains and sent to Toray industries, Japan, for microarray analysis.

5.3.1 Microarray analysis of Ssa2-4

We carried out analysis on microarray data by comparing genes that were up or down regulated in Ssa2-4 in comparison to Ssa1. To test the accuracy of microarray data we initially analyzed the expression of Ssa2-4 in each strain, for example, *SSA2* expression in the Ssa2 strain should be up-regulated in comparison to Ssa1, whereas Ssa2 expression should be down- regulated in strains expressing *SSA3* and *SSA4*. Strains expressing Ssa2 and Ssa4 exhibit high levels of respective genes expressed in comparison to Ssa1 (Table 5.3). Unexpectedly the Ssa3 strain did not exhibit high levels of Ssa3 in comparison to Ssa1. As we have already confirmed Ssa3 is expressed in the strain (Figure 5.7-C), this result may be due to the high homology between Ssa3 and Ssa1 (79%) (Sharma *et al*., 2009), as 70 mer probes were used in microarray analysis it is conceivable that the *SSA3* probe could bind to *SSA1* and give this inaccurate measurement. This analysis is currently being repeated to confirm results.

Strain	*SSA2*	*SSA3*	*SSA4*
Ssa2	48.166	0.278	0.279
Ssa3	0.958	**0.653**	0.966
Ssa4	1.339	1.291	124.937

Table 5.3 Accuracy of microarray analysis. Gene expression levels in Ssa2, Ssa3 and Ssa4 were compared to the Ssa1 control by microarray analysis. Gene levels expressed as a ratio of Ssa1 gene levels, e.g. the reading for *SSA4* from Ssa1 strain was 126.8 and 15838.6 from the Ssa4 strain, therefore *SSA4* is expressed 124.937 times higher in Ssa4 strain.

To analyze the microarray data, we chose genes that were up or down-regulated by two fold or more in Ssa2-4 strains in comparison to Ssa1. There are eleven genes common to Ssa2-4 strains that were up-regulated by greater than three fold (Table 5.4). The gene with the highest up-regulation in the three strains is *LCB4*. The Lcb4 protein is involved in the production of sphingolipids in *S.cerevisiae.* Sphingolipids are essential components of the plasma membrane in all eukaryotic cells. *S. cerevisiae* cells make three complex sphingolipids: inositol-phosphoceramide (IPC), mannose-inositol-phosphoceramide (MIPC), and mannose-(inositol phosphate) 2-ceramide (M(IP)2C). Lcb4 acts as a sphingoid long-chain base kinase, which is responsible for synthesis of long-chain base phosphates, which function as signaling molecules that regulate the synthesis of ceramide (Nagiec *et al*. 1998, Funato *et al*., 2003). Interestingly, sphingolipids have been implicated as playing a role in the heat stress response (Jenkins *et al*., 1997). It is believed that ceramide and other sphingolipid metabolites accumulate during heat stress and signal yeast cells to activate transcription of the *TBS2* gene and accumulate trehalose (Dickson *et al*., 1997). Along with the production of Hsp's, yeast cells have many ways of coping with heat stress, another protective mechanism in *S. cerevisiae* is the accumulation of the disaccharide trehalose, which occurs rapidly following a shift from 25 to 37°C (Trehalose is known to protect proteins and biological membranes from heat denaturation) (Hottiger *et al*., 1994). Previous microarray analysis carried out on heat shocked wildtype and a strain unable to produce sphingolipids, revealed approximately seventy genes that showed differential regulation during first fifteen minutes of heat shock in strain unable to produce sphingolipids (Cowart *et al*., 2003). These genes fell into the groups involved in stress sphingolipids (Cowart *et al*., 2003). These genes fell into the groups involved

ORF/Gene	Gene Function	Ssa2	Ssa3	Ssa4
YOR171C/*LCB4*	Sphingoid long-chain base kinase	8.2	8.4	9.2
YOR064C/*YNG1*	Subunit of the NuA3 histone acetyltransferase complex	6.4	5.5	5.9
YBR120C/*CBP6*	Mitochondrial translational activator of the COB mRNA; phosphorylated	5.3	3.8	4.2
YBR179C/Unknown	Unknown function-not essential	5.0	4.0	3.7
YAL028W/*FRT2*	Tail-anchored endoplasmic reticulum membrane protein, interacts with homolog Frt1p but is not a substrate of calcineurin (unlike Frt1p), promotes growth in conditions of high Na+, alkaline pH, or cell wall stress	4.1	5.1	3.8
YOL105C/*WSC3*	Partially redundant sensor-transducer of the stress activated PKC1-MPK1 signaling pathway	3.9	5.2	5.1
YKL195W/*MIA40*	Essential protein of the mitochondrial intermembrane space	3.7	6.5	6.1
YDR335W/*MSN5*	Karyopherin involved in nuclear import and export of proteins	3.5	3.3	3.6
YMR078C/*CTF18*	Required for sister chromatid cohesion	3.1	3.8	5.0
YDR202C/*RAV2*	Subunit of RAVE complex which promotes assembly and reassembly of the holoenzyme	3.1	2.9	3.1
YPL015C/*HST2*	Cytoplasmic member of the silencing information regulator 2 (Sir2) family of NAD(+)-dependent protein deacetylases	3.0	2.3	2.2

Table 5.4 Up-regulation of genes common in Ssa2-4 strains. Analysis of microarray data revealed eleven genes that are commonly up-regulated in strains expressing Ssa2, Ssa3 or Ssa4. Expression of genes in each strain was compared to expression in Ssa1. Functions of genes were obtained from the *Saccharomyces* genome database (SGD).

in stress response and cell cycle regulation. This is indicative that sphingolipid production broadly influences the expression of genes involved in diverse cellular pathways. However, previous results demonstrated that sphingoid bases do not induce the expression of Hsp70, Hsp90 or Hsp104 (Dickson *et al*. 1997). However, more recent microarray analysis revealed that several stress response genes are dependent

on sphingolipid generation for their induction, including the Hsp70 member, Ssa4 (Cowart *et al*., 2003). The up-regulation of *LCB4* in strains expressing Ssa2, Ssa3 and Ssa4 may indicate that in the absence of Ssa1 or other Ssa members, *S.cerevisiae* utilizes other cellular components such as sphingolipids to deal with heat stress.

Interestingly, genes involved in cell wall stress appear to be up- regulated in Ssa2, Ssa3 and Ssa4 in comparison to Ssa1. *FRT2* and *WSC3* are up-regulated by more than three fold in each strain. *FRT2* is an endoplasmic reticulum membrane protein that promotes growth in conditions of high Na^+, alkaline PH or cell wall stress. The *WSC3* gene is part of the *WSC* family in yeast, which has functions in maintenance of cell wall integrity and the stress response (Verna *et al*., 1997). It is thought that the *WSC* gene family may play a role in the Pkc1-Mpk1 pathway in yeast (Verna *et al*., 1997). This result may indicate that in the absence of Ssa1 or other Ssa family members, other components involved in response to cell wall stress are up-regulated within the cell. This may further implicate Ssa as playing a role in the cell wall integrity signaling pathway.

There is only one ORF that is commonly down regulated in Ssa2, Ssa3 and Ssa4, YOR387C, which is uncharacterized. It is known that it is regulated by the metal-responsive Aft1p transcription factor and is highly inducible in zinc-depleted conditions (Rutherford *et al*., 2003; Higgins *et al*., 2003).

5.3.2 Microarray analysis of Ssa3-4

We found a wide variety of genes up-regulated in Ssa2-4 in comparison to Ssa1, as Ssa3 and Ssa4 are 88% identical we analyzed genes that were commonly up-regulated in both in comparison to Ssa1. Interestingly, a majority of up-regulated genes appear to be involved in the yeast cell cycle (Table 5.5). Proliferation of cells is mediated

through the cell-division cycle. The cell division cycle consists of four stages; genome duplication (S phase) and nuclear division (M phase) which have intermediate gap phases G1 and G2. Periodic gene transcription regulates cell cycle phases. Many of the genes up-regulated in Ssa3 and Ssa4 (*NRM1, PCL1, YOX1, CLB2, CLN1, CLN2,* and *ACE2),* encode components involved in regulating the cell cycle (Table 5.5). The transcription control of G1/S phase is the most widely studied phase of the cell cycle. At the end of the G1 phase, cells decide whether to commit to cell division. DNA replication is initiated by increased transcript levels of several genes during late G1. In *S.cerevisiae* two transcription factor (TF) complexes activate expression of genes, the MBF TF complex is necessary for sufficient cell cycle regulated transcription (Lowndes *et al*.,1991) whereas the SBF TF complex is necessary to promote G1/S specific transcription in heterologous genes (Andrews and Herskowitz, 1989). The proteins Cln1, Cln2 and Clb2 are involved in regulating expression of both MBF and SBF TF complexes (Bähler 2005). Also up-regulated in Ssa3 and Ssa4 is the Ace2 transcription factor, this is involved in regulating the M/G1 phase in conjunction with Swi5 and specifically is involved in activating a number of genes with roles in cell separation (Dohrmann *et al*., 1992; Bidlingmaier *et al*., 2001). During the G1/S phase the SBF TF complex binds to promoters of several transcription factors, one of which is Yox1, which may play a role in regulating the periodic expression of genes during the G1/S phase that may be involved in expression of genes for proteolysis, spindle pole duplication, and DNA synthesis (Horak *et al*., 2002). The Nrm1 TF is also involved in the G1/S phase of the cell cycle,

ORF/Gene	Gene Function	Ssa3	Ssa4
YNL300W/*TOS6*	Glycosylphosphatidylinositol-dependent cell wall protein	11.3	3.9
YNR009W/*NRM1*	Transcriptional co-repressor of MBF (MCB binding factor)	5.9	3.2
YNL289W/*PCL1*	Cyclin, interacts with cyclin-dependent kinase Pho85p; member of the Pcl1,2-like subfamily, involved in the regulation of polarized growth and morphogenesis and progression through the cell cycle	5.8	4.4
YML027W/*YOX1*	Homeodomain-containing transcriptional repressor, binds to Mcm1p and to early cell cycle boxes (ECBs) in the promoters of cell cycle-regulated genes expressed in M/G1 phase; expression is cell cycle-regulated	5.6	2.4
YDL227C/*HO*	Site specific endonuclease required for gene conversion at MAT locus	5.4	1.8
YPR119W/*CLB2*	B-type cyclin involved in cell cycle progression	4.9	2.0
YMR199W/*CLN1*	G1 cyclin involved in regulation of the cell cycle	4.1	3.1
YPL256C/*CLN2*	G1 cyclin involved in regulation of the cell cycle	3.8	4.9
YDR309C/*GIC2*	Protein of unknown function involved in initiation of budding and cellular polarization	3.8	2.7
YBR136W/*MEC1*	Genome integrity checkpoint protein and PI kinase superfamily member	3.6	2.7
YGR108W/*CLB1*	B-type cyclin involved in cell cycle progression;	3.3	1.0
YBR088C/*POL30*	Proliferating cell nuclear antigen (PCNA), functions as the sliding clamp for DNA polymerase delta; may function as a docking site for other proteins required for mitotic and meiotic chromosomal DNA replication and for DNA repair	3.2	2.7
YLR131C/*ACE2*	Transcription factor that activates expression of early G1-specific genes	3.1	1.6
YBR162C/*TOS1*	Covalently-bound cell wall protein of unknown function; identified as a cell cycle regulated SBF target gene	3.1	1.5
YER162C/*RNR1*	Major isoform of the large subunit of ribonucleotide-diphosphate reductase; the RNR complex catalyzes rate-limiting step in dNTP synthesis, regulated by DNA replication and DNA damage checkpoint pathways via localization of small subunits	3.0	2.0

Table 5.5 Up-regulation of genes common in Ssa3 and Ssa4 strains. Analysis of microarray data revealed eleven genes that are commonly up-regulated in strains expressing Ssa3 or Ssa4. Functions of genes were obtained from the *Saccharomyces* genome database (SGD).

it acts as a MBF specific co-repressor and is required for repression of MBF regulated transcription during exit from G1 phase (de Bruin *et al.*, 2004). Increased expression of components involved in regulation of the cell cycle in *S.cerevisiae* in cells

individually expressing Ssa3 and Ssa4, suggests that the expression of components that regulate the cell cycle require the presence of Ssa1 or a combination of other Ssa members in order to have regular expression. These results may also indicate why growth rates of cells individually expressing Ssa3 or Ssa4 have a reduced growth rate in comparison to cells expressing Ssa1 (Table 5.1).

5.3.3 Up-regulation of genes involved in metal homeostasis

In addition to the up-regulation of genes involved in the cell cycle, we also observed that that there was an up-regulation of genes involved in metal homeostasis common to strains expressing Ssa3 or Ssa4 (Table 5.6). Genes involved in Copper and Iron homeostasis appear to be most prominently up-regulated in strains solely expressing Ssa3 or Ssa4. In yeast, Copper is a necessary cofactor for several enzymes, however

ORF/Gene Name	Gene function	*SSA3*	*SSA4*
YPR124W/ *CTR1*	High-affinity copper transporter of the plasma membrane, mediates nearly all copper uptake under low copper conditions; transcriptionally induced at low copper levels and degraded at high copper levels.	2.810	2.254
YDL198C/ *GGC1*	Mitochondrial GTP/GDP transporter, essential for mitochondrial genome maintenance; has a role in mitochondrial iron transport.	5.356	3.581
YJL217W/ *REE1*	Cytoplasmic protein involved in the regulation of enolase (ENO1); mRNA expression is induced by calcium shortage, copper deficiency (via Mac1) and the presence of galactose (via Gal4).	4.890	1.932
YFR055W/ *IRC7*	Putative cystathionine beta-lyase; involved in copper ion homeostasis and sulfur metabolism	3.400	2.952
YOL152W/ *FRE7*	Putative ferric reductase with similarity to Fre2; expression induced by low copper levels.	4.944	2.905

Table 5.6 Up-regulation of genes involved in metal homeostasis. Analysis of microarray data revealed five genes that are commonly up-regulated in strains expressing Ssa3 or Ssa4. Genes are all involved in metal homeostasis. Expression of genes in each strain was compared to expression in Ssa1. Functions of genes were obtained from the *Saccharomyces* genome database (SGD).

excess accumulation of copper results in hydroxyl radicals that can damage the cell at the level of nucleic acids, proteins and lipids (Halliwell and Gutteridge, 1984). Therefore, cells have developed homeostatic mechanisms to control intracellular accumulation and distribution of copper (Askwith and Kaplan, 1998; Puig and Thiele, 2002). In *S. cerevisiae*, copper ions regulate gene expression through the two transcriptional activators, Ace1 and Mac1. Mac1 activates the expression of genes such as *CTR1, REE1* and *FRE7* (which are both up-regulated in Ssa3 and Ssa4 strains), under conditions of low copper levels. In yeast, the proteins Ctr1 and Ctr3 are transporters that regulate the import of copper. Ctr1 is specifically responsible for uptake of copper in low copper environments (Yonkovich *et al*., 2002). Expression of *CTR1* is increased by more that two fold in both the Ssa3 and Ssa4 strain. Expression of *FRE7* is also induced by the transcription factor Mac1 during periods of low Copper levels. Fre7 acts as a copper reductase (Martins *et al*., 1998).

Iron is also an important nutrient for yeast, deficiency of Iron would have an affect on synthesis of ATP and DNA. In excess Iron is toxic, therefore its intracellular levels are tightly controlled. Normal Iron homeostasis relies on the presence of Copper. Two genes, *FRE7* and *GGC1,* involved in Iron homeostasis are up-regulated in strains expressing Ssa or Ssa4. Ggc1 is a GTP transporter and recent studies have shown that GTP in the mitochondrial matrix is involved in organellar Iron homeostasis (Gordon *et al*., 2006).

Interestingly, all genes involved in copper homeostasis that are up-regulated in Ssa3 and Ssa4 strains are down-regulated in the Ssa2 strain. To analyze whether growth of strains expressing Ssa1, 2, 3 or 4 are affected we supplemented YPD agar with

Copper ($CuSO_4$) or Iron ($FeSO_4$) and analyzed affects. We used ranges of $CuSO_4$ (10-14mM) (Figure 5.12) and $FeSO_4$ (2-4mM) (Figure 5.13) (recommended to us by Dr. Simon Avery, personal communication). As genes involved in Copper and Iron homeostasis are up-regulated in strains expressing Ssa3 or Ssa4 and down-regulated in Ssa2, it is conceivable to think that plating these strains on various concentrations of $CuSO_4$ and $FeSO_4$ may produce different phenotypes. The various concentrations of $CuSO_4$ and $FeSO_4$ appeared to have no significant affect on the growth rates of any of the strains tested.

5.5 Genome sequencing

As we have utilized the same strain throughout this entire study (G402), we decided to extract and sequence genomic DNA from G402 to assess whether there are single-nucleotide polymorphisms (SNPS) present in genes that may be related to results from this study. Using next generation sequencing, we have identified approximately 6,500 high quality SNPs in G402 genomic DNA in comparison to the reference strain S288C. Using an Illumina GAII, we obtained approximately 40X genome coverage. From the isolated SNPs, there were approximately 1,500 non-synonymous amino acid changes. DNA sequencing was carried out in St James Hospital in conjunction with Trinity college, Dublin. A summary of the SNP data is presented in Table 5.7. Detailed sequence analysis data are supplemented as a hard copy (CD).

Inactivating stop codon mutations (ISCMs) were located in a number of genes in G402 (Table 5.8). The *ade2* nonsense mutation, which a cause a 507 amino acid truncation in Ade2 (catalyzes a step in the *de novo* purine nucleotide biosynthetic pathway) was correctly identified in the sequence data for G402, which provides us with confidence that data are correct.

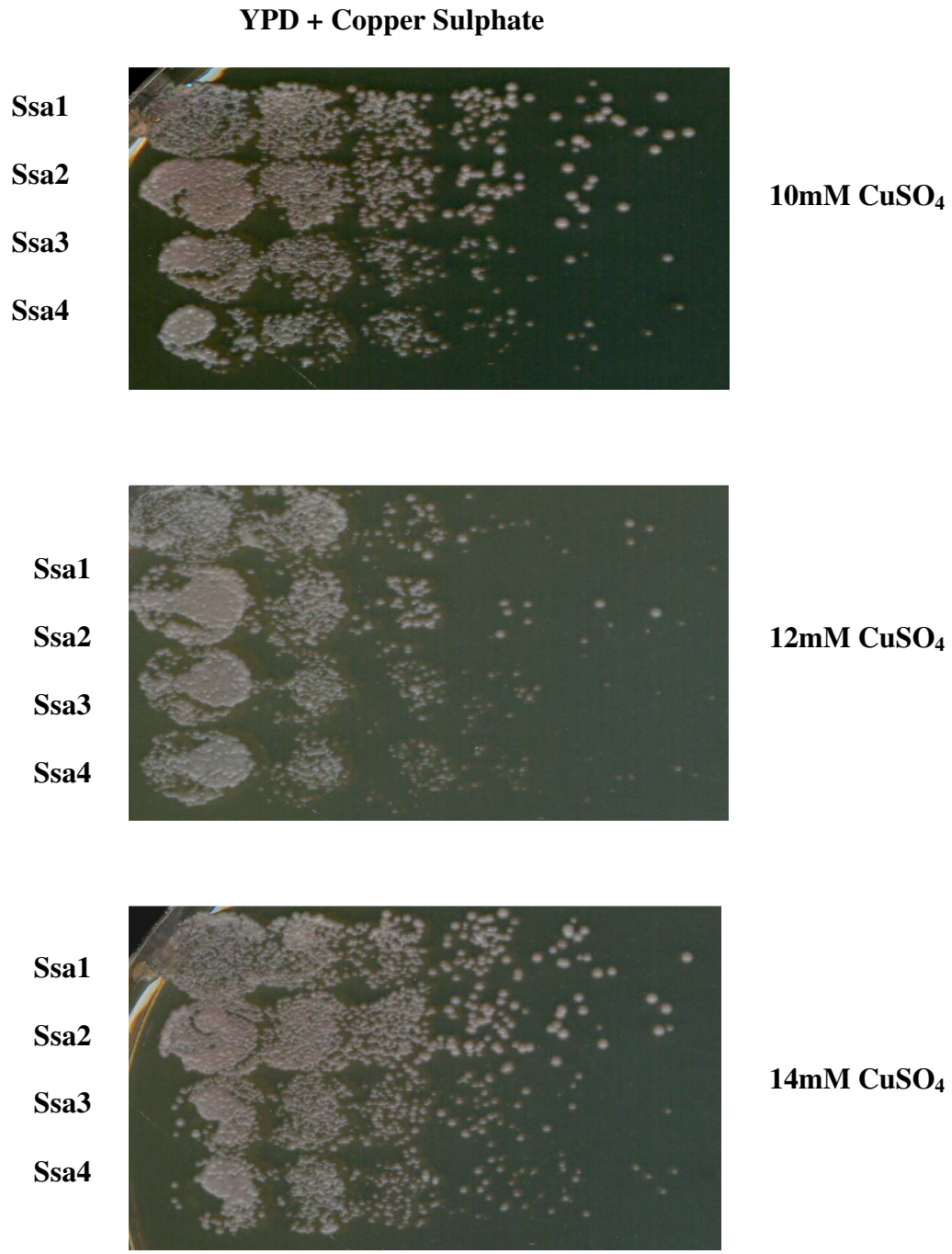


Figure 5.12. Investigation of Ssa1-4 growth on $CuSO_4$. YPD agar was supplemented with various concentrations of $CuSO_4$ post autoclaving. Cells incubated at specific temperatures for 48 hours on YPD plus various concentrations of $CuSO_4$. Cells were diluted by a 1/5 serial dilution. Spots labeled accordingly.

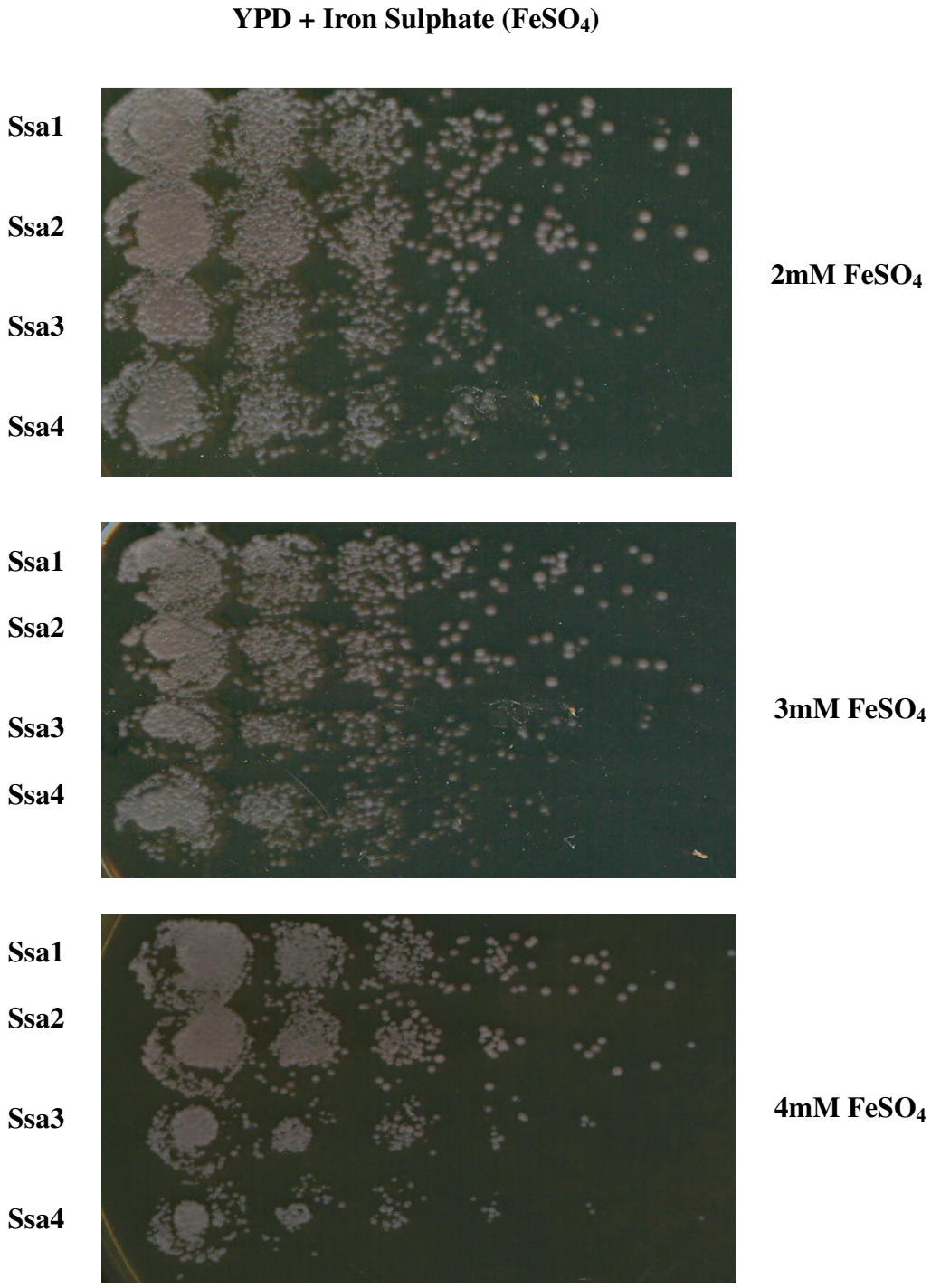


Figure 5.13. Investigation of Ssa1-4 growth on $FeSO_4$. YPD agar was supplemented with various concentrations of $FeSO_4$ post autoclaving. Cells incubated at specific temperatures for 48 hours on YPD plus various concentrations of $FeSO_4$. Cells were diluted by a 1/5 serial dilution. Spots labeled accordingly.

The largest truncation, 653 amino acids was caused by a premature stop codon in YBR074W, which is a putative metalloprotease. The *INP52* gene also contains a 532 amino acids, caused by a premature stop codon. This gene is a member of a conserved family of phosphoinositides phosphatases, it is a 136kD membrane protein and its deletion is viable (Stolz. *et al.,* 1998). *INP52*, encode member of a conserved family of phophoinositde phosphatases that contain an inositol polyphosphate 5-phosphatase domain. This domain in this enzyme specifically hydrolyzes phosphates at position 5 of inositol rings. The Afi1 protein acts as a docking factor for Arf3 and both play roles in maintaining normal budding pattern and actin patch polarization (Tsai *et al*., 2008). The Crt10 protein has a one amino acid truncation, caused by a premature stop codon. The Ctr10 protein is involved in the transcriptional regulation of *RNR2* and *RNR3.* Ribonucleotide reductase (Rnr) catalyzes the rate-limiting steps in dNTP synthesis. In addition to the presence of ISCMs being present in G402, we also located alterations to natural stop codons (Table 5.9). Eight genes were found to have alterations to their natural stop codons in the G402 strain including Nat4 which is an acetyltransferase protein responsible for acetylation of the histones H4 and H2A (Song *et al*. 2003). In G402, a Nat4 stop codon is present at 286aa; this was identified as being an Arginine (R) resulting in an addition of 53 amino acids to the C-terminus of the protein. This could potentially alter yeast molecular and biological processes. Other genes containing alterations to natural stop codons are listed in Table 5.9.

Chromosome	Size (bp)[a]	ORFs[a]	Total number of SNPs	SNPs in ORFs	Non- synonymous amino acid changes
I	230,208	92	156	122	57
II	813,178	400	885	518	212
III	316,616	158	229	157	65
IV	1,531,919	737	37	26	21
V	576,869	272	44	30	21
VI	270,148	124	34	15	7
VII	1,090,947	515	624	342	121
VIII	562,643	277	10	6	3
IX	439,885	206	508	303	98
X	745,741	351	379	267	91
XI	666,454	312	518	312	117
XII	1,078,175	500	649	459	151
XIII	924,429	452	656	380	138
XIV	784,333	390	670	396	177
XV	1,091,289	528	967	614	225
XVI	948,062	455	78	54	34
mito	85,779	19	83	57	4
Totals	12,070,897	5,788	6,527	4,058	1,542

Table 5.7 SNPs present in G402 compared to reference strain S288C. Genomic DNA was extracted from G402 and seqeunced by an Illumina GAII by a sequencing group in Trinity college Dublin. A total of 6,527 SNPs were present in G402 with 1,542 being non-synonymous changes.

Systematic name	Gene name	Biological function	Chromosomal SNP position	Nonsense change and consequence
YBR074W	-	Putative metalloprotease	387,247	Q323TAA 653 amino acid truncation
YNL106C	*INP52*	Polyphosphatidylinositol phosphatase, dephosphorylates a number of phosphatidylinositols (PIs) to PI	422,546	W651TAG 532 amino acid truncation
YOL063C	*CRT10*	Protein involved in transcriptional regulation of *RNR2* and *RNR3*	207,394	Q957TAA 1 amino acid truncation
YOR128C	*ADE2*	Adenine Biosynthesis	566,003	E64TAA 507 amino acid truncation
YOR129C	*AFI1*	Arf3 polarization-specific docking factor, required for the polarized distribution of the ADP-ribosylation factor	566,898	E887TAA 6 amino acid truncation

Table 5.8 ORFs in G402 containing single internal nonsense mutations.
Information obtained from *Saccharomyces* Genome Database (SGD).

The presence of ISCMs and alterations to natural stop codons in genes may have implications to the strains molecular and biological activity.

From the isolated SNPs, there were approximately 1,500 non-synonymous amino acid changes in G402. To analyze whether any of these non-synonymous changes may have had an affect on our results we looked for the presence of non-synonymous changes in factors that may affect Ssa1 activity, prion propagation or the CWI signaling pathway. The components that influence regulate Ssa1 function and influence prion propagations such as Hsp104, Sti1, Cpr7, Ydj1, Fes1 and Sse1 do not appear to have any non-synonymous amino acid changes present. This indicates that Ssa1 mutant phenotypes in relation to Ssa1 function and prion propagation are most likely due to presence of the mutation in Ssa1 and not in any other factors affecting these functions. We also analyzed components of the CWI signaling pathway such a

Systematic name	Gene name	Biological function	Chromosomal SNP position	Nonsense change and consequence
YGR067C	Unknown	Putative protein of unknown function	622,405	TAA795K
YKR103W	NFT1	Putative transporter of the multidrug resistance-associated protein (MRP) subfamily	649,061	TAG1219Y
YML062C	MFT1	Subunit of the THO complex, which is a nuclear complex comprised of Hpr1p, Mft1p, Rlr1p, and Thp2p, that is involved in transcription elongation and mitotic recombination	147,504	TGA393L
YMR069W	NAT4	N alpha-acetyl-transferase, involved in acetylation of the N-terminal residues of histones H4 and H2A	406,850	TGA286R
YNR066C	Unknown	Putative membrane-localized protein of unknown function	753,725	TAA437E
YNR069C	BSC5	Protein of unknown function, ORF exhibits genomic organization compatible with a translational readthrough-dependent mode of expression	761,123	TAG490Y
YOR031W	CRS5	Copper-binding metallothionein, required for wild-type copper resistance	389,186	TAG9E
YPR071W	Unknown	Putative membrane protein; YPR071W is not an essential gene	687,533	TAG212Y

Table 5.9 ORFS in G402 containing alterations to natural stop codons. Information obtained from *Saccharomyces* Genome Database (SGD)

Bck1, Pkc1, Rlm1, Mkk1 and 2 for non-synonymous amino acid changes. The only component of the CWI signaling pathway that contains non-synonymous amino acid changes is Pkc1, which contained four; F81C, K621R, S623P and P789A. The presence of these four amino acid changes may have an affect on the function of Pkc1 *in vivo* and therefore may have influenced some of the phenotypes we observed in relation to the CWI signaling pathway.

Utilizing the G402, sequencing data we were able to create a phylogenetic tree to gain knowledge of the relationship between the G402 yeast strain and other commonly yeast strains. Figure 5.15 illustrates the phylogenetic distribution of yeast strains that have been generated by the *Saccharomyces* Genome re-sequencing project. Analysis of this phylogeny was carried out by Dr David Fitzpatrick. This shows that G402 is closely related to the reference strain S288c that is a commonly used yeast strain in research.

5.6 Discussion

Due to the high conservation between the cytosolic Hsp70 Ssa family, it was postulated that the Ssa family were functionally redundant (Kabani and Martineau, 2008). However, our results and several other reports suggest that there is functional specialization among the Ssa family. The Ssa family differs by expression patterns; Ssa2 is constitutively expressed at high levels, Ssa1 is also constitutively expressed but at lower levels than Ssa2, Ssa3 and Ssa4 are not expressed during vegetative growth but expression increases upon encounters with stress (Boorstein and Craig, 1990b, Boorstein and Craig,1990a). We observed that deletion of all four members is lethal but can be complemented by over- expressing individual members, which suggests redundant functions. Ssa3 and Ssa4 do not seem to support growth as well as

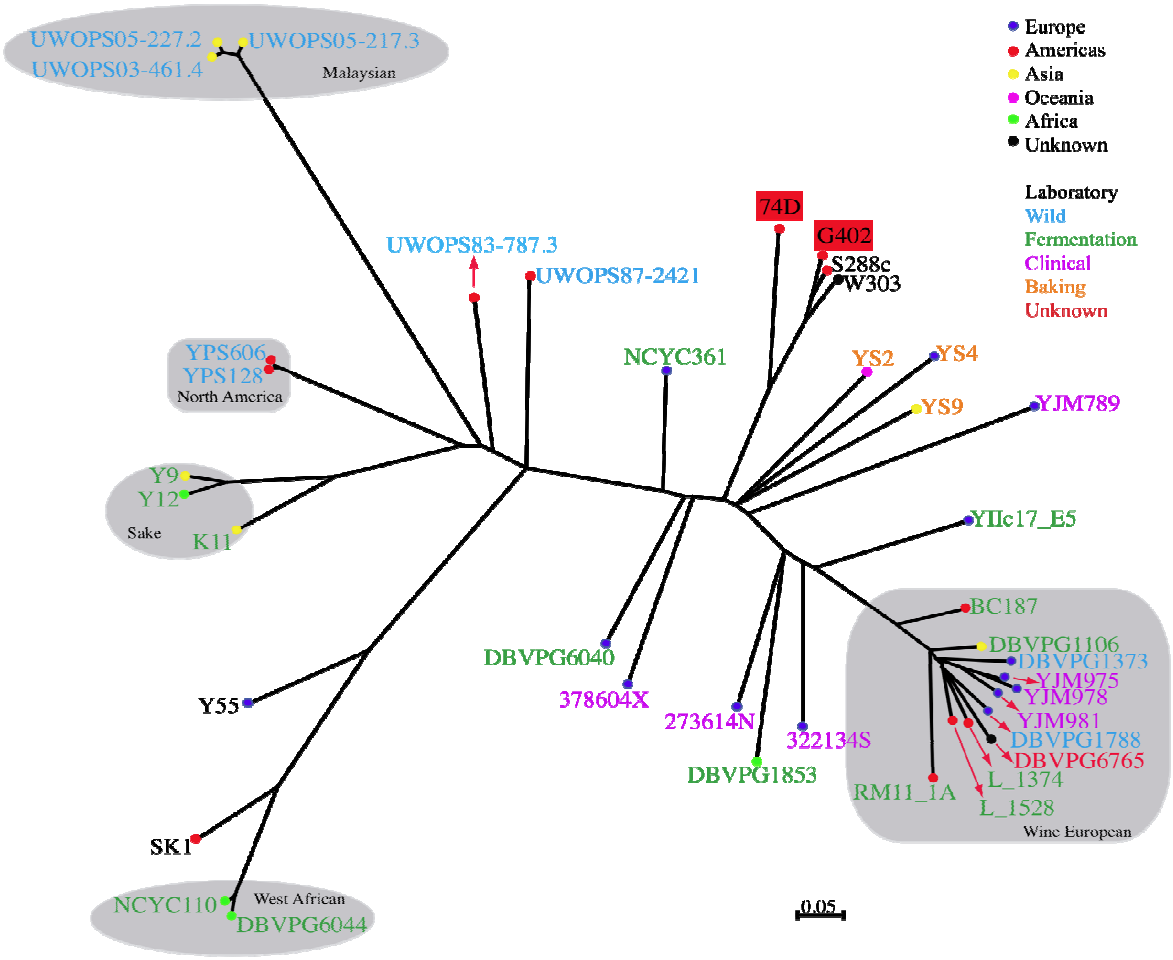


Figure 5.15. Phylogenetic distribution of yeast species. 74D is another commonly used yeast strain for [PSI^{+}] analysis. This phylogeny was generated by Liti *et al.*, (2009).

Ssa1 and Ssa2 and both strains expressing Ssa3 or Ssa4 are non-functional at elevated temperatures suggesting that functions carried out by Ssa1/2 are not fully complemented by Ssa3/4. Individual Ssa's also appear to vary in their ability to help refold proteins *in vivo.*

Propagation of [*PSI*$^+$] also appears to be affected differently by different members of the Ssa family with overexpression of Ssa3 being the most effective for [*PSI*$^+$] maintenance and propagation, this agrees with previous results from the Masison group (Sharma *et al*., 2009). Other groups have also found different effects of Ssa members on [*URE3*] propagation; overproduction of Ssa1 but not Ssa2 cures [*URE3*] whereas a mutation in Ssa2 but not Ssa1 impairs [*URE3*] propagation (Schwimmer and Masison ,2002, Roberts *et al*., 2004). Individually expressing Ssa3 or Ssa4 weakens [*URE3*] propagation. Differences with regard to prion propagation may be due to differences in sequence, which may determine how Ssa members interact with prions. For example a screen undertaken to find components that bind to the prion form of Sup35 found that Ssa1/2 efficiently bind to the prion domain of Sup35 in [*PSI*$^+$] cells but had a weak interaction with Sup35 in [*psi*$^-$] cells (Bagriantsev *et al.,*2008). Ssa3/Ssa4 were not isolated in the screen as binding to Sup35, indicating difference in binding to prions between Ssa members. Aside from degree in sequence specificity, it is possible that individual members of the Ssa family are regulated differently. Ssa proteins are recruited and activated by J proteins (Hsp40/DnaJ family) and regulated by nucleotide exchange factors such as Fes1 and Sse1. It is conceivable that Ssa isoforms have different preferences or affinities for particular J proteins and NEF's which in turn may explain different prion phenotypes when members of the Ssa family are individually expressed.

Up and down regulation of numerous genes in cells individually expressing Ssa member's further highlights functional specialization among individual Ssa members. Previous microarray screens on wildtype and Δ*ssa1*/Δ*ssa2* strains post a mild heat shock identified genes involved in up-regulation of genes involved in protein synthesis in Δ*ssa1*/Δ*ssa2* that were suppressed in wild-type (Matsumoto *et al*., 2005). Furthermore, genes involved in ubiquiton-proteosome protein degradation were up regulated in Δ*ssa1*/Δ*ssa2* strain whereas genes involved in the UPR were up regulated in the wild-type strain, suggesting that mechanism for denatured protein rescue differs in the absence of Ssa1 and Ssa2 (Matsumoto *et al*., 2005). Interestingly, we found the gene that had the highest up regulation and common in Ssa2, Ssa3 and Ssa4 is involved in the production of sphingolipids. Sphingolipids are thought to be involved in the heat stress response in yeast (Jenkins *et al*., 1997). It is believed that ceramide and other sphingolipid metabolites accumulate during heat stress and signal yeast cells to activate transcription of the *TBS2* gene and accumulate trehalose (Dickson *et al*., 1997). Recent studies have shown that trehalose is required for the transcription and maintenance of Heat shock factor one (Hsf1) in a highly active state during response to heat shock (Conlin and Nelson, 2007). This may indicate that in the absence of Ssa1 or by individually expressing Ssa2, Ssa3 or Ssa4, cells may depend on alternative mechanisms to deal with heat stress. Groups of genes involved in regulation of the yeast cell cycle and metal homeostasis appear to be commonly up regulated in cells individually expressing Ssa3 and Ssa4. This adds further weight to the argument that there is functional specialization among cytosolic Hsp70 Ssa members.

As we performed biochemical analysis throughout this project utilizing a His (6X) tagged version of Ssa1 and various Ssa1 mutants, we decided to analyze the affects of

fusing a His (6X) tag to the N-terminal of Ssa1 *in vivo*. Cells were viable when expressing Ssa1-His(6X) as the sole source of Ssa, growth rates seemed to be slightly affected indicating that the His(6X) tag has a slight affect on Ssa1 function although cells were not 37^{o}C t.s indicating Ssa1 still has the ability to recover proteins exposed to heat shock. Interestingly, cells expressing Ssa1 His (6X) appear to be able to maintain [*PSI*$^{+}$] propagation better than cells expressing wildtype Ssa1. The presence of the His (6X) tag may affect binding of Ssa1 to substrates, for example if Ssa1- His (6X) binding to aggregated Sup35 is affected it may mean Hsp104 has more access to disaggregate Sup35 polymers resulting in the production of larger numbers of prion seeds. Fusing a His (6X) to Ssa1 in the presence of PBD mutations such as L483W and F475S appears to make the protein non-functional whereas fusing a His (6X) to Ssa1 in the presence of ATPase domain mutations appears not to affect cell viability. We carried out sequencing analysis of the G402 strain to investigate whether mutations present in the strain background may be affecting phenotypes of Ssa1 mutants that we observed throughout the project. We observed five ISCMs, one of which is purposely present in the *ADE2* gene, to detect the presence of the [*PSI*] prion and eight altered natural stop codons. Truncations or elongations of proteins may affect their molecular or biological function in G402, which then may have an affect on phenotypes observed in that strain. There are 1,500 non-synonymous amino acid changes present in G402. We found no changes present in any of the main components that may affect prion propagation, which leads us to conclude that phenotypes we observed are solely due to the presence of mutations in the ATPase domain, the PBD or truncations of the CTD in Ssa1. Interestingly we found four non-synonymous amino acid changes in the CWI signaling component Pkc1. The CWI signaling pathway consists of a linear pathway in which activation of components is

dependent on phosphorylation by previous component in pathway. Pkc1 initiates the phosphorylation cascade that eventually results in the up regulation of genes involved in cell wall repair. In the presence of wild-type Ssa1 G402 exhibits no phenotypes related to the CWI signaling pathway, indicating that the four non-synonymous amino acids are not affecting phosphorylation of Bck1 by Pkc1.

Investigation of the affects of individually expressing Ssa1, Ssa2, Ssa3 and Ssa4 has provided us with new insight into the functional specialization of each member of the Ssa family with regards Hsp70 function and prion propagation. Microarray analysis highlights specific genes that are regulated differently when members of the Ssa family are expressed individually further highlighting functional specialization. We also purpose that fusing an N-terminal His (6X) tag to Ssa1 has no major affects on Ssa1 function.

6.0 Final Discussion

The *de novo* formation, propagation and maintenance of the [*PSI*$^+$] prion in *S.cerevisiae* is dependent upon many cellular factors. By carrying out mutational analysis on the various domains of the cytosolic Hsp70 members Ssa1-4, we further implicate these chaperones and their co-chaperones as playing a crucial role in prion propagation. As Hsp70 is a highly conserved protein throughout eukaryotes, these results may be transferable to mammalian systems.

Previous Ssa1 mutagenesis screens, highlighted the ATPase domain as being a highly important region for the propagation of the [*PSI*$^+$] prion (Jung *et al.,* 2000, Jones and Masison, 2003, Loovers *et al*.,2007). The previously isolated PBD mutant, *SSA1-21* (Jung *et al*., 2000), highlighted that mutations in PBD could affect prion propagation. By carrying out random mutagenesis on an Ssa1 construct lacking the ATPase domain, we successfully isolated a number of PBD mutations that impair [*PSI*$^+$] propagation. The isolation of the Ssa1 PBD mutants highlights the importance of this domain with respect to prion propagation. Amongst PBD mutations isolated was the novel mutant Ssa1^{F475S}, the first Ssa1 mutation to affect both prion propagation and Ssa1 function. From structural analysis we observed that the F475S mutation is closely located to the previously isolated PBD mutant *SSA1-21* (Jung *et al*., 2000).

There are a number of ways in which the Ssa1^{F475S} mutation may be impairing [*PSI*$^+$] propagation. Previous studies have shown that in cells expressing the *SSA1-21* mutant, there is a 10-fold decrease in the number of transmissible prion seeds but only a two to three fold reduction in the amount of aggregated Sup35 (Jung *et al.,* 2000). Further studies revealed that in cells expressing this mutant, aggregates are larger as there are

more polymers per aggregate; therefore, there are less free polymers available for efficient Sup35 recruitment, which may inhibit prion propagation (Song *et al.,* 2005). The F475S may be having a similar affect on aggregate size. Another plausible reason $Ssa1^{F475S}$ may impair [*PSI*$^+$] propagation, is that this mutation may cause Ssa1 to bind more avidly to its substrates. It has recently been shown that Ssa1/2 interacts with Sup35 resistant polymers (Bagriantsev *et al.,* 2008). Increased binding may affect the Ssa1 function of binding to Sup35 and dismantling polymers from Sup35 aggregates, resulting in defective prion propagation.

The identification of second-site suppressor mutations of $Ssa1^{F475S}$ that suppress impairment of [*PSI*$^+$] (P636S) and restore Ssa1 function (A394V, P432S and V477I), may provide us with further insight into how this mutation impairs these functions. The P636S mutation is located within a conserved CTD motif in Ssa1 that is involved in the binding of TPR co-chaperones such as Sti1 to Ssa1. Disruption to this motif in the presence of the PBD mutations $Ssa1^{F475S}$ and *SSA1-21*, restores [*PSI*$^+$] propagation. It therefore appears that a weak interaction between Ssa1 and Sti1 is needed in order for the prion to propagate. The A394V mutation is located in the linker region between the ATPase domain and the PBD, which is involved in inter-domain communication, suggesting that the F475S mutation may have an affect on inter-domain communication that may cause it to become non-functional.

As a majority of mutants that impair prion propagation, isolated from random mutagenesis screens are located in the ATPase domain, we decided to carry out further analysis on a subset of previously isolated mutants (G73D, G287D and T295I) (Loovers *et al*., 2007). Structural analysis of these mutants reveals that they are

located in regions of importance of Ssa1, the G73D mutant is located in a region for inter-domain communication in the *E.coli* Hsp70, DnaK (Revington *et al*., 2005). The G287D and T295I mutants are located in regions important for the binding of NEFs Ssa1 that are important for the regulation of the Hsp70 ATPase cycle. Interference with the binding of Ssa1 and its NEFs may result in prolonged substrate binding of Ssa1. Prolonged substrate binding may result in Ssa1 binding more avidly to Sup35, restricting access of Hsp104 to disaggregate Sup35 polymers or it may affect the role of Ssa1 in dismantling polymers from Sup35 aggregates. This suggests that both inter-domain communication and interactions of Ssa1 with NEF's is essential for prion propagation. The presence of these mutations does not appear to have a significant affect on cellular functions of Ssa1 suggesting that these residues may be exclusively involved in prion propagation.

Although numerous mutagenesis screen have identified mutations in the ATPase domain and PBD of Ssa1 that impair prion propagation, mutations in the CTD have not been isolated that have an effect on prion propagation. We therefore hypothesized that severe truncations may have an affect rather than single allele alterations. We found that truncations to the CTD of Ssa1 did not appear to affect prion propagation in [*PSI*$^{+}$] cells, but had a severe affect on the function of Ssa1. The absence of the highly conserved TPR binding motif did not appear to have an affect on prion propagation further suggesting that interactions of Ssa1 with Sti1 and Hsp90 are not required for prion propagation.

The cytosolic Hsp70 Ssa family is composed of four members, Ssa1-4, it was originally hypothesized that the members of this family were functionally redundant. By expressing each member individually as the sole source of Ssa, it is clear that members exhibit functional specialization with respect to growth and prion

propagation. This functional specialization between members of the Ssa family may be due to differences between members which may determine how they interact with Sup35. Alternatively, it is possible that individual members of the Ssa family are regulated differently. Ssa proteins are recruited and activated by J proteins (Hsp40/DnaJ family) and regulated by nucleotide exchange factors such as Fes1 and Sse1. It is conceivable that Ssa isoforms have different preferences or affinities for particular J proteins and NEF's which in turn may explain different prion phenotypes when members of the Ssa family are individually expressed.

Furthermore, by carrying out microarray analysis, it appears that numerous genes are regulated differently in the presence of individual members of the Ssa family. Groups of genes involved in regulation of the yeast cell cycle and metal homeostasis appear to be commonly up regulated in cells individually expressing Ssa3 and Ssa4.

A common feature we found in a group of Ssa1 mutants ($Ssa1^{G73D}$, $Ssa1^{F475S}$, Δ20 and Δ40), was a t.s phenotype at elevated temperatures that is rescued by growth on 1M sorbitol. This is a common phenotype present in mutations of components of the cell wall integrity signaling pathway. This result may implicate Ssa1 as playing either a direct or indirect role in this pathway. Other chaperones such as Hsp90, Sse1 and Hsp40 have previously been implicated as playing a crucial role in the pathway and it is plausible Ssa1 may be implicated into pathway through interactions with these chaperones.

As Hsp70 is a highly conserved protein throughout eukaryotes, locating mutations that affect prion propagation without affecting the function of Hsp70 may be potential therapeutic and drug targets for the treatment of prion diseases.

6.1 Future work

In order to decipher a mechanism of how Hsp70 mutants are affecting prion propagation further studies must be carried out. With respect to the PBD mutant $Ssa1^{F475S}$, it may be necessary to carry out studies to determine prion seed numbers and Sup35-GFP studies to analyze the size of Sup35 polymers in cells expressing the mutant in comparison to wild-type. As the fusion of a His (6X) tag to the N-terminal of Ssa1 is non-functional, it is necessary to find an alternative protein purification method for the purification of this mutant. Previous studies have purified Ssa1 and mutants from the yeast strain G402 by anion exchange chromatography, ATP affinity chromatography, liquid chromatography and size exclusion chromatography (Needham and Masison, 2008). Once a purification system is established testing of substrate binding may be carried out by measuring equilibrium binding of fluorescein labeled A7 peptide. This would provide insight into whether the F475S mutation increases binding avidity of Ssa1 to substrates which would have an affect on prion propagation. Further analysis may also be carried out on second-site suppressors to assess whether they restore functions affected by F475S. For example if F475S increases binding of Ssa1 to substrates, does the second-site suppressors restore substrate binding to a normal level. The same theory could be applied to the ATPase activity rate of Ssa. Binding assays may also be preformed on the ATPase mutants G73D, G287D and T295I to assess whether the presence of these mutations increase Ssa1 substrate binding.

As previously discussed, we observed a number of mutants that are t.s at elevated temperatures and are rescued by growth on 1M sorbitol indicating that Ssa may play a part in the CWI signaling pathway. However, from genome sequencing data we discovered four mutations present in the Pkc1 protein, which is a major component of

this pathway. To test whether mutations are the cause of this phenotype and not strain background, mutants should be tested in the commonly used yeast strain BY4741. In order to achieve this, the Ssa family must be deleted in this background so mutants may be expressed as the sole source in the strain. Phenotypes of strains expressing Ssa mutants will then be tested at elevated temperatures and compared to phenotypes observed in the G402 background. Sensitivity of mutants may also be tested by growth on cell wall damaging drugs such as caffeine, SDS and congo red. Yeast-two hybrid assays may also be carried out to analyze whether Ssa1 or mutants bind any components involved in the CWI signaling pathway.

From microarray analysis of strains individually expressing members of the Ssa family we observed increased expression of numerous genes including genes involved in sphingolipid synthesis, metal homeostasis and the yeast cell cycle. At present microarray analysis is being repeated on strains individually expressing Ssa1-4. RT-PCR should be carried out on genes with varied expression to confirm results. To assess whether up-regulation of components involved in sphingolipid synthesis is indicative that this alternative pathway is up-regulated as an alternative mechanism to deal with stress, mutations to impair the sphingolipid biosynthesis pathway may result in cells individually expressing Ssa2-4 not being able to deal with stress as efficiently. As Hsp70 is a highly conserved protein, mutations that impair prion propagation in yeast could be introduced into the mammalian Hsp70 homologue Hsc70 and test their affects on Scrapie propagation in a mammalian system.

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