A LC-MS/MS-Based Approach to Studying the *Saccharomyces cerevisiae* Cca1 Protein

Tian Lai Guan

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By: Tian Lai Guan

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Signed by the final Examining Committee:

_____ Chair

Dr. Cameron Skinner

_____ Examiner

Dr. Ann M. English

_____ Examiner

Dr. Christopher L. Brett

_____ Supervisor

Dr. Paul B. M. Joyce

Approved by _____

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Dean of Faculty

ABSTRACT

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Tian Lai Guan

The enzyme ATP (CTP): tRNA-specific tRNA nucleotidyltransferase (tRNA-NT) is an essential enzyme in eukaryotes and some prokaryotes and plays a critical role in tRNA maturation. This enzyme is responsible for the addition of AMP and CMP residues to the 3'-ends of tRNA molecules to allow for their aminoacylation and subsequent use in protein synthesis. The tRNA-NT (Cca1) in *Saccharomyces cerevisiae* (yeast), although encoded by a single nuclear gene (*CCA1*), functions in multiple cellular compartments (cytosol, nucleus, and mitochondrion). Here we used mass spectrometry to explore both *cis*- and *trans*-acting factors that could provide clues to the importance of the function and localization of this enzyme in *Saccharomyces cerevisiae*. We characterized post-translational modifications on the native enzyme and analyzed the proteomes of native and temperature-sensitive (*ts*) yeast strains at permissive (20°C and 30°C) and quasi-restrictive (33°C) temperatures.

Our experiments identified amino acids cysteine 307, lysine 312 and tyrosine 317 in one α-helix as sites of post-translational modifications in early log phase showing acetylation, acetylation and phosphorylation, respectively. As these modifications were present, this may suggest that these post-translational modifications play a role in protein function or localization.

As we have identified a mutation in the gene coding for tRNA-NT that leads to a temperature-sensitive phenotype, we set out to do a comparison of the proteome profiles of this strain and the native yeast strain from which it was derived. This study revealed specific differences between the native and *ts* strains and between the different temperatures tested. Levels of stress-response proteins (Ctt1, Ddp1, Gad1, Prx1, Sod2, Tdh1, Uga1, and Uga2) were increased in the variant but not the native strain at 33°C but not in either the native or the variant at any other temperature tested (20°C or 30°C). This suggests that in the *ts* strain, the stress response is induced to combat the loss of activity associated with the variant tRNA-NT. In addition, a number of mitochondrial or ribosomal proteins such as Aim24, Atp20, Cox2, Cox5A, Mhr1, Nuc1, Rip1, Drs1, Edc3, Hca4, Nog2, Nsa2, Rlp7, Sod1, Sof1, Tsr4, Utp23 whose levels differ in the native and variant proteomes were identified, suggesting pathways to be explored to explain the *ts* phenotype.

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ABBREVIATIONS

2-D gel	Two-dimensional gel electrophoresis
ATP	Adenosine triphosphate
Bp	Base pair
CCA	Cytidine-cytidine-adenosine
CTP	Cytidine triphosphate
DNA	Deoxyribonucleic acid
GST	Glutathione S-transferase
HPLC	High-performance liquid chromatography
IgG	Immunoglobulin G
KDa	Kilodalton
MS	Mass spectrometry
NES	Nuclear export signal
NLS	Nuclear localization signal
NPC	Nuclear pore complex
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PN	Proteostasis networks
PTM	Post-translational modification
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SC medium	Synthetic complete medium
SDS	Sodium dodecyl sulfate
TAP tag	Tandem affinity purification tag
TEV	Tobacco Etch Virus
TOR	Target of Rapamycin
tRNA NT	tRNA nucleotidyltransferase
ts	Temperature-sensitive
wt	Wild-type

1. INTRODUCTION

Transfer RNAs (tRNAs) play a vital role in protein synthesis (Figure 1.1). They are responsible for delivering amino acids to the ribosome for translation. In eukaryotic cells, tRNAs are transcribed from DNA as precursors that must be further processed to generate functional tRNAs (Bjork *et al*, 1987). One enzyme required in this processing is ATP(CTP): tRNA-specific tRNA nucleotidyltransferase (Deutscher, 1973) that post-transcriptionally adds the 3'-terminal cytidine-cytidine-adenosine sequence, which is needed to define the site of aminoacylation.

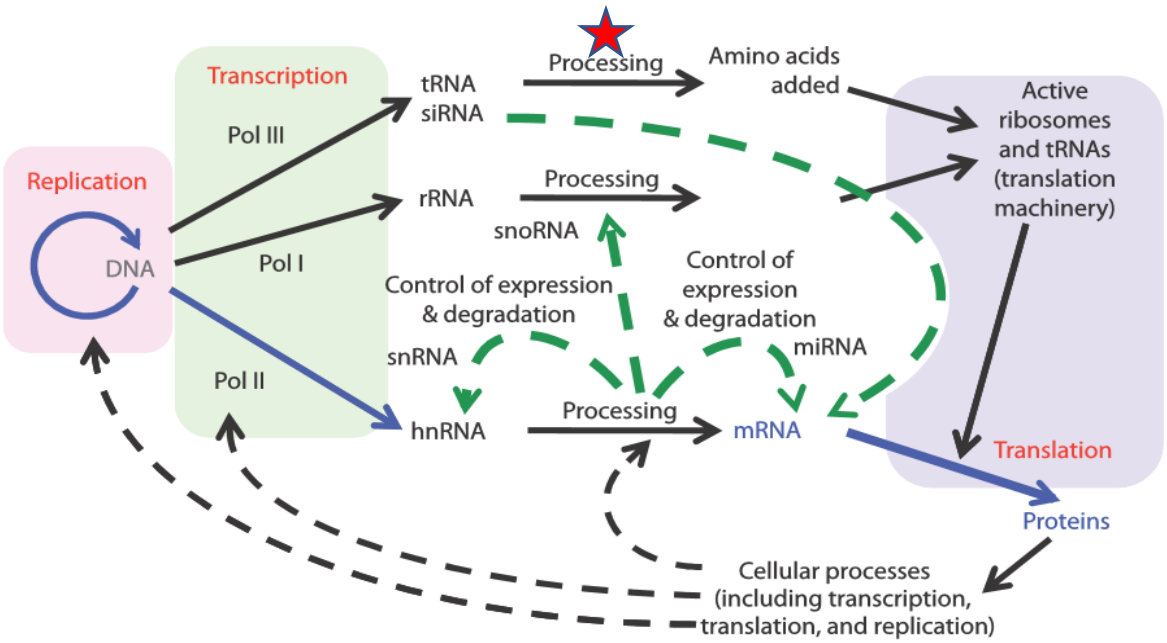


Figure 1.1 Expanded version of the central dogma (Rogers, 2012)
A red star indicates the tRNA nucleotidyltransferase functional process.

Consequently, tRNA-NT plays a key role in tRNA maturation and must function in all intracellular locations where tRNAs are produced or used in protein synthesis (nucleus, mitochondrion, cytosol, and in plants, plastids).

1.1. Overview of tRNA nucleotidyltransferase structure and function

All tRNA-NTs belong to the polβ-type nucleotidyltransferase superfamily (Aravind and Koonin, 1999) and are divided into two classes based on conserved sequence motifs: Class I

confined to Archaea and Class II present in Bacteria and Eukaryota (Vörtler *et al*., 2009). Class II enzymes contain five well-conserved motifs A-E (Figure. 1.2) in the amino-terminal portion of the protein that play roles in substrate recognition, binding and catalysis (Cho *et al*., 2002). We have shown (Shan *et al*., 2008) that mutations in regions associated with these conserved motifs can generate a temperature-sensitive (*ts*) phenotype in yeast. Recently, a number of mutations in or near these conserved motifs have been associated with human diseases (Chakraborty *et al*., 2014; Sasarman *et al*., 2015; Giannelou *et al*., 2015; Wedatilake *et al*., 2016; Frans *et al*., 2016; Deluca *et al*., 2016) highlighting the importance of these sequences in tRNA-NT structure, function and/or localization, and reflecting how small changes to these sequences can lead to changes in cells resulting in deleterious phenotypes.

We recently have shown that the temperature-sensitive phenotype in yeast arises from a hypomorphic effect reflecting reduced activity of the tRNA-NT, *i.e*., the variant protein functions less well at any temperature tested but the reduced growth rate is only evident at the restrictive temperature (Goring *et al*., 2013). Moreover, we also showed that the temperature-sensitive phenotype can be suppressed by overexpressing the variant protein (Goring *et al*., 2013).

Moreover, a number of mutations in the human TRNT1gene, coding for the human tRNA-NT have been linked to diseases such as SIFD (Chakraborty *et al*., 2014), retinitis pigmentosa (Sasarman *et al*., 2015; De Luca *et al*., 2016), and immune dysregulation (Giannelou *et al*., 2015). These mutations lead to amino acid substitution that effect protein stability and activity (Chakraborty *et al*., 2014; Leibovitch *et al*., submitted).

These data, taken with what we observed in yeast, suggest that reduced activity of tRNA-NT may be linked to changes in multiple systems in eukaryotic cells and that these changes lead to metabolic defects.

While the amino-terminal portions of Class II tRNA-NTs show conserved sequences and defined motifs, there is little sequence similarity among the carboxy-terminal portions of these proteins. This less conserved portion of the protein may be involved in tRNA binding and

positioning (Tomita *et al*., 2004; Betat *et al*., 2004; Tretbar *et al*., 2011), but more work is needed to confirm the functions of this region of the protein.

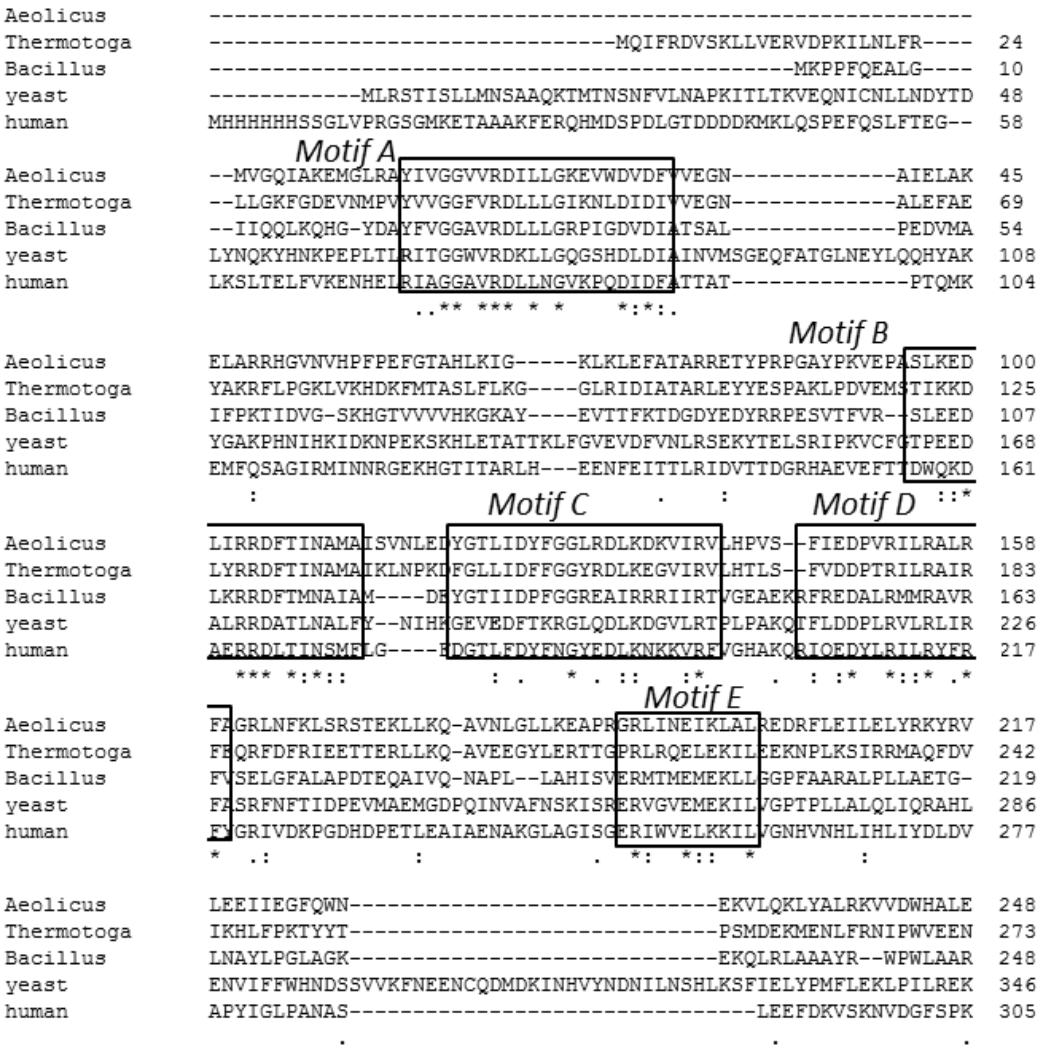


Figure 1.2 Alignment of the head and neck regions of tRNA nucleotidyltransferases (Goring *et al.,* 2013)

Alignment performed by Clustal W (Martin *et al*., 2007). The yeast enzyme and four other class II tRNA nucleotidyltransferases: *Aquifex aeolicus* (Tomita *et al*., 2004), *Thermotoga maritime* (Toh *et al*., 2009), *Bacillus stearothermophilus* (Li *et al*., 2002), and human (Augustin *et al*., 2003) for which crystal structures have been solved are shown. Motifs A-E first identified in the *Bacillus stearothermophilus* enzyme (Cho *et al*., 2002) are boxed and labeled.

* amino acid identity
: strongly conserved amino acid
. weakly conserved amino acid

1.2. Localization of tRNA nucleotidyltransferase

As discussed previously, tRNA-NT must function in all intracellular locations where tRNAs are produced or used in protein synthesis (mitochondrion, nucleus, cytosol and in plants, plastids). Interestingly, unlike most proteins, where a single gene encodes a protein that works in only one compartment of the cell (Carrie *et al*., 2009), a single nuclear gene encodes tRNA-NT that functions in multiple intracellular destinations. In fact, genome sequence analysis of 163 eukaryotic organisms (Leibovitch *et al*., 2013) indicated that 153 carried only a single gene thought to code for tRNA nucleotidyltransferase. This makes tRNA-NT one of a small group of proteins known as "sorting isozymes" (Martin and Hopper, 1994) where a single gene encodes proteins that are distributed to multiple subcellular compartments. For this to occur, these proteins must contain multiple intracellular targeting signals for distribution to multiple intracellular destinations without compromising catalytic activity.

1.3. Mechanisms for targeting sorting isozymes

Studies on tRNA-NTs have helped to identify mechanisms for targeting sorting isozymes to more than one intracellular location. For example, as far back as 1992 (Chen *et al*., 1992), it was evident that the yeast tRNA-NT was a sorting isozyme and that by using multiple transcription and translation start sites (Wolfe *et al*., 1994), different forms of the protein containing or lacking amino–terminal mitochondrial targeting information could be produced (Wolfe *et al*., 1996). More recently, we showed a similar situation in Arabidopsis where different transcription and translation start sites produced tRNA-NT proteins with or without both mitochondrial and plastid targeting information (Schmidt von Braun *et al*., 2007). In addition, we showed that in Arabidopsis the carboxy-terminal portion of the protein could also affect localization (Schmidt von Braun *et al*., 2007; Leibovitch *et al*., 2013). While this provides evidence for where the mitochondrial and plastid targeting signals are found on tRNA-NT, in neither of these cases was a potential nuclear localization signal (NLS) identified, although it was shown (Wolfe *et al*., 1996) that adding an SV40 NLS to the yeast tRNA-NT caused a growth defect and that modifying the carboxy-terminal portion of the Arabidopsis protein could alter its nuclear distribution (Leibovitch *et al*., 2013). This suggests that some feature other than

the amino-terminal sequence is responsible for the distribution of this protein between the nucleus and the cytosol. Possible mechanisms for defining the distribution of sorting isozymes are illustrated in Figure 1.3 (Yogev and Pines, 2011).

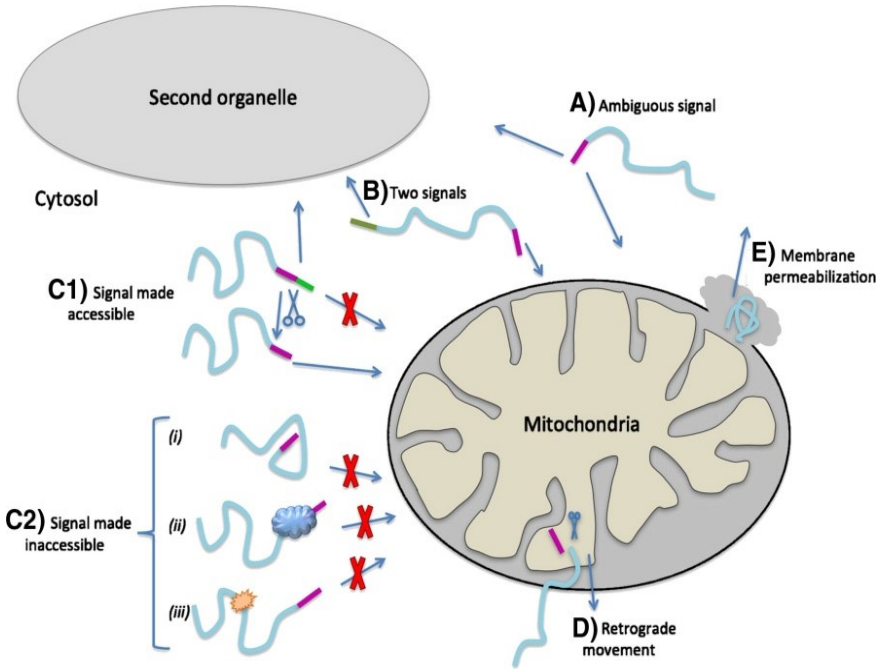


Figure 1.3 Possible modes of distribution of sorting isozymes

Five different mechanisms have been identified in eukaryotic cells: (A) An ambiguous targeting signal is recognized by different organelles. (B) Competition between two targeting signals on the same protein. (C) Post-translational modification of the targeting signal accessibility. (C1) Nascent chain cleavage exposing a cryptic targeting signal. (C2) Inaccessibility of a signal caused by (i) folding, (ii) binding to cellular factors or (iii) modification of the polypeptide. (D) Retrograde movement, cytosolic protein is exported after import into an organelle. (E) Release of proteins from an organelle's intermembrane space by membrane permeabilization (Yogev and Pines, 2011).

While having a defined targeting signal provides a simple way to direct proteins to specific destinations in the cell, at times it may be important to be able to regulate the distribution of a protein between different intracellular destinations. For example, we showed that in the yeast *Candida glabrata* the amount of transcript (potentially encoding the form of tRNA-NT with its amino-terminal mitochondrial targeting signal and targeted to mitochondria) was greater when the yeast were grown on a non-fermentable carbon source and required mitochondrial respiration for energy production than when the yeast were grown on glucose (a

fermentable carbon source) such that mitochondrial respiration was not required for growth (Hanic-Joyce and Joyce, 2002).

One established way in which specific targeting signals can function as regulated targeting signals is through post-translational modifications (mode C2iii in Figure 1.3). Studies have shown that the intracellular distribution of some proteins may be regulated by post-translational modifications. For example, the distribution of mammalian NADH-cytochrome b5 reductase between the mitochondrion and the endoplasmic reticulum is mediated by N-myristoylation (Colombo *et al*., 2005). Protein targeting to chloroplasts can be mediated by targeting signal phosphorylation and dephosphorylation (Waegemann and Soll, 1996; Lamberti *et al*., 2011). In fact, phosphorylation and dephosphorylation can also alter the distribution of proteins (*e.g*., the non-receptor tyrosine kinase, Ab1) to the nucleus, cytoplasm, ER and mitochondria. In this case tyrosine phosphorylation increases mitochondrial localization (Williamson *et al*., 2002; Alvarez *et al*., 2004; Derkinderen *et al*., 2005). As another example, mitochondrial protein import of Tom22 was shown to be regulated by phosphorylation with phosphorylation mediated by cytosolic kinases (Schmidt *et al*., 2010, 2011).

The regulation of the distribution of proteins shared between the cytosol and the nucleus has been studied most extensively. Proteins which move between these two destinations may play roles in transcription, post-transcriptional processing (*e.g*., RNA maturation pathways) and specific nuclear functions (*e.g*., DNA replication, gene regulation, *etc*.) and therefore are of considerable interest. Classical nuclear import involves the transport through the nuclear pore complex (NPC) of proteins possessing a nuclear localization signal (NLS) with the aid of helper proteins, importins. Nuclear export is mediated in a similar manner with a specific nuclear export signal (NES), exportins, *etc*. (Mattaj and Englmeier, 1998; Macara, 2001; Pemberton and Paschal, 2005).

One of the best characterized post-translational modifications involved in nuclear import/export is phosphorylation and dephosphorylation of specific amino acids in proteins to be targeted to or from the nucleus (see Nardozzi *et al*., 2010 for review). For example, the nuclear import of several proteins, *e.g*., lamin B2, SV40 T-antigen, and yeast transcription factor SWI5

(Jans *et al*., 1991; Moll *et al*., 1991; Rihs *et al*., 1991; Hennekes *et al*., 1993) is inhibited by phosphorylation of a serine or threonine residue near the NLS on the protein. Moreover, this targeting can be precisely regulated by phosphorylation (Jans and Hübner, 1996).

Acetylation also has been identified in proteins involved in nuclear import (Bannister *et al*., 2000) and has been shown to alter protein distribution in eukaryotic cells (Zhao *et al*., 2006; Lui *et al*., 2012; Cao *et al*., 2017; Li and Yu, 2017). Even proteins that typically would not be thought to have a nuclear function (*e.g*., glyceraldehyde-3-phosphate dehydrogenase) are directed to the nucleus upon acetylation (Ventura *et al*., 2010). In some instances, protein localization is defined by a combination of phosphorylation and acetylation (Wang *et al*., 2004).

To date a single post-translational modification has been reported on the yeast Cca1 (Albuquerque *et al*., 2008). These authors reported phosphorylation of the serine residue at position 21 just after the third in frame start codon in Cca1 (see Figure 1.2) in cells that had been grown to an OD_{600} of 0.7 and treated with 0.05% methyl methanesulfonate (MMS) for three hours.

It would be interesting to determine whether or not the distribution or activity of tRNA-NT is mediated by this or other post-translational modifications.

1.4. *Saccharomyces cerevisiae* as a model organism

As a biological model organism, *Saccharomyces cerevisiae* is ideal for studying tRNA-NT because it combines the advantages of both prokaryotes and eukaryotes. For instance, like prokaryotes, it has a short doubling time (90 min) in glucose medium (Brewer *et al*., 1984) and is a unicell but with a cellular organization similar to multicellular eukaryotic organisms including humans. In addition, *S. cerevisiae* has the first fully sequenced eukaryotic genome (Goffeau *et al*., 1996) and contains ~6000 genes, compared with around 25 thousand protein coding genes in humans (Duina *et al*., 2014). Moreover, there is considerable information already available on the cell cycle and other processes and metabolic pathways in this organism. For example, there are multiple large-scale surveys on the requirements for specific genes (Ross-Macdonald *et al*.,

1999, Christopher *et al*., 2016), the levels of expression of those genes under various conditions (Puig and Perez-Ortin, 2000; Carreto *et al*., 2010), localization of gene products (Kumar *et al*., 2002; Huh *et al*., 2003), amounts of gene products present in the cell (Raué, 1994), and interactions between specific gene products (see Boone *et al*., 2007 for review). Moreover, the *S. cerevisiae* proteome has been well-characterized (Schwikowski *et al*., 2000; Griffin *et al*., 2002; Léger *et al*., 2016; Lawless *et al*., 2016) and changes in the proteome have been mapped under multiple different conditions (Prokisch *et al*., 2004; Picotti *et al*., 2009).

1.5. Temperature-sensitive (*ts*) mutants of *Saccharomyces cerevisiae*

A temperature-sensitive (*ts*) phenotype implies that the phenotype is dependent on the organism being moved to a restrictive temperature which is elevated as compared to the permissive temperature. In the example discussed here, specific mutations in the *Saccharomyces cerevisiae CCA1* gene allow the yeast to grow at 20°C (the permissive temperature) but not at 37°C (the restrictive temperature). Normally, wild-type yeast cells can grow from 10°C to 37°C (Miller *et al*., 1979). Mutations in many different genes can lead to a *ts* phenotype, for instance, Hartwell initially isolated around 400 *ts* mutants in his ground-breaking research (Hartwell, 1967). These mutations mapped to genes coding for proteins responsible for essential processes such as the cell division cycle, cell wall formation, and the synthesis of proteins, ribonucleic acid (RNA), and deoxyribonucleic acid (DNA) (*e.g*., Hartwell, 1967; Hartwell and McLaughlin, 1967; Hartwell *et al*., 1973; Matsuzaki *et al*., 1988). These *ts* mutants provided critical insights into gene and cell processes. Since Horowitz recognized the temperature-sensitive mutation in the 1950s (Horowitz, 1950), studies have relied on the *ts* phenotype as a useful approach either to identify essential gene sets for various aspects of biology or to understand the function of these essential genes (*e.g*., Edgar and Lielausis, 1964; Hartwell, 1967; Pringle, 1975; Pringle and Hartwell, 1981; Kawakami *et al*., 1992; Tan *et al*., 2009; Ben-Aroya *et al*., 2010).

1.6. *Saccharomyces cerevisiae* tRNA-NT and the temperature-sensitive phenotype

Previous studies in our lab identified a single amino acid substitution in Cca1 that resulted in the generation of a temperature-sensitive phenotype (Shan *et al*., 2008). We have

explored how this amino acid change alters tRNA nucleotidyltransferase structure, stability, and function. We showed that replacing the glutamic acid residue at position 189 in the native enzyme by lysine or phenylalanine led to a temperature-sensitive phenotype where the cells were unable to grow at the restrictive temperature (37°C) (Shan *et al*., 2008). Furthermore, we showed that these mutations lead to reduced tRNA-NT activity which ultimately resulted in the *ts* phenotype as by overexpressing the variant protein, we could suppress the *ts* phenotype (Goring *et al*., 2013). Based on these observations, we suggested that reduced tRNA-NT activity lead to a smaller pool of tRNAs with intact 3'-termini (Aebi *et al*., 1990) which lead to reduced efficiency of translation and ultimately the *ts* phenotype (Goring *et al*., 2013). In contrast, it also has been shown that the same glutamate to lysine substitution in the yeast tRNA-NT results in an alteration in the stability of some mRNAs when cells are shifted from the permissive to the restrictive temperature (Peltz *et al*., 1992). More information is required to make the connection between reduced tRNA-NT activity and the growth phenotype that arises from it.

We believe that what we learn from yeast may be used to explore what is happening in humans where single amino acid substitutions have been linked to disease phenotypes (*e.g.*, Chakraborty *et al*., 2014). As the human and yeast tRNA-NTs are homologues and single point mutations in either can define deleterious phenotypes (*ts* in yeast and disease in humans), it is possible that these variants may affect the same mechanism(s) or interrupt near identical pathways. To address this question, a novel approach is needed, which could reveal the relationship between tRNA nucleotidyltransferase and other proteins *in vivo*. For this purpose, we designed a nano-HPLC tandem Orbitrap mass spectrometric method to apply a whole cell proteomics approach to explore the yeast cell proteome in wild-type and *ts* strains at permissive and quasi-restrictive temperatures.

1.7. A label-free nano-HPLC tandem Orbitrap MS/MS proteomics approach

Proteomics, which is generally defined as the large-scale study of the characterization of the complete protein complement of a cell, tissue, or organism (a proteome), investigates the location, abundance/turnover, and post-translational modifications of proteins in a proteome (Abluquerque *et al*., 2008; Claydon and Beynon, 2012; Christiano *et al*., 2014). It also may

provide information to help to identify protein-protein interactions, and proteins in specific metabolic pathways (Zhu *et al.,* 2001; Koller *et al*., 2002; Kleffmann *et al*., 2004). Compared with other proteomic techniques such as two-dimensional gel electrophoresis (2DE), two-hybrid analysis, and protein microarrays; mass spectrometry (MS) can handle the complexities associated with more comprehensive proteome analyses (Han *et al*., 2008). As we know, there is still much to learn about the properties of the proteome since it represents a complex and integrated system. To get deeper insights into the composition, structure, function and control of the proteome, and shed light on complex biological processes and phenotypes, powerful mass-spectrometry-based technologies can be very helpful (see Aebersold and Mann, 2016 for review).

Mass spectrometry is a powerful technology which provides both qualitative and quantitative analyses that can be used to enhance our understanding of complex and dynamic processes. As a current state-of-the-art analytical technique, mass spectrometry provides the necessary depth for informative proteome analysis. High-resolution MS combined with computational analysis has successfully achieved quantitative characterization of both cellular and organellar proteomes (Walther and Mann, 2010). A typical mass-spectrometric approach involves multiple stages (Aebersold and Mann, 2003). First, the proteins of interest are isolated from the cell, tissue or organism of interest grown under the conditions of interest. This typically involves SDS-polyacrylamide gel electrophoresis as a final step. Subsequently, the proteins are digested with an appropriate protease to generate peptides for analysis as MS analysis of whole proteins is less sensitive than peptide MS and determining the mass of multiple peptides is more informative than a single protein mass (as multiple proteins may share the same mass). At this point, the masses of the peptides are determined, usually after separation by liquid chromatography prior to exposure to the electrospray ion source. The mass spectrum of the peptides may be taken once (MS1) or in tandem (MS/MS) and the spectra acquired and stored for matching against available protein sequence databases to identify the peptides and ultimately the proteins defined by these peptides. This bottom-up proteomics method allows for protein identification through comparison with an existing proteome database (in this case, *Saccharomyces cerevisiae*) downloaded from UniProt. In addition, based on the signal intensities of unique peptides, the levels of detected proteins can be quantified. For example, this type of

approach was used both to identify and quantitate components of the major histocompatibility complex (MHC) (Caron *et al*., 2015). Finally, by comparing individual peptides in this way, post-translational modifications (PTMs) on peptides can be identified. For example, Zhang and colleagues used an MS approach to identify 27 different kinds of PTMs and 53 specific PTM sites (including 13 that had never been reported previously) in 30 GST-fused yeast kinase proteins (Zhang *et al*., 2010).

1.8. The aim and approach of this study

My project had two main goals linked through the Cca1 protein and mass spectrometry. I wanted to explore both the cis- and trans- acting factors that may mediate the structure, activity and localization of Cca1 protein in yeast and to determine what role a temperature-sensitive phenotype may have on these. Our hypothesis includes 1) that PTM(s) may be detected on Cca1 in yeast cell during the early log phase; 2) that reduced activity of Cca1 protein may lead to protein levels increasing/decreasing in certain functional groups, for instance, proteins involved in protein synthesis process, or heat stress response proteins, which might cause the cell to present the *ts* phenotype. Thus, mass spectrometry was used 1) to detect any post-translational modifications on yeast Cca1 in early log phase and 2) to identify any changes in specific protein levels that may mediate or be mediated by changes in Cca1 function at permissive and restrictive temperatures.

2. MATERIALS AND METHODS

2.1. Strains, growth media and reagents

Saccharomyces cerevisiae strains, except for the commercially-prepared Yeast TAP-Tagged *CCA1* strain YSC1178 (Id: YER168C) purchased from Dharmacon, were kindly provided by Dr. Pamela Hanic-Joyce. Primary antibody: Anti-Cca1 antibody for Western blotting was kindly provided by Dr. Erin Redmond (University of Ottawa). Pierce Goat Anti-Rabbit IgG, (H+L), Peroxidase conjugated (Product number: 31460, Lot number PI208014,

Thermo Scientific). Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, Cat. Number: 32209)

Table 2.1 Yeast *Saccharomyces cerevisiae* strains used in this study

Yeast Strain	Relevant Genotype	Genetic Background
BY4743		MATa/α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0*
CCA1-TAP	*CCA1*TAP-Tagged	W303-1B
NT33-5	*cca1-E189K*	MATα *ade2 his3 leu2 trp1 ura3*
W303-1B		MATα *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*
W303 Heterozygous Diploid		MATa/Matα *ade2/ade2 can1/can1 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3*
YSC1178		MATa *his*3-Δ1 *leu*2-Δ0 *ura*3-Δ0 *met*15-Δ0 RMD9-TAP [rho+]

This study used growth media and buffers listed in Table 2.2. Unless otherwise indicated all chemicals and reagents were from Bioshop. Ampicillin (sodium salt, BioTech grade) was typically prepared as 100 mg/ml stock solutions and filtered through a 0.2 µm sterilized syringe filter and stored at -20°C in 1 ml aliquots. DTT (dithiothreitol) was prepared as a 1 M stock solution and stored at -20°C in 1 ml aliquots. PMSF (phenylmethane sulfonyl fluoride) protease inhibitor (Thermo Scientific™, Cat. Number: 36978) was prepared at 100 mM in isopropanol and stored at -20°C in 1 ml aliquots. Complete™ Protease inhibitor cocktail tablets provided in EASYpacks (Roche). Bio-Rad protein assay dye reagent concentrate, 450 ml (Bio-Rad, Cat. Number 5000006). CHAPS (Bio-Rad, Cat. Number 1610460)

Table 2.2 Recipes for media and buffers

Medium or buffer	Components
YT (Sambrook et al., 1989)	0.8% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl (plates: 1.5 % agar)
SC (-URA) (Kaiser et al., 1994)	0.67% yeast nitrogen base without amino acids, 2% glucose, 20 mg/l: adenine, L-histidine-HCl, L-arginine-HCl, L-methionine, 30 mg/l: L-leucine, L-isoleucine, L-lysine-HCl, 50 mg/l: phenylalanine. Autoclave. when cool, add 10 ml/l 100×T-mix (plates: 1.5 % agar)
100×T-mix (Kaiser et al., 1994)	0.2% tryptophan, 0.3% tyrosine, 2% threonine, adjust to pH10 with 5M NaOH, filter sterilized

Medium or buffer	Components
YPD (Kaiser et al., 1994)	2% peptone, 2% glucose, 1% yeast extract (plates: 1.5% agar)
Luria Bertani (LB) (Miller, 1992)	1% Bacto-tryptone, 0.5% Bacto-yeast extract, 1% NaCl (plates: 1.5 % agar)
5×-PAGE loading buffer (Sambrook et al., 1989)	250 mM Tris-HCI (pH 6.8), 30% glycerol, 10% SDS, 5% β-mercaptoethanol, 0.02% bromophenol blue
10× SDS-PAGE running buffer (pH 8.3) (Sambrook et al., 1989)	Tris 30 g/l, glycine 144 g/l, SDS 10 g/l
SDS-PAGE Resolving gel (10%) (Sambrook et al., 1989)	10% acrylamide (29:1 acrylamide: bis acrylamide, 0.1% SDS, 0.1% APS, 0.375 M Tris-HCI (pH 8.8), 0.1% TEMED
SDS-PAGE Stacking gel (6%) (Sambrook et al., 1989)	6% acrylamide (29:1 acrylamide: bis acrylamide), 0.1% SDS, 0.1% APS, 0.125 M Tris-HCI (pH6.8), 0.1% TEMED
5×TBE (Modified from Sambrook et al., 1989)	1 M Tris, 1 M Boric acid, 20 mM EDTA (pH8)
10×TBS (Modified from Towbin et al., 1979)	137 mM Sodium Chloride, 20 mM Tris, 0.1% Tween-20. Supplied at pH 7.6
Towbin transfer buffer (Towbin et al., 1979)	25 mM Tris-HCl, 192 mM Glycine, 20% Methanol, 0.01% SDS
PBS (Sambrook et al., 1989)	137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4
50×TAE(Tirs-acetate-EDTA) (Slibinskas et al., 2013)	2 M Tris-HCl, 1 M acetic acid, 50 mM EDTA
IEF buffer (Slibinskas et al., 2013)	7 M Urea, 2 M Thiourea, 2% CHAPS, 2% ampholytes, 0.02% bromophenol blue, (75 mM DTT, add just before use)
Equilibrium buffer (Slibinskas et al., 2013)	50 mM Tris-HCl (pH8.5), 2% SDS, 6 M urea, 30% glycerol, 0.02% bromophenol blue, (75 mM DTT and 150 mM iodoacetamide, add just before use respectively)
Extract buffer (Ghaemmaghami et al., 2003)	50 mM Tris-HCl (pH8.0),150 mM NaCl, 0.2% NP-40, 10% glycerol, containing one complete tablet without EDTA (Roche) per 10 ml, 1 mM PMSF and 1mM DTT (add just before using)
Breaking buffer (Harju et al., 2004)	2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0

Table 2.3 Oligonucleotides used in this study

Oligo	Sequence (5' → 3')
SCCALF	TAA TAT **ACT AGT** ATG CTA CGG TCT ACT ATA TCT CTA C
TAPR	TAT ATT **CTC GAG** CCT CAC TGA TGA TTC GCG TC

The restriction sites used in SCCALF (*Spe*I) and TAPR (*Xho*I) are shown in bold and the start and stop codons are underlined.

2.2. Construction of Cca1 overexpression system

2.2.1. Plasmid used to construct Cca1-TAP tagged fusion protein for expression in yeast (Figure 2.1)

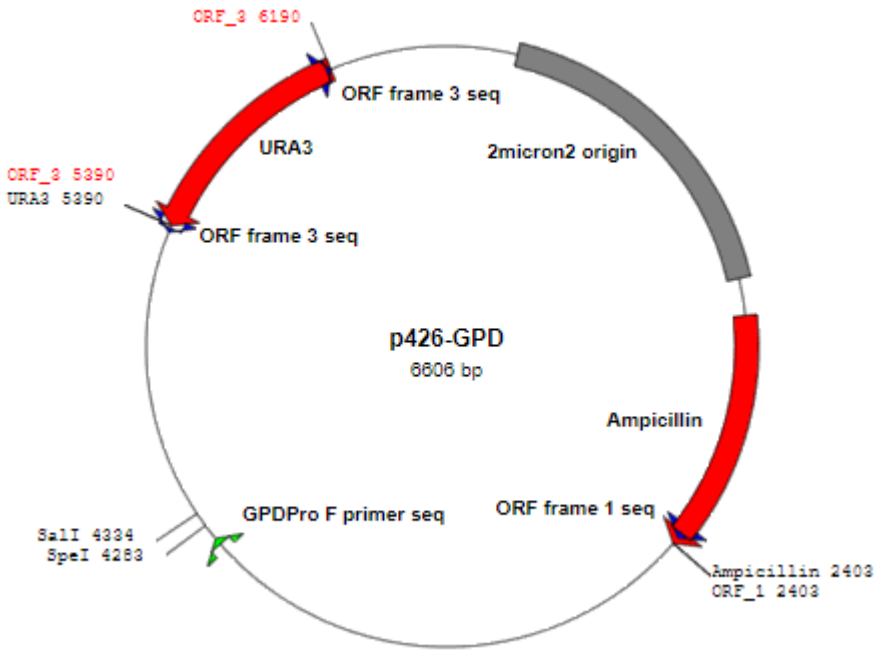


Figure 2.1 Plasmid p426 into which was cloned the *CCA1*-TAP open reading frame

Restriction enzyme sites in the multiple cloning site are indicated in blue. The Cca1-TAP open reading frame was cloned between the *Sal*I and *Spe*I (*Bcu*I) restriction sites for expression under the control of the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter.

2.2.2. Extracting DNA from the *CCA1*-TAP strain YSC1178 (Harju *et al*., 2004)

The *CCA1*-TAP-containing yeast strain was grown overnight on a YPD plate at 30°C. Cells (2 cm^2) were scraped from the plate, transferred to an Eppendorf tube and resuspended in 200 µl of breaking buffer (Harju *et al*., 2004). Subsequently, 200 µl of acid washed glass beads and 200 µl of phenol were added with vortexing for 3 min and centrifugation at 12 000 ×g for 5 min. After centrifugation, the aqueous phase was transferred to another Eppendorf tube and the phenol extraction repeated. The aqueous phase (~400 µl) was collected and 1 ml of cold 99% ethanol was added with vortexing. The sample was centrifuged as above, the supernatant

discarded, and the pellet resuspend in 400 µl of dH_2O by vortexing. To this was added 5.3 µl of 7.5 M ammonium acetate and 1 ml of cold 99% ethanol with vortexing and centrifugation as above. The supernatant was discarded, and the pellet washed with 1 ml of 85% ethanol with centrifugation as above. The pellet was dried under vacuum for 20 min and resuspended in 30 µl of dH_2O by vortexing. To check the result, 1.5 µl of sample was examined after electrophoresis on a 0.8% agarose 1×TBE gel (containing 1 µg/ml ethidium bromide) for 1 h at 100 V.

2.2.3. Polymerase chain reaction (PCR) and the purification of PCR products

In order to generate a Cca1-TAP expression system to overexpress *S. cerevisiae* TAP fusion tRNA-NT, *CCA1*-TAP was amplified from extracted DNA (section 2.2.2.) using oligos SCCALF, and TAPR (Table 2.3). Oligonucleotides were obtained from Integrated DNA Technologies. The PCR reactions contained 100 ng yeast genomic DNA, 100 pmol of each oligonucleotide primer, 10 mmol dNTP solution, 1.25 units of Phusion HF DNA polymerase, and dH_2O to a final volume of 50 µl and were performed in a PERKIN ELMER DNA thermal cycler using an initial hot start cycle of 98°C/3 min, followed by 35 cycles of 98°C/15 sec, 52°C/30 sec, 72°C/60 sec. The last cycle was finished at 72°C for 10 minutes to ensure complete extension of PCR products. That PCR products of the expected size were generated was confirmed by electrophoresis of 5 µl of the reaction mix on 0.8% agarose 1×TBE gels at 100 V (section 2.2.2.).

2.2.4. Phenol extraction and ethanol precipitation (Ausubel *et al*., 1995)

An aliquot (44 µl) of the PCR product was diluted to 200 µl with dH_2O and 20 µl of 3 M sodium acetate (pH 5.2) and 200 µl of phenol were added with subsequent vortexing and centrifugation as above. The aqueous phase was collected, and the phenol extraction repeated twice more. Finally, the aqueous phase was collected and extracted twice with equal volumes of water-saturated ether. After removing the ether phase, 400 µl of 99% ethanol was added to the aqueous phase with vortexing, the sample was placed at -70°C for a minimum of 30 min, centrifuged for 30 min and the supernatant discarded. After adding 400 µl of 80% ethanol and inverting the tube a few times, the tube was centrifuged for 5 min and the supernatant discarded.

The pellet was dried under vacuum as above and resuspended in an appropriate volume of sterile dH_2O for use.

2.2.5. Restriction digestions

An appropriate amount of purified PCR product or p426 plasmid was diluted to a final volume of 40 µl to which was added 5µl of 10×TANGO buffer and 2 µl of *Bcu*I and 2 µl of *Sal*I (to vector) or *Xho*I (to PCR product). After gentle mixing, the samples were placed in a 37°C water bath for one hour. The digested samples were separated by agarose gel electrophoresis (2.2.2.) and the desired fragments were excised (2.2.6.).

2.2.6. Fragment purification (Bewsey *et al*., 1991)

After separating the fragments by agarose gel electrophoresis in the dark, fragments were visualized under the preparative light setting on a Foto/Prep®I (FOTODYNE Incorporated) transilluminator and excised. The agarose plug containing the fragment of interest was placed in an Eppendorf tube to which was added 300 µl of phenol (saturated with 0.1 M Tris-HCl, pH 8.0). After vigorous vortexing for 2 min, the sample was placed at -70°C for a minimum of 30 min and thawed at 37°C for a minimum of 10 min. Again, an equal volume of phenol was added, and the procedure repeated. After the second freeze-thaw cycle, 150 µl of dH_2O and 40 µl of 3M sodium acetate (pH 5.2) were added with vortexing and centrifugation for 5 min to separate the aqueous and phenol layers. The aqueous layer was collected and two phenol and two ether extractions followed by ethanol precipitation were carried out as described in 2.2.4. The pellet remaining was resuspended in 10 µl of dH_2O.

2.2.7. Ligations

Insert (100-200 ng) and phosphatase-treated vector (100 ng) DNA were ligated in 1×ligase buffer (MBI) containing 1 unit of T4 DNA ligase (MBI) and 0.25 mM ATP in a final volume of 20 µl at room temperature for 2 hours. Linearized vector alone and dephosphorylated vector without insert were used as controls. Ligation mixes were stored at -20°C until needed.

2.2.8. *E. coli* transformation (Ausubel *et al*., 1989)

Aliquots (10 µl) of the appropriate ligation mixes were added to prechilled Eppendorf tubes containing 100 µl of XL2 *E. coli* competent cells. After incubation on ice for 30 min, the cells were heat shocked at 42°C for 45 sec and returned to the ice. At this point 400 µl of YT medium was added and the tubes were incubated at 37°C for 30 min. Then the tubes were centrifuged at 12 000 ×g for 30 sec, 400 µl of the supernatant removed and the pellet resuspended in the remaining solution prior to plating on YT+Ampicillin medium for incubation overnight at 37°C.

2.2.9. Plasmid purification and confirmation

A Gene Jet Plasmid Miniprep Kit (#K0503, Lot 00184629, Thermo Scientific) was employed to extract plasmid following the manufacturer's instructions. Restriction digestions as described above (section 2.2.5.) were carried out using 1µl of purified plasmid and 1µl *Hin*dIII (MBI) to identify clones containing the insert of interest by agarose gel electrophoresis (section 2.2.5.).

2.2.10. *Saccharomyces cerevisiae* transformation (Dohmen *et al*., 1991)

Plasmid DNA was made sterile by ethanol precipitation (2.2.4.) and resuspension in 50 µl of sterile dH_2O. A 10 µl aliquot of the plasmid DNA was added to a sterile Eppendorf tube containing 100 µl of competent yeast cells (W303-1B) provided by Dr. Pamela Hanic-Joyce. After incubation at 42°C for 2 hours, the solution was spread onto SC (-URA) plates with incubation at 30°C. Colonies were selected from the plate, inoculated into liquid SC (-URA) medium and incubated at 30°C with shaking (225 rpm). An aliquot of the transformed cells was taken and stored at – 70ºC after addition of glycerol to 20%.

2.3. Western blotting

2.3.1. **Whole cell lysis** (Ghaemmaghami *et al*., 2003)

Yeast cells were harvested by centrifugation at 3 000 ×g for 5 min at an OD_{600} value of approximately 1.0 after growth in 1l liquid YPD medium under the appropriate experimental conditions (20°C, 30°C, and 33°C). The resulting cell pellet was resuspended in extract buffer (2 ml of buffer/g wet weight of cells) by vortexing and transferred to a 2-ml screw cap Eppendorf tube. An equal volume of acid washed glass beads (425-600 µm, SIGMA®) was added and the cells were homogenized in a Precellys™ tissue homogenizer (Bertin Technologies) at 6800 rpm for six cycles of 30 sec. Between cycles, tubes were chilled on ice for 5 min. The homogenate was cleared by centrifugation at 14 000 xg for 30 min at 4°C. The supernatant was collected as the total protein extract.

2.3.2. **Measurement of protein concentration by Bradford Assay** (Bradford, 1976)

Protein concentrations were determined by Bradford assay according to the procedure of the supplier (Bio-Rad). In brief, a 200 µl aliquot of Bio-Rad protein assay dye reagent concentrate was mixed with 800 µl of protein sample and water to give a final volume of 1 ml. Absorbance was measured at 595 nm with the Shimadzu™ UV-260 UV Visible Recording Spectrophotometer. A standard curve was produced by measuring absorbance at 595 nm of different amounts of bovine serum albumin (0, 5, 10, 15, 20, 25 µg). The colour development of the mixture of protein and dye reagent was time dependent, so the incubation time of the BSA standard and unknown samples was kept the same (5-10 minutes). The concentration of proteins was determined by linear regression analysis from the standard curve.

2.3.3. **Sodium dodecyl sulfate-polyacrylamide gel electrophoresis** (SD-PAGE)

SDS polyacrylamide stacking (6%) and resolving (10%) gels (5 ml and 2 ml, respectively) were made according to the instructions accompanying the Bio-Rad apparatus and cast between 4 cm by 10 cm gel plates separated by 0.75 mm spacers. Protein samples were

mixed with 5×PAGE loading buffer (see Table 2.2), boiled for 2 min and loaded into the wells of the stacking gel for electrophoresed in pre-chilled running buffer at constant voltage (200 volts) for approximately one hour. After electrophoresis, the gels were removed from the apparatus and stained by the procedure of Wong *et al*. (2000). In brief, gels were stained for one minute in a 1000 KW Microwave oven (Whirlpool) in 100 ml of 0.1% Coomassie blue R-250, 10% isopropanol, 10% acetic acid and destained for one minute under the same conditions in 250 ml of 20% isopropanol, 10% acetic acid followed by gentle shaking for 20 min. Finally, the isopropanol/acetic acid solution was removed from the gel by gentle shaking in 500 ml of dH_2O for three hours. For Western blotting, the gels were used directly without any staining or de-staining.

2.3.4. **Preparing the transfer membrane** (Towbin *et al*., 1979)

For Western blotting, the gel was removed from the SDS-PAGE apparatus and placed immediately into Towbin transfer buffer for 1 min with gentle shaking (Table 2.2). At the same time, 10×7.5 cm^2 Gel Blot paper (Whatman® Item No. 10427812) and the foam pads (Bio-Rad) were prewetted in the transfer buffer and the PVDF membrane was prewetted in 100% methanol. The transfer apparatus was assembled (foam pad-filter paper-gel-membrane-filter paper-foam pad) and placed in the transfer apparatus (gel on the cathode, and membrane on the anode). Transfer in pre-chilled Towbin transfer buffer was carried out at 4°C at constant voltage (30 Volts) overnight.

2.3.5. **Membrane blocking and antibody incubations** (Bronstein *et al*., 1992)

After transfer, the apparatus was disassembled, and the membrane blocked first in 20 ml of 4% w/v skim milk (Carnation® Instant Skim Milk Powder, Nestlé) in 1×TBST (Table 2.2) solution (pre-chilled to 4°C) with gentle shaking at room temperature for 1 hour. After blocking, the blocking solution was replaced with the primary antibody solution, rabbit anti-Cca1antibody (diluted 1:10 000) in 10 ml pre-chilled 4% skim milk in 1×TBST (Table 2.2) solution and gentle shaking at room temperature is performed for 2 hours. After incubation with the primary antibody is complete, the membrane is washed three times for five minutes in 20 ml 1×TBST on

the shaker. After washing, 20 ml of pre-chilled 4% skim milk 1×TBST containing the secondary antibody, goat anti-rabbit IgG (H+L) peroxidase conjugated antibody (Thermo Scientific Prod# 31460 Lot# PI208014) diluted 1: 100 000 was added with gentle shaking at room temperature for 1 hour. After incubation with the secondary antibody the membrane was washed again three times in 20 ml of 1×TBST as above.

2.3.6. **Protein signal development** (Bronstein *et al*., 1992)

Protein signals were developed by adding 2 ml of Pierce™ ECL Western Blotting Substrate solution to the membrane at room temperature for two minutes. The signal intensity was recorded using the Amersham Imager 600 on auto exposure mode. (GE Healthcare Life Sciences).

2.4. Affinity purification of Cca1-TAP fusion purification

Strain Cca1-TAP was grown in 1 l of SC (-URA) medium at 30°C with 225 rpm shaking to an OD_{600} of 1. Cells were collected and lyzed on ice as in section 2.3.1. The cell lysate was loaded at 4°C onto 1 ml of IgG Sepharose™6 Fast Flow resin that had been packed by gravity and pre-rinsed with 10 ml of lysis buffer (Table 2.2). The column was sealed and incubated at 4°C overnight with gentle rotation. The column was unsealed and the flow-through collected by gravity as one 10 ml sample. The column then was washed by gravity with 50 ml of pre-chilled lysis buffer (Table 2.2) which was collected as a single fraction. After washing, the IgG Sepharose beads were transferred to a new tube containing 250 µl of 5×loading dye (Table 2.2) and placed at 37°C for 1 hour to denature and remove any protein associated with the beads. After collecting the beads by centrifugation (12 000 xg, 4°C, 30 min), the supernatant was collected and 25 µl aliquots were used in Western blotting (see section 2.3. above).

2.5. Yeast whole cell protein and post-translational-modification mass spectrometry-based analysis (Xu *et al*., 2009)

2.5.1. Yeast whole cell protein Mass Spectrometry-based analysis (Figure 2.2)

The strains of interest, W303-1b and NT33-5, were grown in YPD medium at various temperatures (20°C, 30°C, and 33°C) to an OD_{600} of 1 and cleared cell lysates were prepared as described in section 2.3.1. An aliquot of this cleared lysate (20 µl) was mixed with 5 µl of 5×loading dye (Table 2.2), boiled for 1 min and loaded onto a 6% SDS-PAGE gel for electrophoresis at 200 V for 20 min (section 2.3.3.). Electrophoresis for such a brief time allows the proteins to be concentrated at the interface between the stacking and separating gels. After staining the gel with Coomassie (section 2.3.3.), the region showing stain was excised and chopped by hand into ~1×1 mm pieces and transferred to an Eppendorf tube. To this tube was added 250-300 µl of 50 mM ammonium carbonate + 10 mM DTT with vortexing and subsequent incubation at room temperature for 30 min. After the time had elapsed, the tube was centrifuged briefly to pellet the gel pieces and the supernatant was discarded and replaced with 250-300 µl of 50 mM ammonium carbonate + 50 mM iodoacetamide with the vortexing and incubation repeated. Again, after centrifugation the supernatant is discarded and replaced this time with 250-300 µl of 50 mM ammonium carbonate with vortexing and incubation at room temperature for 15 min. The supernatant is changed again to 250-300 µl of 25 mM ammonium carbonate + 5% acetonitrile and the incubation repeated. The acetonitrile concentration was increased stepwise with two 30 min incubations in 25 mM ammonium carbonate + 50% acetonitrile and one 10 min incubation in 250-300 µl 100% acetonitrile. The gel pieces, saturated with acetonitrile, were dried in the SpeedVac at 40°C, resuspended in 40-50 µl of 25 mM ammonium carbonate + 0.01µg/µl of trypsin and incubated at 30°C overnight. By adding four volumes of 60% acetonitrile + 0.5% formic acid to the tube and incubating at room temperature for 15 min, the peptides resulting from the tryptic digestion were allowed to diffuse from the gel pieces into the solution. After vortexing and a brief centrifugation step, the supernatant was transferred to a new tube and the 60% acetonitrile + 0.5% formic acid extraction step repeated twice more. The acetonitrile, formic acid solutions were combined in a single tube and dried completely in the SpeedVac at 40°C. The samples were resuspended in 2% acetonitrile prior to analysis.

Analyses were performed on a Thermo EASY nanoLC II LC system coupled to a Thermo LTQ Orbitrap Velos mass spectrometer equipped with a nano-spray ion source. Typically, 2 µl of each sample (50-500 ng of protein) was injected onto a 10 cm × 100 µm column in-house packed with Michrom Bioresources Inc. Magic C18 stationary phase (5 µm particle diameter and 300Å pore size).

Peptides were eluted using a 20-120 min linear gradient at a flow rate of 400 nl/min with decreasing amounts of mobile phase A (96.9% water, 3% acetonitrile, 0.1% formic acid) and increasing amounts of mobile phase B (2.9% water, 97% acetonitrile, 0.1% formic acid).

A full MS spectrum (*m/z* 400-1400) was acquired in the Orbitrap at a resolution of 60000 and the ten most abundant multiple charged ions were selected for MS/MS sequencing in a linear trap with the option of dynamic exclusion. Peptide fragmentation was performed using a collision-induced dissociation at a normalized collision energy of 35% with an activation time of 10 ms.

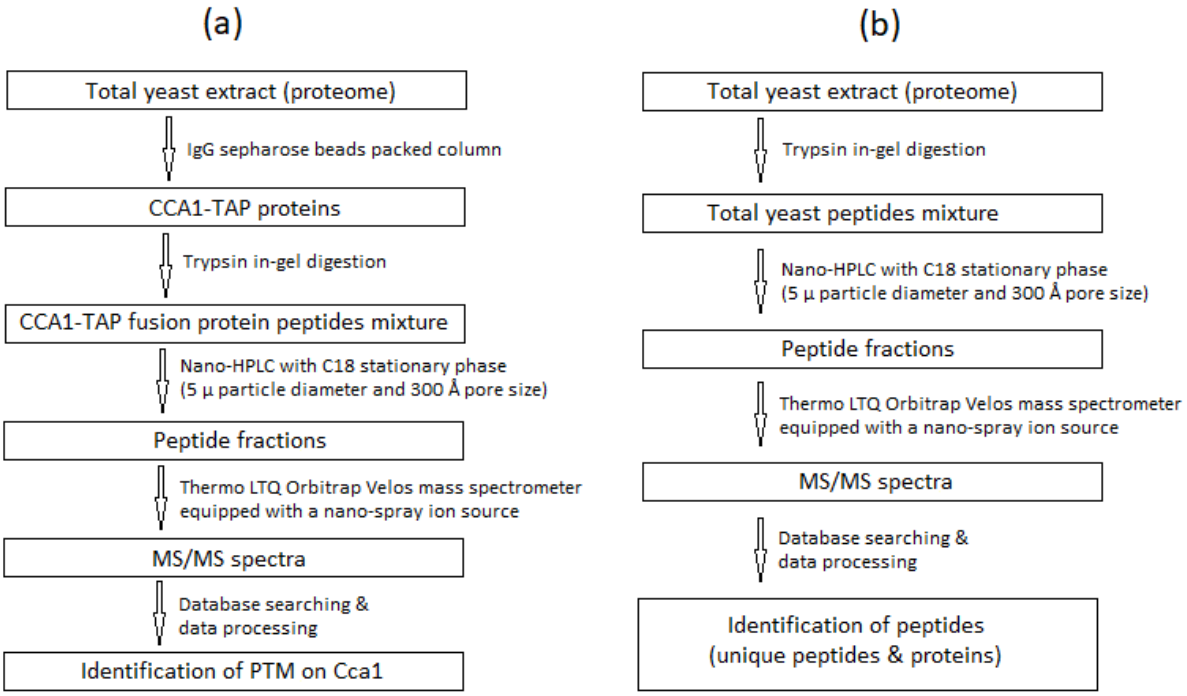


Figure 2.2 The general label-free MS proteomics approach

(a) Post-translational Modification (PTM) identification
(b) The label-free yeast whole cell proteomic approach

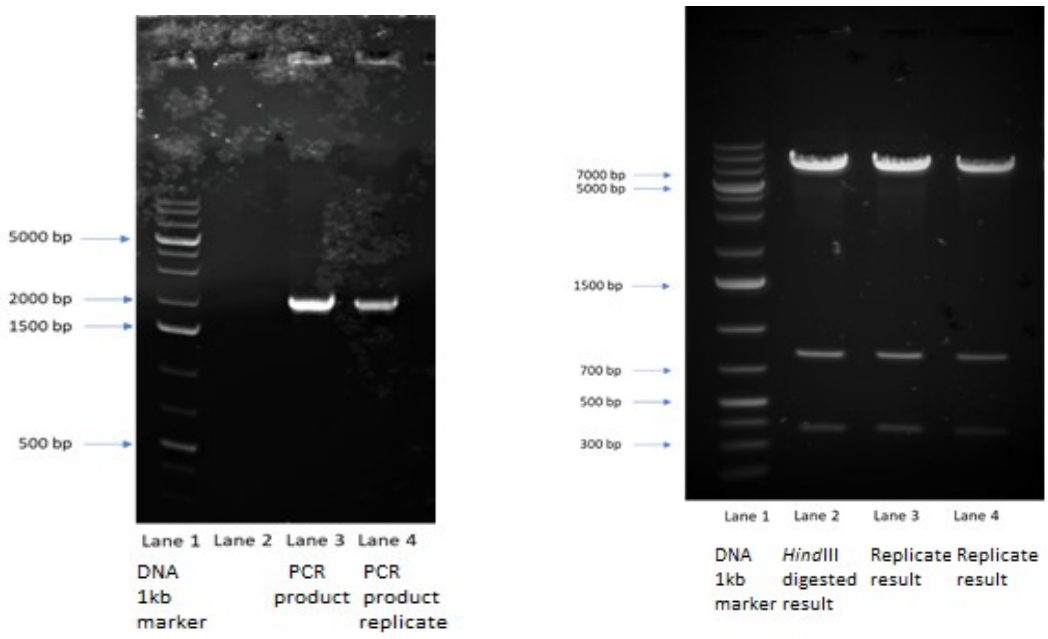
2.5.2. *Saccharomyces cerevisiae* database search

The MS data were processed using Thermo Proteome Discoverer software (v2.1) with the SEQUEST search engine. The search was against the *Saccharomyces cerevisiae* proteome database as downloaded from UniProt. The proteolytic enzyme used to define the peptide database search was chosen as trypsin (full) and the maximum missed cleavage sites were set at three. Mass tolerances of the precursor ion and fragment ion were set at 10 ppm and 0.7 Da, respectively. The search allowed for dynamic modifications on methionine (oxidation, +15.994915 Da) and cysteine (Carbamidomethyl, + 57.021464 Da). Only peptides with high confidence (false discovery rate <1%) were reported.

3. RESULTS

3.1. *CCA1*-TAP fusion plasmid construction and transformation into *Saccharomyces cerevisiae*

The open reading frame coding for a Cca1-TAP fusion protein was amplified from strain YER168C (Dharmacon™ GE Healthcare) by PCR amplification using primers (SCCALF and TAPR). A product of expected size, 2157 bp, was generated (Figure 3.1a), digested with *Bcu*I and *Sal*I and cloned into the vector p426. A diagnostic restriction digestion of the resulting plasmid with *Hin*dIII showed the expected profile (7560 bp, 766 bp, and 386 bp) for the plasmid containing the cloned PCR product (Figure 3.1b). DNA sequence analysis (Genome Quebec Innovation Centre) revealed that the sequence of the insert was correct (Appendix). The successfully constructed plasmid was transformed into yeast strain W303-1B and the fusion protein was successfully produced in high levels as seen from the Western blot (Figure 3.2). The native Cca1 protein shows a mass of between 50 and 75 KDa (Figure 3.2, Lane 2) in good agreement with the predicted molecular mass of 62 KDa. In contrast, the Cca1-TAP tagged fusion protein has a molecular mass of between 75 and 100 KDa (Figure 3.2, Lane 6) in good agreement with the predicted molecular mass of 82 KDa for the fusion protein. It is worth noting that the endogenous levels of Cca1 in *S. cerevisiae* are below the level of detection by Western blotting (Figure 3.2b, Lane 4).



(a) (b)

Figure 3.1 Cloning of *CCA1*-TAP region

(a) Agarose gel electrophoresis of products of polymerase chain reaction (PCR) of the *CCA1*-TAP region of yeast genomic DNA (YSC1178 – YER168c)
(b) *Hin*dIII restriction enzyme digestion to confirm that the plasmids contained the insert of interest

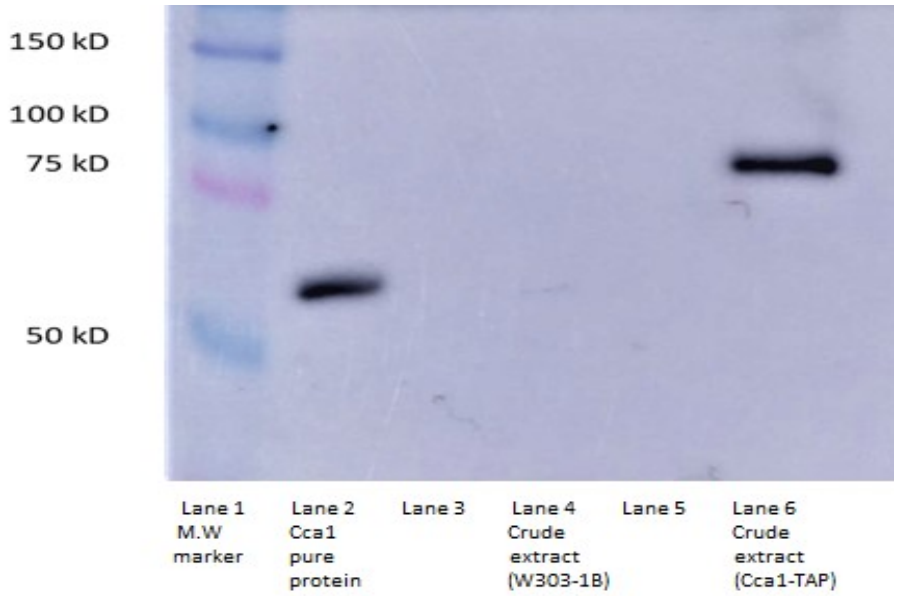


Figure 3.2 Western blot of Cca1 protein

3.2. Mass Spectrometry (MS) results of post-translational modification (PTM) of Cca1

Yeast strain Cca1-TAP was generated to produce large amounts of Cca1-TAP fusion protein by placing the *CCA1-TAP* gene fusion under the control of the GPD promoter in plasmid p426 (Mumberg *et al*., 1995) and transforming this into the haploid yeast strain (W303-1B). This haploid strain was grown in SC (-URA) medium with shaking (225 rpm) at 30°C and a growth curve generated (Figure 3.3). The shape of the resulting curve was typical of growth curves in that it started with an initial lag phase where growth was slow, then showed a log phase with more rapid growth and ended with a stationary phase (Figure 3.3). Based on this growth curve, cells were collected at OD_{600} values of 1.0, the early log phase. These cells were lyzed and the Cca1-TAP fusion protein was purified, digested with trypsin and subjected to MS analysis under the parameters described to search for post-translational modifications. Cells in log phase showed multiple sites exhibiting potential phosphorylation and acetylation on the Cca1 protein (Table 3.1). To show that the identification of these modifications was reproducible the experiment was carried out with three biological replicates. Importantly, the acetylations at C307 and K312 were identified in all three samples while the phosphorylation at Y317 was identified once in three samples (Table 3.1).

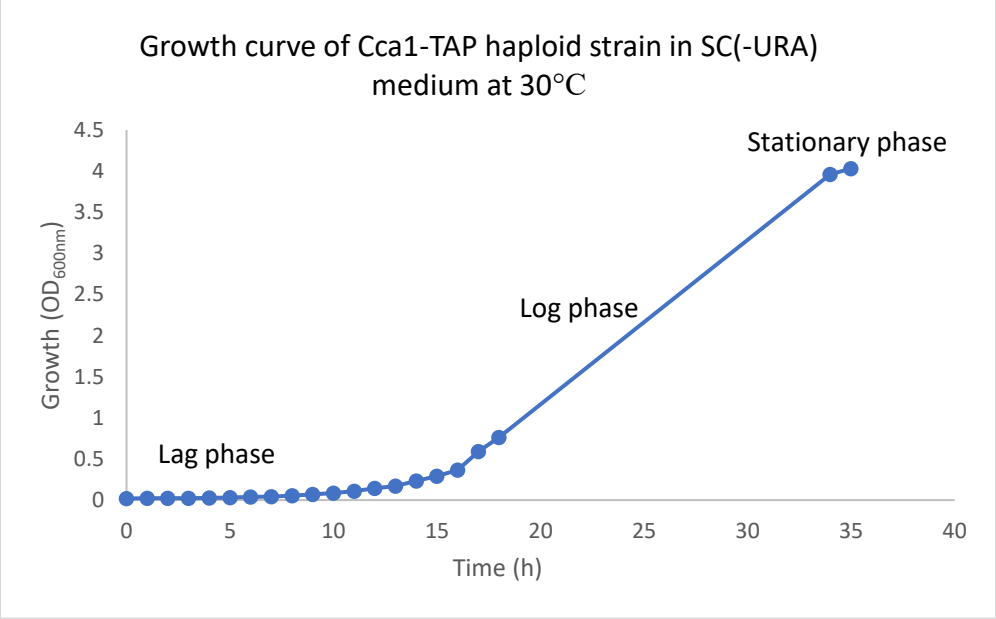


Figure 3.3 Growth curve of Cca1-TAP1 over-expressing haploid strain in SC (-URA) medium at 30°C

Table 3.1 Post-translational modifications identified on the Cca1-TAP protein extracted from the early log phase cells

Position	Target	Modification	PTM Score (%)
307	C	Acetylation	100.00
312	K	Acetylation	89.83
317	Y	Phosphorylation	100.00

The PTMs of Cca1-TAG identified in three separate cell preparations are listed. The amino acid position, modified amino acid, and type of modification are indicated in the first three columns, respectively. The PTM scores and highest peptide confidence values were provided automatically by Thermo Proteome Discoverer (v2.1, SP1) software. The Sequence Motif column shows the position of the modification (lower case and italics) in the original peptide sequence. The acetylation sites were identified in all three replicates, but the phosphorylation only once.

3.3. Yeast cell proteomics

3.3.1. Harvesting wild-type and temperature-sensitive strains at the permissive and quasi-restrictive temperatures

The wild-type strain (W303-1B) or the temperature-sensitive mutant strain (NT33-5, derived from W303-1B) was grown in enriched medium (YPD) at 20°C, 30ºC, or 33ºC to an OD_{600} value of 1.0 (early log phase). The cells then were harvested and lysed as described previously (section 2.3.1.). As we had seen for growth at 22ºC (Shan *et al*., 2008) and for a temperature-sensitive strain bearing the *cca1-E189K* mutation at 37ºC (Shan *et al*., 2008), the *wt* and *ts* strains showed similar growth rates at 20°C and at 30ºC. They reached the appropriate OD_{600} after 18-20 hours at 20°C and after 8-9 hours at 30ºC. As the temperature-sensitive strain did not grow at 37°C, the highest temperature that could be used was 33°C. At this temperature, we were able to harvest *wt* cells after 7-8 hours and the *ts* strain after 48-50 hours.

The ranges listed are defined by three biological replicates for each strain at each temperature. In all cases, the growth curves resembled typical yeast growth curves as seen in

Figure 3.3 except for the *ts* strain at 33°C. In this case, the cell density increased slowly to the 30-h time point at which time it entered a more rapid log growth phase. The abrupt increase in cell density after 30 h suggests that a mutation or mutations accumulated either in the *cca1-E189K* gene (reversion or intragenic suppressor) or elsewhere in the NT33-5 genome (intergenic suppressor) to allow this increase in growth. As the mutation leading to the temperature-sensitive phenotype is a single guanine-to-adenine transition, it is possible that this increased growth reflects a reversion at this position although the growth rate is less than that of the *wt* strain. In spite of the fact that the increased growth rate may relate to additional mutations in the strain, cells were harvested for analysis anyway.

3.3.2. Mass Spectrometric results

Three biological replicate batches were analyzed, and each batch contained six samples (two strains cultured at three different temperatures) with three technical replicates. Table 3.2 summarizes the number of identified total proteins in the three batches. In analyzing the data, the parameters were set such that a protein was accepted only when it had at least two unique peptides identified. The abundance of the protein was calculated and reported by the software, Thermo Proteome Discoverer (v2.1, SP1). The summarized identification results are shown in Table 3.2. These data show that under any experimental condition more than 1000 proteins were seen with the range of proteins identified varying between approximately 1100 and 1300. In each batch, a number of proteins ranging from two to 62 were detected only under a specific condition. There are no obvious correlations between the total number of proteins and the number of unique proteins and there is much variability between replicates of the same condition.

Table 3.2 LC-MS/MS results for wild-type and temperature-sensitive yeast proteomes grown at the permissive and quasi-restrictive temperatures

Strains	The total protein number identified (≥2 unique peptides)		
	20°C	**30°C**	**33°C**
W303-1B	1198	1192	1063
W303-1B	1311	1273	1322

Strains	The total protein number identified (≥2 unique peptides)		
	20°C	**30°C**	**33°C**
W303-1B	1179	1169	1184
wt average	1229	1211	1190
NT33-5	1146	1182	1120
NT33-5	1279	1284	1332
NT33-5	1142	1174	1148
ts average	1189	1213	1200

LC-MS/MS analysis was repeated three times for each sample. Proteins were identified with at least two unique peptides by searching against the Uniprot *Saccharomyces cerevisiae* database (https:// www.uniprot.org/).

3.3.2.1. Comparisons of the proteins identified in the wild-type and temperature-sensitive strains

We had total of 21 698 proteins in 18 samples. Before analyzing the properties of the proteome, we needed to ensure that all data may be used and, therefore, a quality control was performed. First, we decided that if the ratio of a protein abundance was equal to or higher than three as compared between two samples, then this difference in protein abundance would be taken as significant. To account for measurement error, including random error and systematic error, a test was performed with the help of Dr. H. Jiang in CBAMS. In the test, tryptic peptides derived from a single yeast whole cell lysate were analyzed by LC-MS/MS six times. Based on the calculated results, we noticed a 99.9% prediction interval when the ratio of the protein abundance was larger than 2.2, which indicated that a ratio larger than 2.2 was the limit of an estimate of plausible values. We consider a number of 3.0, which the probability of a reported regulated protein is due to measurement errors is <0.1%, as a threshold to filter the ratio of protein abundance between two samples.

Of the more than 1100 proteins detected by MS in each sample, there were 725 that were common to both strains at all three temperatures tested. Based on these 725 common proteins, a comparison of the relative abundance of these proteins in the wild-type strain as compared to the temperature-sensitive strain was carried out for each temperature. We found that the difference in the abundance of the majority of these common proteins was not statistically

significant (difference ratio less than three) between the two strains at each temperature tested (data not shown).

3.3.2.2. Protein expression comparison between wild-type and temperature-sensitive mutant strains

3.3.2.2.1. Permissive temperature (20°C)

We identified 914 proteins common to the wild-type strain and the temperature-sensitive strain at 20°C. Of these proteins, only one (Ypt7, P32939) showed an increased amount (*p* value = 0.19) in the wild-type strain as compared to the temperature-sensitive strain and only one (Utp9, P38882) showed an increased amount (*p* value = 0.34) in the temperature-sensitive strain as compared to the wild-type strain. However, the large *p* value suggests that their difference may not be true.

3.3.2.2.2. Elevated temperature (30ºC)

We identified 933 proteins common to the two strains at 30°C. We found six proteins with higher levels (but none with lower levels) in the temperature-sensitive strain as compared to the wild-type strain (Table 3.3) based on the ratio of the protein abundances. The differences in the abundance ratios of these six proteins between the temperature-sensitive strain and the wild-type strain varied from approximately three to six-fold (Table 3.3).

Table 3.3 Six proteins showing greater amounts in the temperature-sensitive strain (*ts*) as compared to the wild-type strain (*wt*) at 30°C based on protein abundance

Accession	Protein name	Gene	Ratio of abundance (*ts*/*wt*)	*p* value
P00815	Histidine biosynthesis trifunctional protein	*HIS4*	6.2	0.14
P06105	Protein SCP160	*SCP160*	4.6	0.07
P07172	Histidinol-phosphate aminotransferase	*HIS5*	4.2	0.07

Accession	Protein name	Gene	Ratio of abundance (*ts*/*wt*)	*p* value
P38998	Saccharopine dehydrogenase [NAD(+), L-lysine-forming]	*LYS1*	4.2	0.18
P03965	Carbamoyl-phosphate synthase arginine-specific large chain	*CPA2*	4.2	0.03
P07702	L-2-aminoadipate reductase	*LYS2*	3.1	0.02

3.3.2.2.3. Quasi-restrictive temperature (33ºC)

We identified 787 proteins common to the wild-type and temperature-sensitive strains at 33ºC. Of these proteins, nine were at reduced levels in the temperature-sensitive strain as compared to the wild-type strain (Table 3.4), and 14 were at increased levels in the temperature-sensitive strain as compared to the wild-type strain (Table 3.5). With one exception (Gly1), all of the nine proteins showing higher abundance in the wild-type strain as compared to the temperature-sensitive strain are involved in RNA maturation or ribosome assembly.

In contrast, the 14 proteins showing higher amounts in the temperature-sensitive strain as compared to the wild-type strain were primarily related to a stress response, *e.g.*, DNA replication stress (Ape1, Dcs1, Pgm2, Tdh1), oxidative stress (Sod2, Trx2), or a combination of both (Uga1). The remaining seven proteins are involved in catabolic (Idh2) or anabolic processes (Cpa2, Bna1, His4, His5, Lys1, Ser3) (Table 3.5).

Table 3.4 Proteins showing reduced levels in the temperature-sensitive strain as compared to the wild-type strain at 33°C

Accession	Protein	Gene	*wt*/*ts*	*p* value
P37303	Low specificity L-threonine aldolase	*GLY1*	5.8	0.08
P49166	60S ribosomal protein L37-A	*RPL37A*	4.7	0.36
P05748	60S ribosomal protein L15-A	*RPL15A*	4.5	0.00
P0CX84	60S ribosomal protein L35-A	*RPL35A*	3.7	0.01
Q02892	Nucleolar GTP-binding protein 1	*NOG1*	3.5	0.11

Accession	Protein	Gene	*wt*/*ts*	*p* value
P04650	60S ribosomal protein L39	*RPL39*	3.3	0.03
P24000	60S ribosomal protein L24-B	*RPL24B*	3.2	0.03
Q12211	tRNA pseudouridine synthase 1	*PUS1*	3.6	0.22
P35178	Ribosomal RNA-processing protein 1	*RRP1*	3.0	0.08

The range of the ratios is from three to six-fold.

Table 3.5 Proteins showing increased levels in the temperature-sensitive strain as compared to the wild-type strain at 33°C

Accession	Protein name	Gene	*ts*/*wt*	*p* value
P00360	glyceraldehyde-3-phosphate dehydrogenase 1	*TDH1*	17.3	0.02
P00815	histidine biosynthesis trifunctional protein	*HIS4*	6.0	0.17
P14904	vacuolar aminopeptidase 1	*APE1*	5.4	0.02
P38998	Saccharopine dehydrogenase [NAD(+), L-lysine-forming]	*LYS1*	4.1	0.37
P37012	Phosphoglucomutase 2	*PGM2*	4.0	0.07
P47096	3-hydroxyanthranilate 3,4-dioxygenase	*BNA1*	3.8	<0.01
P00447	Superoxide dismutase [Mn], mitochondrial	*SOD2*	3.6	<0.01
P40054	D-3-phosphoglycerate dehydrogenase 1	*SER3*	3.6	0.10
P03965	carbamoyl-phosphate synthase arginine-specific large chain	*CPA2*	3.2	<0.01
Q06151	M7GpppX diphosphatase	*DCS1*	3.2	<0.01
P07172	histidinol-phosphate aminotransferase	*HIS5*	3.2	0.06
P28241	Isocitrate dehydrogenase [NAD] subunit 2, mitochondrial	*IDH2*	3.1	0.12
P17649	4-aminobutyrate aminotransferase	*UGA1*	3.0	0.03
P22803	thioredoxin-2	*TRX2*	2.8	<0.01

The ratio of the abundance varied from three to seventeen-fold.

3.3.2.3. Temperature effects within a single strain

3.3.2.3.1. Wild-type strain

To validate the variation of protein abundances linked to increasing temperatures, we compared the differences in proteins in a single strain at the different temperatures. In the wild-type strain, most of the common proteins showed no statistically significant difference in abundance at all three temperatures. Only ten proteins showed a significant increase in abundance in the wild-type strain at 33ºC as compared to 20°C and 30°C. Of the proteins showing higher levels (p<0.01) at 33°C as compared to the lower temperatures (20°C and 30°C) (Table 3.6), most were involved in some aspect of a stress response, e.g., ATP-dependent RNA helicase Dbp5 which is essential for mRNA export from the nucleus and plays a role in the cellular response to heat stress, Hch1, Hsp10 involved in heat stress, Grx1 involved in oxidative stress, and Tps1 involved in a combination of both. Of the others, Ssa1 is involved in the protein folding response, Ald4 is involved in metabolic processes during anaerobic growth on glucose, Hxk1 is involved in glucose homeostasis, Fas2 is involved in fatty acid biosynthesis and Pnc1 involved in the NAD+ salvage pathway.

Table 3.6 Proteins with increased abundance in the wild-type strain at 30°C and 33°C as compared to 20°C

Accession	Protein name	Gene	*wt* 30/20	*p* value	*wt* 33/20	*p* value
P20449	ATP-dependent RNA helicase dbp5	*DBP5*	3.0	0.05	6.0	<0.01
P46367	Potassium-activated aldehyde dehydrogenase, mitochondrial	*ALD4*	2.0	0.14	5.0	<0.01
P04806	Hexokinase-1	*HXK1*	1.0	0.45	4.0	<0.01
P19097	Fatty acid synthase subunit alpha	*FAS2*	1.0	0.51	4.0	0.29
P53834	Hsp90 co-chaperone HCH1	*HCH1*	2.0	<0.05	4.0	<0.01
P38910	10 kDa heat shock protein, mitochondrial	*HSP10*	2.0	0.06	4.0	<0.01
P10591	heat shock protein SSA1	*SSA1*	2.0	0.42	3.0	0.13
P53184	Nicotinamidase	*PNC1*	2.0	0.43	3.0	0.02
Q00764	Alpha,alpha-trehalose-phosphate synthase [UDP-forming] 56 kDa subunit	*TPS1*	1.0	0.61	3.0	0.07
P25373	Glutaredoxin-1	*GRX1*	2.0	0.2	3.0	0.01

3.3.2.3.2. Temperature-sensitive strain

Similar to the wild-type strain, most of the common proteins in the temperature-sensitive strain showed no significant differences in protein abundance at all three temperatures. However, compared to the small number of proteins, 10, in the wild-type strain showing increased abundance (Table 3.6), there are many more proteins, 54, in the temperature-sensitive strain showing increased abundance at 33°C as compared to 20°C or 30°C (Table 3.7).

In the *ts* strain, the protein showing the greatest increase in abundance was Tdh1, glyceraldehyde-3-phosphate dehydrogenase 1, which showed a 47-fold increase as compared to the level at 20°C (p=0.089) (highlighted in Table 3.7).

Table 3.7 Proteins with increased abundance in the temperature-sensitive strain at 30°C and 33°C as compared to 20°C.

Accession	Protein name	Gene	*ts* 30/20	*p* value	*ts* 33/20	*p* value
P00360	glyceraldehyde-3-phosphate dehydrogenase 1	*TDH1*	1.0	0.106	47.0	0.089
P46367	Potassium-activated aldehyde dehydrogenase, mitochondrial	*ALD4*	2.0	0.089	12.0	0.024
P14904	vacuolar aminopeptidase 1	*APE1*	1.0	0.403	9.0	0.019
P53184	Nicotinamidase	*PNC1*	1.0	0.763	8.0	0.047
P00815	histidine biosynthesis trifunctional protein	*HIS4*	6.0	0.136	7.0	0.16
P34227	Mitochondrial peroxiredoxin PRX1	*PRX1*	2.0	0.011	7.0	0.035
P00447	Superoxide dismutase [Mn], mitochondrial	*SOD2*	1.0	0.115	7.0	0.051
Q06151	M7GpppX diphosphatase	*DCS1*	2.0	0.079	6.0	0.019
P38910	10 kDa heat shock protein, mitochondrial	*HSP10*	2.0	0.185	6.0	0.141
P38715	NADPH-dependent aldose reductase GRE3	*GRE3*	2.0	0.202	6.0	0.011
Q04432	Glutathione-independent glyoxalase HSP31	*HSP31*	1.0	0.925	6.0	0.026
P40106	Glycerol-1-phosphate phosphohydrolase 2	*GPP2*	1.0	0.659	6.0	0.061
P17709	Glucokinase-1	*GLK1*	1.0	0.07	6.0	0.024
P28241	Isocitrate dehydrogenase [NAD] subunit 2, mitochondrial	*IDH2*	2.0	0.168	5.0	0.022
P17649	4-aminobutyrate aminotransferase	*UGA1*	2.0	0.466	5.0	0.028
P10591	heat shock protein SSA1	*SSA1*	2.0	0.338	5.0	0.011
P38075	pyridoxamine 5'-phosphate oxidase	*PDX3*	2.0	0.353	5.0	0.031
P50861	6,7-dimethyl-8-ribityllumazine synthase	*RIB4*	2.0	0.393	5.0	0.046
P33734	imidazole glycerol phosphate synthase hisHF	*HIS7*	3.0	0.306	5.0	0.118

Accession	Protein name	Gene	*ts* 30/20	*p* value	*ts* 33/20	*p* value
P19882	heat shock protein 60, mitochondrial	*HSP60*	2.0	0.209	5.0	0.02
Q00764	Alpha,alpha-trehalose-phosphate synthase [UDP-forming] 56 kDa subunit	*TPS1*	1.0	0.815	5.0	0.05
P47096	3-hydroxyanthranilate 3,4-dioxygenase	*BNA1*	2.0	0.082	5.0	0.016
P06106	Homocysteine/cysteine synthase	*MET17*	3.0	0.07	5.0	0.021
P00890	citrate synthase, mitochondrial	*CIT1*	1.0	0.088	5.0	0.023
P80210	adenylosuccinate synthetase	*ADE12*	2.0	0.1	5.0	0.063
P00729	carboxypeptidase Y	*PRC1*	2.0	0.372	5.0	0.061
Q00955	Acetyl-CoA carboxylase	*ACC1*	2.0	0.388	5.0	0.183
P04840	Mitochondrial outer membrane protein porin 1	*POR1*	2.0	0.146	4.0	0.01
P06101	Hsp90 co-chaperone Cdc37	*CDC37*	2.0	0.26	4.0	0.079
P53834	Hsp90 co-chaperone HCH1	*HCH1*	2.0	0.015	4.0	0.07
P36010	Nucleoside diphosphate kinase	*YNK1*	1.0	0.304	4.0	<0.01
P28834	Isocitrate dehydrogenase [NAD] subunit 1, mitochondrial	*IDH1*	2.0	0.173	4.0	<0.01
P19097	Fatty acid synthase subunit alpha	*FAS2*	1.0	0.538	4.0	0.051
P38115	D-arabinose dehydrogenase [NAD(P)+] heavy chain	*ARA1*	1.0	0.639	4.0	0.013
P17505	Malate dehydrogenase, mitochondrial	*MDH1*	1.0	0.155	4.0	0.014
P37291	Serine hydroxymethyltransferase, cytosolic	*SHM2*	3.0	0.132	4.0	0.154
P04806	Hexokinase-1	*HXK1*	1.0	0.405	4.0	0.058
Q12335	Protoplast secreted protein 2	*PST2*	1.0	0.904	4.0	0.077
P47176	Branched-chain-amino-acid aminotransferase, cytosolic	*BAT2*	2.0	0.278	4.0	0.106
P53912	Uncharacterized protein YNL134C	*YNL134C*	1.0	0.519	4.0	<0.01
P14832	peptidyl-prolyl cis-trans isomerase	*CPR1*	1.0	0.488	4.0	0.065
P25373	Glutaredoxin-1	*GRX1*	1.0	0.922	4.0	0.073
P35719	Uncharacterized protein MRP8	*MRP8*	1.0	0.325	4.0	0.011
P25719	Peptidyl-prolyl cis-trans isomerase C, mitochondrial	*CPR3*	1.0	0.806	4.0	0.058
P38765	Uncharacterized isomerase YHI9	*YHI9*	3.0	0.167	4.0	0.075
P30624	Long-chain-fatty-acid--CoA ligase 1	*FAA1*	2.0	0.15	4.0	0.074
P53312	Succinate--CoA ligase [ADP-forming] subunit beta, mitochondrial	*LSC2*	1.0	0.248	3.0	<0.01
P31539	heat shock protein 104	*HSP104*	1.0	0.613	3.0	0.05
P33416	Heat shock protein 78, mitochondrial	*HSP78*	1.0	0.931	3.0	0.072
P07172	histidinol-phosphate aminotransferase	*HIS5*	4.0	0.072	3.0	0.064
Q12363	Transcriptional modulator WTM1	*WTM1*	1.0	0.672	3.0	0.077
P25294	Protein SIS1	*SIS1*	1.0	0.504	3.0	0.029
P39929	vacuolar-sorting protein snf7	*SNF7*	2.0	0.264	3.0	0.045

Red highlight indicates greatest increased abundance, the yellow highlighted proteins also showed increased abundance in the wild-type stain at the same temperature, the grey highlighted proteins are linked to mitochondria, and the blue highlighted proteins were increased in the temperature-sensitive strain at both 30°C and 33°C.

The remaining proteins showing increased levels can be separated into several groups based on function and protein level: six proteins showed equal abundances at 33°C and 30°C but an increase over their levels at 20°C (blue highlighted in Table 3.7). Of these, five are involved in anabolic processes (His4, His5, His7, Met17, and Shm2) while the final one (Yhi9) is an uncharacterized isomerase. Nine proteins (Ald4, Hxk1, Fas2, Hch1, Hsp10, Ssa1, Pnc1, Tps1, Grx1) also show increased abundance in the wild-type strain at 33°C (yellow highlighted in Table 3.7).

Of the remaining proteins: eleven are linked to mitochondria (Cit1, Cpr3, Hsp60, Hsp78, Idh1, Idh2, Lsc2, Mdh1, Por1, Prx1, and Sod2) (grey in Table 3.7); twelve are stress response proteins linked to DNA replication stress (Cpr1, Mrp8, Sis1, Ynl134C), DNA damage (Ynk1), heat or oxidative stress (Dcs1, Gpp2, Gre3, Hsp104, Hsp31, Pst2, Uga1); nine are involved in anabolic processes (Acc1, Ade12, Bat2, Bna1, Rib4, Ser3, Ara1, Faa1, and Glk1) and three are involved in catabolic processes in the vacuole (Ape1, Prc1, Snf7). The remaining three proteins are involved in protein stabilization and regulation of the cell cycle (Cdc37) or transcriptional modulation with roles in meiotic regulation and silencing (Wtm1) or pyridoxal 5'-phosphate salvage (Pdx3).

3.3.2.4. Unique proteins

While the data till now relate to proteins that were present in one or both strains at one or more temperatures, some proteins were present or absent in only one strain at only one temperature. There was a small subset of 14 proteins present only in the temperature-sensitive strain only at 33°C (Table 3.8). Among these unique proteins, six were related to a stress response, *e.g.*, DNA replication stress (Ddp1, Sol1, Ykl151C), oxidative stress (Ctt1), or a combination of both of these (Gad1, Uga2). The remaining eight proteins are involved in

catabolic (Nqm1, Tda3) or anabolic processes (Ecm4, GLC3, Ynl200C) or have no known function (Gpm2, Ymr090W, Ydr061W).

Table 3.8 Fourteen unique proteins present only in the temperature-sensitive strain at 33°C

Accession	Description	Gene	Function
P06115	catalase T	*CTT1*	Occurs in almost all aerobically respiring organisms and serves to protect cells from the toxic effects of hydrogen peroxide. Cytosolic catalase T; has a role in protection from oxidative damage by hydrogen peroxide
Q99321	Diphosphoinositol polyphosphate phosphohydrolase DDP1	*DDP1*	May eliminate potentially toxic dinucleoside polyphosphates during sporulation,protein abundance increases in response to DNA replication stress
P36156	Glutathione S-transferase omega-like 2	*ECM4*	May be involved in cell wall organization and biogenesis
Q04792	glutamate decarboxylase	*GAD1*	Glutamate decarboxylase; converts glutamate into gamma-aminobutyric acid (GABA) during glutamate catabolism; involved in response to oxidative stress
P32775	1,4-alpha-glucan-branching enzyme	*GLC3*	Glycogen branching enzyme, involved in glycogen accumulation
Q12008	phosphoglycerate mutase 2	*GPM2*	Could be non-functional.
P53228	Transaldolase NQM1	*NQM1*	Transaldolase is important for the balance of metabolites in the pentose-phosphate pathway.
P50278	6-phosphogluconolactonase-like protein 1	*SOL1*	May be involved in regulation of tRNA subcellular distribution, protein abundance increases in response to DNA replication stress
P38758	Putative oxidoreductase TDA3	*TDA3*	Putative oxidoreductase that negatively regulates the retrieval of cargo from late endosomes to the Golgi. Regulates YIF1 and KEX2 localization. Required for fast DNA replication. Putative oxidoreductase involved in late endosome to Golgi transport
P38067	Succinate-semialdehyde dehydrogenase [$NADP^{(+)}$]	*UGA2*	Involved in the GABA shunt pathway as a nitrogen source; part of the 4-aminobutyrate and glutamate degradation pathways
Q12298	uncharacterized ABC transporter ATP-binding protein YDR061W	*YDR061W*	ATPase activity; ATP binding
P36059	ATP-dependent (S)-NAD(P)H-hydrate dehydratase	*YKL151C*	Nicotinamide nucleotide metabolic process
Q04304	UPF0659 protein YMR090W	*YMR090W*	Putative protein of unknown function
P40165	NAD(P)H-hydrate epimerase	*YNL200C*	NADP metabolic process

Twenty-three proteins (Table 3.9) were absent only from the temperature-sensitive strain at 33ºC but were found in either the wild-type or the temperature-sensitive cells at 20°C or 30°C, or in the wild-type strain at 33°C. Again, these proteins have many functions, but the majority are associated with mitochondria (Aim24, Atp20, Cox2, Cox5A, Mhr1, Nuc1, and Rip1) and/or ribosomes (Drs1, Edc3, Hca4, Nog2, Nsa2, Rlp7, Sdo1, Sof1, Tsr4, Utp23).

Table 3.9 The list of 23 proteins detected in one or more of the conditions tested and absent only from the temperature-sensitive strain at 33ºC

Accession	Protein name	Gene	Function
P53909	Adenine deaminase	*AAH1*	Plays an important role in the purine salvage pathway and in nitrogen catabolism
P47127	Altered inheritance of mitochondria protein 24, mitochondrial	*AIM24*	Protein with a role in determining mitochondrial architecture; inner membrane protein that interacts physically and genetically with the MICOS complex and is required for its integrity
Q12233	ATP synthase subunit g, mitochondrial	*ATP20*	Mitochondrial membrane ATP synthase (F1F0 ATP synthase or Complex V) produces ATP from ADP in the presence of a proton gradient across the membrane which is generated by electron transport complexes of the respiratory chain.
P00410	Cytochrome c oxidase subunit 2	*COX2*	Cytochrome c oxidase is the component of the respiratory chain that catalyzes the reduction of oxygen to water.
P00424	Cytochrome c oxidase polypeptide 5A, mitochondrial	*COX5A*	Subunit Va of cytochrome c oxidase; cytochrome c oxidase is the terminal member of the mitochondrial inner membrane electron transport chain
P07258	Carbamoyl-phosphate synthase arginine-specific small chain	*CPA1*	Synthesized by two pathway-specific (arginine and pyrimidine) under separate control
P32892	ATP-dependent RNA helicase DRS1	*DRS1*	ATP-binding RNA helicase involved in ribosome assembly
P39998	Enhancer of mRNA-decapping protein 3	*EDC3*	Stimulates decapping of both stable and unstable mRNA during mRNA decay.
P53045	Methylsterol monooxygenase	*ERG25*	Catalyzes the first step in the removal of the two C-4 methyl groups of 4,4-dimethylzymosterol.
P54781	Cytochrome P450 61	*ERG5*	Required to form the C-22(23) double bond in the sterol side chain.
P20448	ATP-dependent RNA helicase HCA4	*HCA4*	ATP-dependent RNA helicase required for ribosome biogenesis.

Accession	Protein name	Gene	Function
Q07938	S-methyl-5'-thioadenosine phosphorylase	*MEU1*	catalyzes the initial step in the methionine salvage pathway
Q06630	Mitochondrial homologous recombination protein 1	*MHR1*	Component of the mitochondrial ribosome (mitoribosome), a dedicated translation machinery responsible for the synthesis of mitochondrial genome-encoded proteinshas extraribosomal functions, being involved in regulation of mitochondrial DNA recombination, maintenance and repair, and generation of homoplasmic cells
P53615	Carbonic anhydrase	*NCE103*	Involved in protection against oxidative damage.
P53742	Nucleolar GTP-binding protein 2	*NOG2*	Putative GTPase; associates with pre-60S ribosomal subunits in the nucleolus and is required for their nuclear export and maturation
P40078	Ribosome biogenesis protein nsa2	*NSA2*	Involved in the biogenesis of the 60S ribosomal subunit. May play a part in the quality control of pre-60S particles.
P08466	mitochondrial nuclease	*NUC1*	Major mitochondrial nuclease; has RNAse and DNA endo- and exonucleolytic activities; roles in mitochondrial recombination, apoptosis and maintenance of polyploidy; involved in fragmentation of genomic DNA during PND (programmed nuclear destruction)
P08067	cytochrome b-c1 complex subunit Rieske, mitochondrial	*RIP1*	Component of the ubiquinol-cytochrome c reductase complex (complex III or cytochrome b-c1 complex), which is a respiratory chain that generates an electrochemical potential coupled to ATP synthesis. The complex couples electron transfer from ubiquinol to cytochrome c.
P40693	Ribosome biogenesis protein RLP7	*RLP7*	Involved in the biogenesis of the 60S ribosomal subunit
Q07953	Ribosome maturation protein SDO1	*SDO1*	Involved in the biogenesis of the 60S ribosomal subunit and translational activation of ribosomes.
P33750	Protein SOF1	*SOF1*	Protein required for biogenesis of 40S (small) ribosomal subunit, Required for ribosomal RNA processing.
P25040	20S rRNA accumulation protein 4	*TSR4*	Required for processing of the 20S pre-rRNA at site D to generate mature 18S rRNA.
Q12339	rRNA-processing protein utp23	*UTP23*	Involved in rRNA-processing and ribosome biogenesis

4. DISCUSSION AND CONCLUSIONS

4.1. Post-translational modification (PTM) of Cca1 protein

Post-translational modifications (PTMs) refer to biochemical modifications that occur to one or more amino acid residues on a protein following protein biosynthesis. PTMs may include acetylation, glycosylation, phosphorylation, proteolytic cleavage, ubiquitination, etc., and play a fundamental role in protein activity, regulation, translocation, and interaction within the cell. The major challenge in the detection of protein PMTs *in vivo* originates from the low abundance of many cellular proteins. As an example, Cca1 is present at less than 0.01% of total yeast cellular protein (Chen *et al*., 1990) and is at a level so low as to be undetectable by Western blotting (Figure 3.2). To overcome the problem of low endogenous protein levels, the protein can be overexpressed, and/or a sensitive detection method employed. Here we used a combination of both of these approaches. First, to increase the abundance of Cca1, we engineered a high copy number plasmid to overexpress the gene encoding Cca1 fused to a multifunctional TAP tag. The open reading frame coding for the Cca1/TAP fusion protein was cloned downstream of the GPD promoter in p426, a high copy number plasmid (Mumberg *et al*., 1995). This plasmid increased expression of β-galactosidase by approximately 1000-fold as compared to the CYC promoter (Mumberg *et al*., 1995) and we anticipated that it would do the same for Cca1. That the level of Cca1 was increased is evident from the appearance of a strong Cca1/TAP band in our Western blot in the overexpressing strain as compared to the native strain (Figure 3.2).

By expressing this protein with a TAP tag, we hoped to be able to efficiently enrich it from the whole cell extract. The plan was to express sufficient quantities of Cca1 *in vivo* and facilitate the purification of this protein via the fused tag. Once we had isolated the tagged protein we then could analyze it to see if it contained any post-translational modifications. There is no reason to expect that the protein would be completely modified at any one position, so it was necessary to isolate as much protein as possible in case only a small percentage of the protein contained any specific modification. The TAP tag harbours a Protein A module which has affinity for IgG Sepharose beads. Theoretically, very few yeast proteins should interact with the IgG resin, so we should get a good enrichment of the fusion protein. We anticipated that after

washing the resin and removing any non-specifically bound proteins, Cca1 would be conveniently released from the solid support by cleavage with the TEV protease which removes the Protein A tag. Unfortunately, we found that Cca1 was not released from the IgG solid support after addition of the TEV protease. We propose that the TEV cleavage site is inaccessible in the folded Cca1-TAP fusion protein as carrying out the same experiment with multiple different batches of TEV protease including one that was shown to cleave another TAP-tagged protein (kindly provided by Dr. Michael Sacher) gave the same negative result. As we reasoned that few yeast proteins were bound to the IgG beads, we simply treated the resin with SDS-containing buffer to release the Cca1-TAP fusion protein and any other proteins that were bound. The resulting eluate was analyzed by mass spectrometry. Regrettably, about 200 non-specific binding proteins were also retained on the resin together with Cca1. These additional proteins competed with Cca1 and interfered with the detection of the PTMs on our target protein. Nevertheless, our proteomics analysis was moderately successful and allowed the detection of a single phosphorylation site (Y317) and two acetylation sites (C307 and K312) in three biological replicates with high confidence values (PTM scores > 85%) in each case (Table 3.1).

It is recognized that the first 17 amino acids of the peptide sequence translated from the first in-frame ATG of *CCA1* acts as a mitochondrial localization signal (Wolfe *et al.*, 1996). However, it is still unknown how Cca1 is transported into the nucleus. As we know that tRNA nucleotidyltransferase is found in the nucleus as some tRNAs are not released from the nucleus unless they contain the CCA sequence (Simos and Hurt, 1999) we believe that Cca1 must have a nuclear localization signal. While it is possible that Cca1 binds to tRNAs in the cytosol and is transported with them back into the nucleus by a tRNA retrograde import mechanism (Rubio and Hopper, 2011), it seems more likely that Cca1 is the carrier and the tRNA is the passenger. Nuclear accumulation of cytoplasmic tRNA occurs when yeast cells are nutrient deprived (Shaheen and Hopper, 2008) and this retrograde process has been hypothesized to serve as a conserved process to down-regulate translation in response to nutrient availability by reducing the pool of tRNAs available for protein synthesis. If this is the case, then it may also be useful to sequester tRNA nucleotidyltransferase away from cytosolic ribosomes. As retrograde tRNA transport is still not well understood but is certainly regulated in response to conditions such as glucose or amino acid starvation (Whitney *et al*., 2007) there must be some mechanism to

control this process. This mechanism may include tRNA-binding proteins such as Cca1. If Cca1is involved and there is regulation of the nuclear import of this protein, it may be mediated by post-translational modification perhaps linked to different growth conditions.

As phosphorylation is a well characterized way to control nuclear localization (Nardozzi *et al*., 2010), phosphorylation of Cca1 may be linked to its nuclear import or export. Prior to this study, the literature showed a single phosphorylation site (Ser21) near the mitochondrial targeting signal (Albuquerque *et al*., 2008). One could argue that phosphorylation at this position could block mitochondrial targeting as had been seen previously for chloroplast targeting (Lamberti *et al*., 2011) such that less protein is directed to the mitochondrion and remains in the cytosol available for targeting to the nucleus. We hoped to show this phosphorylation site and to see if any other sites of phosphorylation could be found. Our study did not reveal phosphorylation at position 21, but this may reflect the different treatments that the cells received. Albuquerque *et al*. (2008) harvested their cells after a three-hour treatment with 0.05% methyl methanesulfonate (MMS) which damages DNA. While it may be disappointing that we did not see the same modification as Albuquerque *et al*. (2008) this may support the idea that

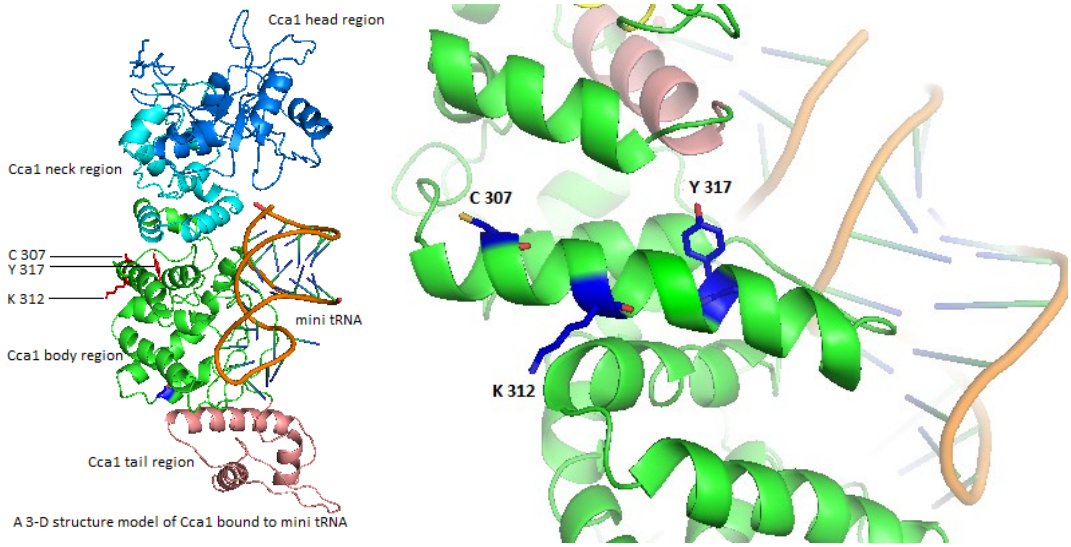


Figure 4.1 The 3-D structural model of Cca1
On the left-is the modeled structure of *S. cerevisiae* Cca1 bound to a mini tRNA. Cca1 is depicted as four regions: the head region (blue), neck region (indigo), body region (green), and tail region (pink). The post-translational modification (PTM) sites (Y317, K312 and C307) determined from MS analysis are located in Cca1's body region. On the right, is a close-up which highlights the position of the PMT sites in the helical body domain. (Picture provided by Dr. M. Leibovitch).

post-translational modifications are involved in different cell processes. While we did not see phosphorylation at position 21, we did find a phosphorylated tyrosine at position 317. The potential significance of phosphorylation at this position will be discussed later.

In addition to a role of phosphorylation in nuclear localization, there are also several reports indicating that acetylation is involved in protein localization, especially for nuclear import and export (Sadoul *et al*., 2011 for review). In addition, Zhao reported that Lys10 near the N-terminus of CtBP2 was critical for nuclear localization (Zhao *et al*., 2006). We identified two acetylation sites (C307 and K312) in close proximity to the Y317. In fact, a 3-D structural model of Cca1 designed based on the structure of related tRNA nucleotidyltransferases showed that these three amino acids could be found on the same face of an α-helix in the "body" domain of the protein (Figure 4.1). This face of the helix is solvent-exposed such that these three amino acids would be accessible for modification. That the three amino acids in the protein showing the highest degree of reproducible post-translational modification are found on the same face of one α-helix is interesting. Given the location of these modifications (on the side of the protein away from the active site) it is unlikely that they play a direct role in substrate binding or catalysis. However, this surface exposed sequence is exactly where one would expect a protein such as an importin or exportin to bind to facilitate nuclear import or export. What makes this region of Cca1 particularly interesting is that it represents either an insertion into the yeast protein or a deletion from other tRNA nucleotidyltransferases (Figure 4.2). That this sequence is present in

```
Aeolicus    LEEIIEGFQWN----------------------------EKVLQKLYALRKVVDWHALE 248
Thermotoga  IKHLFPKTYYT----------------------------PSMDEKMENLFRNIPWVEEN 273
Bacillus    LNAYLPGLAGK----------------------------EKQLRLAAAYR--WPWLAAR 248
yeast       ENVIFFWHNDSSVVKFNEENCQDMDKINHVYNDNILNSHLKSFIELYPMFLEKLPILREK 346
human       APYIGLPANAS--------------------------------LEEFDKVSKNVDGFSPK 305
```

Figure 4.2 The region found immediately after conserved motif E in yeast and four other class II tRNA nucleotidyltransferases: *Aquifex aeolicus* (Tomita *et al*., 2004), *Thermotoga maritime* (Toh *et al*., 2009), *Bacillus stearothermophilus* (Li *et al*., 2002), and human (Augustin *et al*., 2003) as taken from Figure 1.2: Alignment performed by Clustal W (Martin *et al*., 2007).
The numbers indicate the amino acid position of the last amino acid represented in the sequence.
The sites of acetylation and phosphorylation are shaded in yellow and green, respectively.

Cca1 but absent from other tRNA nucleotidyltransferases including the human homologue suggests that this region is not important for the CCA-adding activity of the protein but may play some other function.

The continuation of this research could take one of two main tracks: A) determining the importance of the modified residues identified to the structure, function or localization of Cca1, or B) determining what proteins may interact with this region of the protein. With respect to track A, experimental strategies worth pursuing could include site-directed mutagenesis to remove one or all of these residues to see what effect this has on the activity of the enzyme *in vitro* or on the phenotype or protein localization *in vivo*. We also could isolate Cca1 from different stages of growth of yeast to see if the presence or absence of these PTMs correlates with specific growth stages. For example, perhaps during fermentation, less Cca1 is required in the mitochondrion and the presence or absence of these PTMs is linked to targeting of Cca1 to mitochondria. It would be interesting to isolate Cca1 from yeast fermenting glucose (*e.g.*, in day one) and those carrying out aerobic respiration in stationary phase (*e.g.*, day seven) to compare the PTMs contained on Cca1 as was done previously for yeast Ccp1p (Kathiresan and English, 2017).

In terms of demonstrating what proteins interact with this region of the protein, it would be interesting to test commercially available catalysts (kinases, acetylases, etc.) to see whether or not they function *in vitro* with purified Cca1. Defining the specific kinase or acetylase may provide insight into which pathway these modifications of Cca1 are linked. Following that, pull down assays or co-immunoprecipitations from whole cell extracts prepared from yeast at different stages of growth could be carried out. This would tell us not only what proteins are associated with Cca1, but also if the interactions are growth stage dependent.

As the TEV protease was not able to cleave the protease cleavage site engineered into the TAP tag, we will use a different tag such as the hexa-His sequence or a glutathione S-transferase domain to increase the enrichment of Cca1. As in this experiment we were forced to use a denaturing agent to release both Cca1 and any proteins interacting with it and also all proteins non-specifically binding to the resin, it is difficult to say which are interacting with

Cca1. Adding an additional purification step would help to solve this problem. We currently are engineering a plasmid which will express a fusion protein containing a glutathione S-transferase domain at the carboxy terminus of the Cca1-TAP tagged fusion protein. With this we can carry out an initial round of purification using a resin with bound GST which should remove many contaminating yeast proteins and then use the IgG resin to indicate more specific proteins bound to Cca1.

In summary, this study has identified three amino acids showing post-translational modification in yeast Cca1. These three amino acids cluster on the surface-exposed face of an α-helix. This site allows access for the appropriate kinases and acetylases (and potentially phosphatases and deacetylases) that are involved in generating these PTMs. Moreover, the presence of these PTMs on a region of the protein that is not found in other tRNA nucleotidyltransferases suggests that this region is involved in an activity other than CCA addition. It will be interesting to explore this region of Cca1 to see its role in protein structure and function and to see whether other proteins (or perhaps even other nucleic acids) interact with Cca1 here.

4.2. Temperature-sensitive phenotype and Cca1 mutation

Temperature-sensitive phenotypes have been used for many years to define and study essential genes in *Saccharomyces cerevisiae* (Edgar and Lielausis, 1964; Hartwell, 1967; Pringle, 1975; Pringle and Hartwell, 1981; Kawakami *et al*., 1992; Tan *et al*., 2009; Ben-Aroya *et al*., 2010). Here we have studied a temperature-sensitive yeast strain derived from ts352 (Aebi *et al*., 1990) that contains a single guanine-to-adenine transition in the *CCA1* gene leading to the conversion of the glutamate at position 189 to lysine in tRNA nucleotidyltransferase (Shan *et al*., 2008). This temperature-sensitive phenotype was defined by the inability of these cells to grow at the restrictive temperature of 37°C while they grew equally as well as the native strain at the permissive temperature (22°C). Our comparative biophysical and biochemical studies of both the native and variant proteins revealed that this amino acid substitution resulted in a structural change in the protein that reduces enzyme activity (both at the permissive and restrictive temperatures), decreases the melting temperature of the protein and alters its stability at the

restrictive temperature (Shan *et al*., 2008). Using a second site suppressor of the *ts* phenotype within the *cca1-1* mutant gene, we further showed that the *ts* phenotype does not arise from thermal instability of the variant tRNA nucleotidyltransferase, but instead from the inability of a partially active enzyme to support growth only at higher temperatures (Goring *et al*., 2013). In fact, we argued that the *ts* phenotype may result simply because the defective enzyme cannot generate functional tRNAs at a sufficient rate for protein synthesis at the restrictive temperature, but we cannot exclude that it may involve other differences in the features of cells grown at higher temperatures (Goring *et al*., 2013). To explore more fully the differences between yeast cells carrying the wild-type or *cca1-1* gene, we set out to explore and compare the proteomes of strains carrying the *CCA1* allele or the *cca1-E189K* allele at permissive, intermediate and quasi-restrictive temperatures. Based on these differences in growth rate we hypothesized that we would see differences in the levels of components of the protein synthetic machinery as fewer tRNAs should result in a reduction in protein synthesis. Moreover, if tRNA-NT plays a role in retrograde import of tRNAs and this shuttling of tRNAs from the cytosol to the nucleus is linked to nutrient deprivation (Shaheen and Hopper, 2008), then perhaps we also will see down-regulated translation in response to a reducing the pool of functional tRNAs available for protein synthesis. If the pool of functional tRNAs is reduced not because of nutrient starvation directly but because they lack complete CCA termini, then perhaps a set of response pathways typical to glucose or amino acid starvation (Whitney *et al*., 2007) may be initiated in response to the reduced activity of tRNA-NT. This may include different types of stress response pathways or mechanism to protect the reduced amount of proteins that are made.

As expected from our previous results (Shan *et al*., 2008), the *wt* strain (W303-1B) showed an exponential growth rate that was greater at 30ºC than at 20ºC and slightly greater at 33ºC than at 30ºC (Table 4.1). While the *ts* strain (NT33-5) had approximately the same exponential growth rate as the *wt* strain at 20ºC, it grew more slowly than the *wt* strain at 30ºC (Table 4.1) and essentially not at all at 33ºC (at least for the first 30 hours). As mentioned previously the growth observed after 30 h probably represents a reversion or second site suppression event, which does not restore the growth rate to *wt* levels.

The reduced growth rate seen at the restrictive temperature for the *ts* strain (10% that seen at the permissive temperature) likely reflects that this strain has become petite. We previously have shown that reduced tRNA nucleotidyltransferase activity in the mitochondrion of yeast cells leads to the petite phenotype and reduced respiratory competence (Shanmugam *et al*., 1996) and numerous human disease phenotypes linked to mutations in the TRNT1 gene coding for tRNA nucleotidyltransferase have been linked to reduced mitochondrial protein synthesis (Chakraborty *et al*., 2014; Sasarman *et al.,* 2015; Liwak-Muir *et al*., 2016).

Peltz *et al.* (1992) who showed changes in specific mRNA stability when *cca1-1* cells where shifted to the restrictive temperature. Peltz *et al*. (1992) noted an increase in the stability of certain mRNAs and suggested that this was due to a decrease in the ability of these cells to carry out translation (as the population of functional tRNAs was reduced) such that some specific mRNAs are protected from degradation by association with the ribosome. Taken together these data may suggest that if polysomes exist with mRNAs bound for longer times as they wait for a functional charged tRNA to arrive at the ribosome to allow translation to continue, other mRNAs may accumulate in the cytosol.

While Peltz *et al*. (1992), observed approximately 5-fold increases in the half-lives of CDC*4*, *PAB1* and *TCM1* transcripts, we did not detect any Cdc4 in our samples and the Pab1 and Tcm1 proteins showed no significant differences in their abundance between the *wt* and the *ts* strain grown at 33°C (the ratio of abundance of Pab1 and Tcm1 were 1.4 (p=0.17) and 1.7 (p=0.08), respectively). Therefore, we could not provide a direct connection between mRNA and protein levels and the *ts* phenotype. Perhaps differences in experimental methods may have led to the variation in results; in the current study cells were gown at 33°C to an OD_{600} of one while Peltz *et al*. (1992) simply shifted the culture temperature from 24°C to 36°C for one hour. Finally, we also must consider that the *ts* cells that we examined also may represent a strain that has reverted or picked up a suppressor mutation and/or become petite.

Given the differences in growth rates observed, it was necessary to harvest the cells for proteome analysis at different time points to reflect an OD_{600} of 1, for example, after 18-20 hours at 20°C or 8-9 hours at 30ºC for the *wt* strain.

Table 4.1 Exponential growth rate of each strain at various temperatures

Temperature (^{O}C)	Growth Rate (ΔOD_{600}/h)	
	W303-1B	**NT33-5**
20	0.27	0.30
30	0.78	0.17
33	0.82	0.03

To define differences in the two strains and at the different temperatures, we chose to use a label -free mass spectrometric approach to explore the proteomes of these cells at these different temperatures. A comparison of the different proteins and their amounts may suggest proteins or pathways that are affected by the changes in temperature or the decreased efficiency of the tRNA-NT. We hypothesized that we would see some proteins at lower levels in the *ts* strain at the elevated temperature as we have postulated that protein synthesis works less well as the decreased activity of the mutant tRNA-NT reduces the population of tRNAs with the complete CCA sequence required (Goring *et al*., 2013). We also thought that we may see increases in the levels of other proteins as the cell tries to cope with the reduced level of proteins resulting from this defect.

Initially, we explored 2D gel electrophoresis (2DE) to compare the relative abundance of proteins expressed from *wt* cells and cells harbouring the *E189K ts* mutation. Proteins were separated based on their isoelectric point (in the first dimension) and by size (in the second dimension), detected by staining and excised from the polyacrylamide gel for MS analysis. A number of spots were analyzed including those that appeared unique, that is, were detected in one sample and not the other. Surprisingly, however, we observed no major differences in the types and levels of proteins expressed in the *wt* versus the *ts* cells. This result, coupled with the realization that low abundance proteins might not be readily detected on stained polyacrylamide gels prompted us to analyze the yeast whole proteome directly by mass spectrometry.

A high-resolution nano-HPLC tandem MS mass spectrometric comparison of the abundance of whole cell protein levels at 20°C, 30°C, and 33°C should allow us to identify candidate proteins whose cellular levels are altered at the different temperatures or between the *wt* and *ts* strains. Our MS approach was a label-free “bottom up” proteomic technique: the yeast whole-cell protein population was digested with trypsin, and the sequence of the resulting

peptides were determined from the analysis of their ionized fragmentation pattern. A specific protein was identified based on the detection of the peptides that are unique to that protein. To obtain results with high confidence, the data were filtered such that proteins were identified only if they showed ≥2 unique peptides. The advantage of this MS-based approach was that it could be used to quantify the amount of a specific protein in any sample (Han *et al*., 2008) so that we could determine if the abundance of a specific protein had changed between samples. If the ratio of the protein abundance in one sample as compared to a second sample was greater than three, then we consider that as a real difference. In this study, an equal amount of total protein (100 μg) from three biological samples of each strain was analyzed to obtain replicate MS analysis results. Comparing the distribution of the protein abundance ratios, we found that most of the cellular proteins common to both *wt* and *ts* strains were present at similar levels regardless of the strain sampled or at what temperature the cells were grown. We then set out to establish if the protein species or the abundance of the common proteins varied: 1) in a given strain as the temperature was changed (to 20°C, 30°C, or 33°C), or 2) between *wt* and *ts* strain at any given temperature.

4.2.1. Different proteomes in wild-type and temperature-sensitive strains

Our experiments suggested no major differences in growth rate between the *wt* and *ts* strains when they were grown at the permissive temperature (20ºC) or the intermediate temperature (30ºC) although both strains grew more quickly at 30ºC than at 20ºC suggesting that the protein synthetic machinery was sufficient to keep up with the increased growth required at the higher temperature. This fits with our hypothesis (Goring *et al*., 2013) that even though the *ts* strain has a tRNA-NT with reduced activity, its remaining activity is sufficient to meet the protein synthetic needs of the cell at these temperatures. When we examined the proteomes of the *wt* and *ts* strains at these two temperatures we found no major differences in the protein patterns shown. Both strains showed similar numbers and types of proteins as each other at both temperatures. These observations are consistent with our hypothesis that both strains can perform sufficient protein synthesis to keep the cells alive at these temperatures.

In contrast, at the quasi-restrictive temperature the *ts* strain showed a much different growth phenotype than did the wild-type strain. If our hypothesis is correct, then we should

expect to see different protein profiles when we compare the *wt* to the *ts* strain under these conditions, so we focused initially on this comparison. Although the overall number of proteins in the two strains was similar, the relative amounts of many of these proteins were different between the two strains. Additionally, fourteen proteins were detected only in the *ts* strain at 33ºC, and twenty-three proteins were missing only from the *ts* strain at 33ºC. When the proteins which were present only in the *ts* strain at 33ºC, or which were only absent from the *ts* strain at 33ºC, or which showed increased or decreased levels as compared to the *wt* strain at 33ºC were analyzed two consistent themes became evident. Proteins which showed increased levels in the *ts* strain were linked generally to stress responses and metabolic pathways while those that showed reduced levels in the *ts* strain were primarily mitochondrial or ribosomal proteins. These observations are in good agreement with our hypothesis that reduced tRNA-NT activity leads to fewer functional tRNAs and a disruption of protein synthesis such that translation cannot meet the needs of the cell. This reduced translation is then manifest in a stress response and a change in metabolic pathways. Linking protein synthesis and the stress response implicates proteostasis (protein homeostasis), the state of proteome balance (Hipp *et al*., 2014). Proteostasis results from the balancing of protein synthesis and protein degradation and a loss of proteostasis has been implicated in ageing and disease (Ruan *et al*., 2017). We postulate that the *ts* phenotype may result from a disruption of proteostasis, particularly a reduction in the protein synthetic component. As described above, this is entirely consistent with our observations that both ribosomal and mitochondrial proteins showed reduced levels in the *ts* strain at the quasi-restrictive temperature as compared to the *wt* strain. Moreover, proteostasis can be altered in response to environmental stresses (Labbadia and Morimoto, 2015) and we also see an increase in stress response proteins in the *ts* strain at the quasi-restrictive temperature. To further explore the links between tRNA-NT, proteostasis and the stress response we will look more specifically at some of the classes of proteins whose levels are altered at the quasi-restrictive temperature in the *ts* strain as compared to the *wt* strain or to the *ts* strain at the permissive temperature.

4.2.2. Proteins involved in ribosome (or RNA) structure or synthesis

Most of proteins showing reduced levels in the *ts* strain at the quasi-restrictive temperature (Drs1, Edc3, Hca4, Nog1, Nog2, Nsa2, Pus1, Rpl37A, Rpl15A, Rpl35A, Rpl39,

Rpl24B, Rrp1, Sdo1, Sof1, Tsr4, Utp23) are ribosomal proteins or are involved in ribosome synthesis (Table 3.4, Table 3.9). Specifically, Drs1 is the nucleolar DEAD-box protein required for ribosome assembly and function (Horsey *et al*., 2004), Edc3 has a role in mRNA decapping (Kshirsagar and Parker, 2004), Hca4 is the DEAD box RNA helicase and involved in 18S rRNA synthesis (Liang *et al*., 1997), Rrp1 (Horsey *et al*., 2004) and Nog1 (Kallstrom *et al*., 2003) are required for 60S ribosomal subunit biogenesis, Nog2 associates with pre-60S ribosomal subunits in the nucleolus and is involved in the nuclear export and maturation of these subunits (Saveanu *et al*., 2001), Nsa2 is involved in the processing of the 27S pre-rRNA, Rlp7 plays a key role in processing precursors RNAs of the large ribosomal subunit (Horsey *et al*., 2004), Pus1 introduces pseudouridines into the U2 snRNA and also acts on tRNAs and some mRNAs, and additionally, as a nuclear protein, it appears to be involved in tRNA export (Simos *et al*., 1996; Massenet *et al*., 1999; Grosshan *et al*., 2001; Carlile *et al*., 2014). The remaining proteins, Rpl37A, Rpl15A, Rpl35A, Rpl39, Rpl24B (Venema and Tollervey, 1999), are all components of the large ribosomal subunit. Sdo1 plays an essential role in ribosome maturation (Menne *et al*., 2007), Sof1 is response to the biogenesis of 40S (small) ribosomal subunit (Venema and Tollervey, 1999), Tsr4 is involved in the processing of the 20S pre-rRNA to generate mature 18S rRNA (Li *et al*., 2009), and Utp23 is involved in 40S ribosomal subunit biogenesis (Hoareau-Aveilla *et al*., 2012). Taken together, these results clearly implicate reduced ribosome assembly in the *ts* phenotype. Whether this results directly from a reduction in the amount of mature tRNAs or more indirectly from a response to stress induced by reduced tRNA-NT activity (Mazouzi *et al*., 2014; Mittal *et al*., 2016) remains unclear.

4.2.3. Degradation pathway protein levels increasing

As proteostasis requires a balancing of protein synthesis and protein degradation we also looked for proteins that may be involved in degrading proteins or protecting proteins from aggregation and degradation. Interestingly, in the *ts* strain at the quasi-restrictive temperature we detected increased levels of both heat shock proteins (Cdc37, Hsp10, Hsp104, Hsp60, Hsp78, Ssa1) which are involved in chaperone complex formation and protein folding/unfolding to protect from degradation (Table 3.7), and vacuolar proteins (Ape1, Prc1, and Snf7) (Table 3.5, Table 3.7) involved in targeting proteins for degradation.

We found increases in both mitochondrial (Hsp10, Hsp60, Hsp78) and cytosolic (Cdc37, Hsp104, Ssa1) chaperones which play roles in eliminating aggregation and in protein refolding. At the quasi-restrictive temperatures, the levels of these proteins were increased by up to seven-fold as compared to their levels at the permissive temperature (Table 3.7) indicating that some type of unfolded protein response (Fu and Gao, 2014; Jovaisaite *et al*., 2014) had been upregulated. Again, it is still unclear as to whether this response is a direct result of reduced tRNA-NT activity altering protein synthesis or whether it results indirectly as a stress response initiated by the reduced tRNA-NT activity. The mitochondrial unfolded protein response is a stress response pathway that has been implicated in health and disease (Jovaisaite *et al*., 2014) and activation of this pathway in yeast may be linked to the *ts* phenotype.

We propose that perhaps reduced protein synthesis in the *ts* strain and the subsequent reduction in the levels of some important proteins signals an apparent shortage of energy and nutrients inside the cell. This leads to the increase in the levels of proteins such as, Ape1, which is present in nine-fold abundance (p=0.019), and which is elevated during nitrogen starvation (see review by Cebollero and Reggiori 2009; Torggler *et al*., 2017). Ape1, as a major cargo protein, plays a key role in the cytoplasm-to-vacuole targeting (Cvt) pathway (Shintani and Klionsky, 2004). It is involved in the protein catabolic autophagy-like process in the vacuole (Scott *et al*., 1996; Baba *et al*., 1997), which serves to eliminate misfolded proteins (Quinones *et al.,* 2012) and degrade cytosolic components in response to starvation (Lynch-Day and Klionsky, 2010). Also along this line, Prc1, a broad-specificity C-terminal exopeptidase, which is involved in non-specific protein degradation in the vacuole (Van Den Hazel *et al*., 1996) also shows elevated levels in the *ts* strain grown at 33°C. Moreover, Snf7 which is required for localization of the Bro1 vacuolar protein sorting factor to endosomes (Odorizzi *et al*., 2003) which may travel to lysosomes where the proteins that they carry can be degraded. Again, the increased levels of these proteins associated with protein degradation is consistent with the idea that reduced tRNA-NT activity mimics nutrient starvation and activates pathways to degrade some proteins to recycle the amino acids into other proteins. Also, consistent with our hypothesis that reduced Cca1 activity mimics starvation conditions, we saw increased levels of Gad1, Uga1, and Uga2 for which previous studies (Rossignol *et al*., 2003) have shown increased gene expression during stationary phase under nitrogen starvation conditions.

4.2.4. Dysfunctional mitochondria in *ts* strain at 33ºC

Given that Cca1 must function both in the cytosol and the mitochondrion we expect that reduced Cca1 activity at 33ºC will also result in reduced mitochondrial protein synthesis and subsequently mitochondrial dysfunction. These indeed appears to be the case as seven mitochondrial proteins (Aim24, Atp20, Cox2, Cox5A, Mhr1, Nuc1, and Rip1) were not detected in the *ts* strain at 33ºC but were found in both strains under all other conditions (Table 3.9). As with the cytosolic ribosome one of these missing proteins, Mhr1, is mitochondrial ribosomal protein (Ling *et al*., 2000) suggesting that reduced Cca1 activity may also affect the mitochondrial ribosome. Other missing proteins such as Atp20, subunit *g* of the mitochondrial F1F0 ATP synthase (Davies *et al*., 2012), Cox2, subunit II of cytochrome *c* oxidase, Cox5A, subunit Va of cytochrome *c* oxidase (Taanman and Capaldi, 1992) and Rip1, a subunit of the mitochondrial cytochrome *bc*1 complex (Ljungdahl *et al*., 1989) are components of the electron transport chain and ATP synthase. While Atp20, Cox5A and Rip1are encoded by nuclear genes, Cox2 is encoded on the mitochondrial genome again suggesting that both cytosolic and mitochondrial translation are reduced. In contrast to these proteins which play a role in oxidative phosphorylation and ATP synthesis there are other proteins which play a less direct role in mitochondrial ATP production and are involved in mitochondrial structure and maintenance. For example, Aim24 plays a role in determining mitochondrial architecture (Harner *et al*., 2014), while Nuc1 as a major mitochondrial nuclease with a role in the maintenance of polyploidy (Zassenhaus and Denniger, 1994). Taken together, the loss of these proteins involved in the respiratory components of the mitochondrion and mitochondrial structure and maintenance suggests a loss of mitochondrial function. If this is the case, then ATP production must come from other sources. Consistent with this, the level of the glycolytic enzyme glyceraldeyde-3-phosphate dehydrogenase, Tdh1, increases 17-fold (p=0.02) in the *ts* strain as compared to the *wt* strain at 33ºC, and 47-fold higher (p=0.089) in the *ts* strain grown at 33ºC as compared to 20°C. This increase in Tdh1 levels may lead to an increase in the glycolytic rate such that in the absence of respiratory competent mitochondria these cells may survive by fermentation. More importantly, Tdh1 has been reported to be synthesized in response to NADH-reductive stress (Valadi *et al*., 2004), when cells enter stationary phase, or in conditions of glucose starvation (Boucherié *et al*., 1995), or in heat-shocked cells (Delgado *et al*., 2001). So, the dramatic

increases in Tdh1 activity may not suggest an attempt to increase glycolysis but instead a more general response to the stress that reduced Cca1 activity seems to generate. Although these cells were harvested in early log phase and should not be at stationary phase or under starvation conditions, we argue that the reduced activity of Cca1 leads to a proteostatic pattern that reflects stationary phase or starvation conditions. Again, whether this results directly from reduced levels of protein synthesis or due to an indirect effect of reduced Cca1 activity on the stress responses is still unclear. Oxidative stress is known to affect both translation and protein turnover (Vogel *et al*., 2011; Keller, 2006) so perhaps the reduced levels of protein synthesis result from increased oxidative stress and not directly from reduced levels of Cca1. To explore this possibility, we looked for changes in the levels of proteins linked to oxidative stress.

4.2.5. Reactive oxygen species (ROS) accumulation

As a result of mitochondria dysfunction, reactive oxygen species (ROS) increase inside the cell (Leadsham *et al*., 2013; Murphy, 2013). Although we did not measure the levels of ROS directly, we have identified increased levels of some proteins (Table 3.5 and Table 3.7) which are involved in the cell's response to oxidative stress, *e.g*., Ctt1 which plays a protective role in oxidative damage by hydrogen peroxide (Lushchak and Gospodaryov, 2005), Nqm1 which is responsive to oxidative stress and chronological cell aging (Michel *et al*., 2015), and Gad1, Uga1, and Uga2 which are all involved in the GABA shunt pathway which plays a role in the response to stress and signalling (Bach *et al*., 2009). In *S. cerevisiae* cells, Gad1 and Uga2 in addition to being upregulated in response to nitrogen starvation (section 4.2.3) also are elevated in response to oxidative stress (Coleman *et al*., 2001). More recently, Cao *et al*. (2013) reported that the GABA shunt pathway plays a crucial role in inhibiting the accumulation of ROS, which is caused by heat damage. So, it appears that the reduced Cca1 activity leads to an increase in stress responses including oxidative stress perhaps through the loss of mitochondrial function.

4.2.6. Proteostasis networks (PN) maintain protein homeostasis in the cell

The proteostasis network contains components involved in protein synthesis, protein trafficking, the unfolded protein response, and protein degradation machineries including the

ubiquitin proteasome and lysosomal autophagy pathway (Sklirou *et al*., 2018). Our data suggest that proteostasis is perturbed in the *ts* strain at 33ºC with protein synthesis reduced (section 4.2.2) and protein degradation upregulated (section 4.2.3). In the *ts* strain the decrease at 33ºC of proteins such as Drs1, Edc3, Hca4, Nog1, Nog2, Nsa2, Pus1, Rpl37A, Rpl15A, Rpl35A, Rpl39, Rpl24B, Rrp1, Sdo1, Sof1, Tsr4, and Utp23 involved in ribosome assembly and function suggests a decrease in protein synthesis and the increase in proteins such as Ape1, Prc1, and Snf7 level suggests that protein degradation has increased. Moreover, the concomitant increase in heat shock and ubiquitination proteins (Cdc37, Hsp10, Hsp60, Hsp78, Hsp104 and Ssa1) further reflects the destabilization of proteostasis. We suggest that this alteration of proteostasis leads ultimately to the *ts* phenotype observed.

4.2.7. No significant difference in the expression of TOR pathway proteins

It is difficult to discuss the role of proteostasis is cell viability without implicating the Target of Rapamycin (TOR) signalling pathway. Since the TOR signalling pathway plays a key role in sensing nutrient or oxygen status and promoting appropriate changes in cell growth, proliferation, survival, and protein synthesis (Yuan *et al*., 2013), we tried to identify any proteins that would suggest the involvement of TOR in the *ts* phenotype. Our data showed no increases in expression of the well-characterized TOR pathway proteins Tap42, Mks1, Ure2, Gln3, and Gat1 (Raught *et al*., 2001) and in fact, we did not even detect Tor1 or Tor2. We did identify Kog1, a subunit of the TOR1 complex (Loewith and Hall, 2011), in the *ts* strain at 33ºC once in the three replicates and Slm1, a subunit of the TOR2 complex (Loewith and Hall, 2011), twice in three replicates but in both strains at all three temperatures. Thus, there is not enough evidence in our data to support a role for the TOR pathway either in the presence or absence of the *ts* phenotype.

4.2.8. Conclusions

Living cells protect themselves against stress including heat stress by making proteins that stabilize the structure of functional proteins in the cell. In our case, growth at 33°C was not sufficiently high to severely jeopardize the development of *wt* cells although we detected several stress response proteins in this strain that increased in abundance as compared to cells grown at

lower temperatures (Table 3.6). Our results support the idea that ROSs may play a role in the activation of the response to elevated temperature in yeast (Zhang *et al*., 2015). The evidence for this is the increased protein abundance of the GABA shunt pathway proteins and various antioxidation proteins. Furthermore, the accumulation of ROS is linked to dysfunctional mitochondria (Trancikova *et al*., 2004), which is supported by our proteomics study revealing that many mitochondrial proteins were below the level of detection in the *ts* cells grown at 33°C. Thus, mitochondrial dysfunction could originate from the Cca1 variant and the petite phenotype associated with it. When the temperature is elevated there is a need for an increase in mitochondrial respiratory efficiency (Postmus *et al*., 2011), which in turn increases the rate of mitochondrial protein synthesis. However, because of the defect in Cca1, we argue that the mitochondria in the *ts* cell do not contain sufficient quantities of mature tRNA to sustain effective protein synthesis. Under these circumstances, mitochondria are damaged by the shortage of viable Cca1 protein and mitochondrial DNA is damaged or lost leading to the petite phenotype (Doudican *et al*., 2005). Simultaneously, high concentrations of ROS are released into the cytosol from mitochondria, stimulating the cell to express more stress response proteins.

The defect in Cca1 leads to a shortage of mature tRNAs and reduces protein synthesis in the cytosol and mitochondria. Two things happen in response to this (Fig. 4.3) reduced protein synthesis in the cytosol which mimics starvation or stress conditions and leads to further reductions in protein synthesis and an increased stress response leading to a loss of proteostasis, and reduced protein synthesis in the mitochondrion which reduces mitochondrial function, increases oxidative stress and the associated stress responses and decreases cell viability. Increased oxidative stress may then deleteriously affect cytosolic protein synthesis further disrupting proteostasis and exacerbating the detrimental effects of the reduced activity of Cca1.

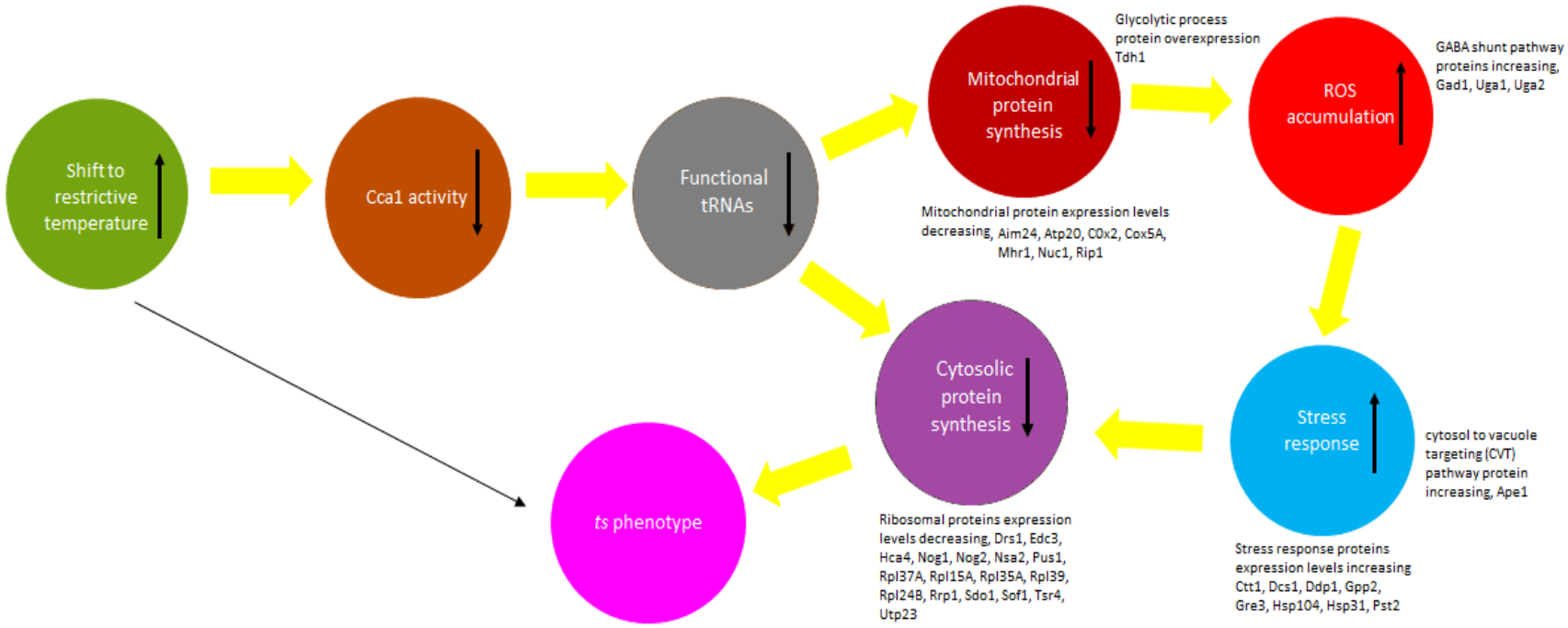


Figure 4.3 Proposed model for how reduced Cca1 activity leads to the temperature-sensitive phenotype

5. FUTURE WORK

To more fully explore the role of PTMs in Cca1's function, structure and localization, further experiments are required. These may include site-directed mutagenesis experiments to see if modifying any or all of C307, K312, or Y317 alters enzyme activity *in vitro* or localization or the phenotype *in vivo*. It also would be interesting to see the effect of removing this entire region of the protein (which is absent from many other tRNA nucleotidyltransferases) on activity, structure, and localization. This may provide insight into what role these extra amino acids play in the yeast protein. Finally, a more efficient purification protocol should be developed so that this protein can be used to pull down proteins (or nucleic acids) that interact with Cca1, and accompany by a top-down MS approach. It would then be interesting to compare the protein profiles pulled down by the native enzyme and the one lacking the region containing C307, K312, or Y317, and discover more modification or sites. Now that we have shown that we can identify PTMs in Cca1, it would be interesting to see if these PTM differ through different growth phases. Are the modifications present only in early log phase, or late log phase or throughout stationary phase, or cell fractionation to identify the PTMs in Cca1in subcellular compartment. This information may help us to define the roles of these modifications.

With respect to the comparison of the proteomes of the *wt* and *ts* strains, it would be important to repeat the experiments using the *cca1-E189F* strain. This strain is analogous to the *cca1-E189K* strain and shows the same *ts* phenotype, but is much less likely to revert. This would allow us to see if the anomalous growth curve that we saw for the *ts* mutant was due to a reversion or to a second site suppressor. If it is a second site suppressor, then a more detailed analysis of the protein profiles resulting may lead toward an understanding of proteins involved in tRNA metabolism. Also, we should focus on the specific protein groups identified in these results, such as those involved in protein degradation, oxidative stress, or mitochondrial structure and function, which might provide insights into proteostasis networks.

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APPENDIX

Sequence of Cca1-TAP

agtaaatgatgacacaaggcaattgacccacgcatgtatctatctcattttcttacacct
tctattactttctgctctctctgatttgaaaaaagctgaaaaaaaaggttgaaaccagtt
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cgccttcatccaaagtttaaaagatgacccaagccaaagcgctaacctttagcagaagc
taaaaagctaaatgatgctcaggcgccgaaagtagacgcgaatcatcaggagctgactca
tcaatn

Cca1 gene sequence is between the yellow highlighted regions; and the TAP sequence is between the red highlighted regions.