

Translation Homeostasis Contributes to SIFD Pathobiology in Yeast

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Abstract

Protein phosphorylation is an essential regulatory mechanism employed by many key pathways in the eukaryotic cell. This thesis explored two examples of protein phosphorylation, one in translation and one in cell cycle regulation.

TRNT1 is the RNA polymerase that adds the 3' CCA nucleotides to all tRNA and is required for aminoacylation, tRNA quality control, and protection of the translation machinery (Aebi et al., 1990; Vörtler & Mörl, 2010; Wellner et al., 2018). Mutations in *TRNT1* cause SIFD, Sideroblastic Anemia With B-Cell Immunodeficiency, Periodic Fevers, And Developmental Delay, a rare mitochondrial disease (Chakraborty et al., 2014). In addition to oxidative phosphorylation defects previously reported (Liwak-Muir et al., 2016; Sasarman et al., 2012; Wedatilake et al., 2016), I show that defects in TRNT1/Cca1 induce the eIF2 α -P translation stress response to slow cytoplasmic translation but is only beneficial when mitochondrial translation is required. This data suggests that impairment of TRNT1/Cca1 function results in an imbalance of translation homeostasis and that altering the balance between mitochondrial and cytoplasmic translation may be an effective therapy for SIFD.

In another example of protein phosphorylation regulation, Cdc25-dependent dephosphorylation of Cyclin dependent kinase (Cdk1) is a universally conserved mechanism regulating mitotic entry. In budding yeast, Cdk1 is dephosphorylated when the *cdc25* homologue, *MIH1*, is deleted, indicating an additional phosphatase functions redundantly with Mih1. I identified two phosphatases, Ptp1 and PP2A^{Rts1}, that function in conjunction with Mih1. This redundancy suggests novel mechanisms for how cells may activate pools of Cdk1 at different times and in different cellular locations.

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List of Abbreviations

AID	auxin-inducible degron
amUPR	accumulation unfolded protein response pathway
ARS	amino acyl-tRNA synthetase
ATP	adenosine triphosphate
BSA	bovine serum albumin
BME	β -mercaptoethanol
CAK	cyclin activating kinase
CBP	calmodulin binding peptide
CDK	cyclin dependent kinase
CFU	colony forming units
CFW	calcofluor white
CTP	cytidine triphosphate
CNS	central nervous system
CWI	cell wall integrity pathway
DNA	deoxyribonucleic acid
DTT	dithiothreitol
DMF	dimethylformamide
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ER	endoplasmic reticulum
ETC	electron transport chain

EV	empty vector
G1	gap 1
GAAC	general amino acid control pathway
GDP	guanosine diphosphate
GST	glutathione S-transferase
GTP	guanosine triphosphate
HOG	high osmolarity glycerol pathway
HYG	hygromycin
IgG	immunoglobulin G
ISR	integrate stress response pathway
KCl	potassium chloride
M-phase, M	mitosis
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
MEKK	mitogen-activated protein kinase kinase kinase
MELAS	mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes
MERFF	myoclonic epilepsy with ragged-red fibers
mRNA	messenger RNA
mtDNA	mitochondrial DNA
mtUPR	mitochondrial unfolded protein response pathway
ORF	open reading frame
OXPHOS	oxidative phosphorylation

PCR	polymerase chain reaction
PDR	pleiotropic drug resistance pathway
PMSF	phenylmethylsulfonyl fluoride
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
RNA	ribonucleic acid
RNAi	interference RNA
ROS	reactive oxygen species
RRF	ragged red fibers
rRNA	ribosomal RNA
RTD	rapid tRNA decay pathway
RTG	retrograde response pathway
S-phase	synthesis phase
SCF	Skp, Cullin, F-box containing complex
SD	Synthetic depleted
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SIFD	sideroblastic anemia, b-cell immunodeficiency, periodic fever, developmental delay
TAP	tandem affinity purification
TBS	tris buffered saline
TBS-T	tris buffered saline + 0.1% Tween-20
TCA	tri-carboxylic acid cycle

TOR	target of rapamycin pathway
tRNA	transfer RNA
tRNA-NT	tRNA nucleotidyl transferase
UPR	unfolded protein response pathway
YNB	yeast nitrogen base
YPD	yeast extract peptone media + 2% dextrose

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Chapter 1: General Introduction

1.1. Mitochondrial diseases

Mitochondrial diseases are a large group of inborn errors in mitochondrial energy metabolism that are both clinically and genetically heterogeneous. The vast majority of protein functioning in the mitochondrial are nuclear encoded, with the nuclear DNA providing 1000 to 1500 proteins compared to 13 encoded on the mtDNA for mammals (Goldstein & McCusker, 1999). Epidemiological studies estimate the frequency of mitochondrial diseases being 1 in 2000, and those resulting from mutations in nuclear encoded proteins being most common given that only 15 to 20% are a result of mtDNA mutations (Longtine et al., 1998). Of the over 150 nuclear gene mutations resulting in mitochondrial disease, approximately 70% are conserved in yeast (Koopman et al., 2012). It has been estimated that 15% of proteins that function in the mitochondria are dual-localized, functioning in more than one cellular compartment (Foster et al., 2006; Kumar et al., 2002). This co-localization of the proteins creates difficulties in identifying the causes of pathobiology from individual or multiple cellular compartments.

Mitochondrial diseases generally show phenotypes in tissues with high-energy requirements such as muscle, neurons, endocrine, heart, retinal, and auditory systems. Clinical presentations include skeletal muscle myopathy, cardiomyopathy, seizures, strokes, ataxia, peripheral neuropathy, blindness, deafness, GI mobility dysfunction, liver failure, bone marrow dysfunction, and pancreatic exocrine and endocrine dysfunction (Calvo & Mootha, 2010; DiMauro & Davidzon, 2005; DiMauro et al., 2006; Soumalainen et al., 1995). In addition, diseases of aging (type 2 diabetes, Parkinson's disease, atherosclerotic

heart disease, stroke, Alzheimer's disease, and cancer) have been associated with defects in mitochondrial function (Moreira et al., 2010).

This range of pathobiologies is not surprising given that mitochondria, in addition to producing 80% of the cellular ATP, are responsible for fatty acid oxidation, ketogenesis, ketolysis, the Krebs cycle, the urea cycle, amino acid metabolism, pyrimidine biosynthesis, heme synthesis, cholesterol metabolism, steroid synthesis, calcium homeostasis involved in neurotransmission, and apoptosis (Calvo & Mootha, 2010; Friedman & Nunnari, 2014). Thus, although the obvious consequence of mitochondrial malfunction is reduction of oxidative phosphorylation (OXPHOS) causing reduced cellular ATP levels, other outcomes may also result, including: alteration of cellular pH; imbalances in phosphate, calcium, potassium, and sodium homeostasis; redox imbalances; or ROS level changes (Friedman & Nunnari, 2014; Nicholls et al., 2013; Suomalainen & Battersby, 2017). Mitochondria also function as integrators of cellular signaling and mitochondrial malfunction can activate several pathways of mitochondrial stress response signaling and deregulate of mitochondrial apoptotic pathways (Quirós, et al, 2016; Suomalainen & Battersby, 2017). Defective OXPHOS does not account for the variable and pleiotropic clinical manifestations of the wide range of mitochondrial disorders, indicating that cellular stress responses triggered from lack of nuclear-mitochondrial coordination likely also play a role (Battersby & Richter, 2013; Suomalainen & Battersby, 2017).

1.1.1. Defects in mitochondrial translation

In mammals, 37 genes are encoded by the mitochondrial DNA, 24 are involved in mtDNA translation (two rRNAs and 22 tRNAs) and the remaining thirteen encode subunits of the respiratory chain. For a review on mitochondrial transcription and translation see

Pearce et al., 2017 or Richter-Dennerlein et al., 2015. Mutations in the 200 to 300 nuclear-encoded genes that function in mitochondrial translation are proposed to be the primary cause of respiratory chain deficiencies in patients that do not present with mitochondrial depletion or mtDNA mutations (Jacobs & Turnbull, 2005; Pearce et al., 2013). Defects in mitochondrial translation can be caused by mutations in the mtDNA encoded tRNA or mitochondrial ribosome components, or in the nuclear encoded components of the mitochondrial ribosome, RNA modifying enzymes, aminoacyl tRNA-synthetases, and initiation, elongation, or termination factors (Pearce et al., 2013; 2017; Rotig, 2011).

Studies in cell culture have shown that stalling mitochondrial translation results in stress response signaling prior to defects in OXPHOS, indicating that the loss of respiration was subsequent and not causative of the mitochondrial retrograde response (Battersby & Richter, 2013; Richter et al., 2013; Yun & Finkel, 2014). Prolonged induction of the stress response resulted in degradation of mitochondrial ribosomes and mRNA (Richter et al., 2013). This stress response was not simply due to decreased translation because inhibition of mitochondrial translation did not produce the same response, and paradoxically, stalling ribosome assembly while inhibiting mitochondrial translation suppressed the stress response induction (Richter et al., 2013; Richter et al., 2015). It was proposed that stalled ribosomes would produce abnormal translation products into the mitochondrial inner membrane, generating stress that would lead to a loss of mitochondrial membrane potential (Battersby & Richter, 2013).

Because the vast majority of the proteins functioning in the mitochondrial are encoded in the nucleus, the maintenance of translation homeostasis is required for mitochondrial translation and to prevent the accumulation of pre-proteins proteins while at the same time ensuring adequate protein supply (Battersby & Richter, 2013; Eisenberg-Bord

& Schuldiner, 2017; Raimundo, 2014; Suhm & Ott, 2016; Topf et al., 2016; Yun & Finkel, 2014).

1.1.2. *Saccharomyces cerevisiae* mitochondrial translation

Yeast can survive as rho⁰ petites with no mtDNA (and therefore no mitochondrial translation) and is a robust model to study translation, especially in relation to stress responses (Altmann & Linder, 2010). In yeast, eight proteins are encoded on the mtDNA, seven integral membrane proteins of the electron transport chain (Cox1, Cox2, Cox3, Cytb, Atp6, Atp 8, Atp9) and a component of the mitochondrial small ribosome subunit (Var1) (Merz & Westermann, 2009; Smits et al., 2010; Towpik, 2005). There are 24 mitochondrial encoded tRNA and two rRNA (21S rRNA and 15S rRNA) (Turk et al., 2013). Additionally, mitochondrial transcripts are polycistronic and individual genes from one polycistronic form can have different levels of product, including tRNA levels, with some tRNA 40x more abundant than others (Turk et al., 2013).

Mitochondrial translation initiation requires nuclear-encoded mIF2 (conserved with cytoplasmic eIF2 complex) to bind initiator tRNA and in a GTP/GDP dependent manner and positioning of the tRNA at the ribosome (Garofalo et al., 2003; Roll-Mecak et al., 2000; Smits et al., 2010; Towpik, 2005). mIF2 is not regulated by phosphorylation as observed for the cytoplasmic counterpart eIF2 α -P (Towpik, 2005). Mitochondrial mRNA are uncapped, and thus not regulated by eIF4E cap-binding complex as observed in the cytoplasm (Grohmann et al., 1978; Smits et al., 2010). Yeast use nuclear-encoded gene-dependent activators for initiation and elongation of mt-mRNA translation (Herrmann et al., 2013; Smits et al., 2010; Turk et al., 2013). Protein translation is activated by these activators at the matrix membrane to allow for co-translational membrane insertion (Chacinska &

Boguta, 2000; Fiori et al., 2003; Fox, 2012; Herrmann et al., 2013). This may also be true of some nuclear-encoded mitochondrial proteins given the presence of mitochondrial-protein specific cytoplasmic ribosomes at the mitochondrial outer membrane (Lesnik et al., 2014; MacKenzie & Payne, 2004; Segev & Gerst, 2018; Szyrach, et al., 2003).

1.2. tRNA

Yeast contain 275 cytoplasmic tRNA genes with more than 1% of the genome used to encode and process them into functional molecules (Hopper & Phizicky, 2003). Translation fidelity is dependent on correct tRNA amino acylation, with fidelity decreasing during times of nutrient or environmental stress (Dittmar et al., 2005; O Farrell, 1978; Pan, 2013). The levels of charged tRNA can drop in starved cells, specific to the tRNA (Dittmar et al., 2005; Zaborske et al., 2009). The selection of tRNA during translation elongation is considered the rate-limiting step, with variation in tRNA concentrations leading to changes in translation rate (Gingold & Pilpel, 2011; Varenne et al., 1984). Additionally, imbalances in tRNA and amino acid pools can result in mischarging of tRNA and subsequent substitutions (Dittmar et al., 2005; Pan, 2013). It was also shown that transcripts with pauses that cause vacant A-sites have high competition between near-cognate tRNAs and transcripts with rare codons have decreased translation rates and increased frameshift frequency (Fluitt et al., 2007; Gingold & Pilpel, 2011; Rosenberg et al, 1993; Sipley & Goldman, 1993). The concentrations of tRNA and cognate aminoacyl synthetases need to be balanced in the cell to avoid mischarging and subsequent decrease in tRNA fidelity (Swanson et al., 1988).

tRNA are very stable molecules with half lives comparable to rRNA and ranging from two to three days. The stability of tRNA depends on interaction with proteins, such as

tRNA nucleotidyl transferase (tRNA-NT), aminoacyl synthetases (ARSs), base modification enzymes, or the ribosome; this is defined as Channeling Theory where aminoacylated tRNA is directly transferred to elongation factors and ribosomes without disassociation (Belostotsky et al., 2012; Negrutskii & Deutscher, 1991). Elevating levels of these proteins is one of the few potential therapeutic approaches to tRNA related disorders (Pearce et al., 2013).

1.2.1. 5' tRNA end processing

The 5' leader sequence is processed by RNaseP, an essential endoribonuclease ribozyme complex highly conserved in all kingdoms of life (Frank & Pace, 1998a; Schon, 1999; Walker & Engelke, 2006). RNaseP activity has been identified in the nucleus, cytoplasm, mitochondria, and chloroplasts in all cells and organelles (Schon, 1999). Substrate recognition is dependent on the tertiary structure of pre-tRNA in the acceptor-stem and T-hairpin domains by RNaseP, the specific 5' sequence is not required in recognition (Frank & Pace, 1998b). It was generally accepted that the catalytic component of RNaseP in both prokaryotes and eukaryotes was RNA, with eukaryote protein components further stimulating catalysis (Kikovska et al., 2007). Interestingly, mitochondrial RNaseP does not contain any RNA components and RNaseP activity was reconstituted by three polypeptides in *E. coli*, leaving doubt to RNA being the catalytic component of the complex (Holzmann et al., 2008). To date no human diseases have been identified due to mutations in RNaseP (Walker & Engelke, 2008).

1.2.2. 3' tRNA end processing

3' pre-tRNA processing occurs following 5' pre-tRNA processing *in vivo* (Dubrovsky et al., 2004; Pellegrini et al., 2003). Endoribonuclease tRNaseZ cleaves tRNA at the discriminator base N73 to leave a 3'-hydroxyl group on the pre-tRNA (Redko et al., 2007; Schiffer et al., 2002; Vogel et al., 2005). Length and secondary structures of the 3' leader sequence and pre-tRNA base modifications have no effect on tRNaseZ activity (Mohan et al., 1999; Schiffer et al., 2002). The 5' end of tRNA is processed first because the 5' leader sequence inhibits 3' tRNaseZ activity due to the tRNA acceptor arm conformation (la Sierra-Gallay et al., 2005; Minagawa et al., 2008; Redko et al., 2007). Once C, CC, or CCA is added by tRNA-NT, tRNaseZ is increasingly unable to cleave the 3' end of the tRNA, with the most inhibition following the addition of the first C residue (la Sierra-Gallay et al., 2005; Mohan et al., 1999; Pellegrini et al., 2003). tRNaseZ inhibition by 3' CCA is due to interactions between C74 and a loop protruding from the pre-tRNA body (Mohan et al., 1999; Redko et al., 2007; Wilusz et al., 2008).

While cleavage of the 3' leader sequence in eukaryotes occurs primarily at the anti-determinant base by the endoribonuclease tRNaseZ, additional 3' exonucleases may be involved (Furter et al., 1992; Huang et al., 2006; Maraia & Bayfield, 2006; Yoo & Wolin, 1997). From yeast to mammals, cells lacking the La protein have the 3' pre-tRNA trailer removed by exonucleases, supporting the role of La in tRNA 3' end protection (Wolin & Cedervall, 2002; Yoo & Wolin, 1997). In yeast lacking La (yeast *LHP1*), the 3' trailer is removed by an exonuclease up to a stem common to most pre-tRNA by base pairing between the 5' and 3' trailers (Lee et al., 1997; Yoo & Wolin, 1997). The 5' end of the pre-tRNA is then removed by RNaseP, after which the remainder of the 3' trailer is removed (Yoo & Wolin, 1997).

1.2.3. tRNA base modifications

The RNA Modification Database lists a total of 92 different post-transcriptional modifications in the 5800 tRNA gene sequences from 111 analyzed species, including archaeal, bacteria, eukaryotes, chloroplasts, mitochondria, and viruses (Phizicky & Alfonzo, 2010; Sprinzl & Vassilenko, 2005). Overall, there was found to be an average of 8 modifications per species tRNA (the group of tRNA with a particular anti-codon); yeast are found to have 25 of the 92 identified modifications with an average of 12 modifications per tRNA species (Phizicky & Alfonzo, 2010; Sprinzl & Vassilenko, 2005). This introduction will not discuss these modifications in detail; reviews can be found in (Hopper, 2013; Phizicky & Alfonzo, 2010; Phizicky & Hopper, 2010), and for mitochondrial tRNA in (Bohnsack & Sloan, 2017; Suzuki, 2014). Overall, modifications to the tRNA are important in translation, translation fidelity, tRNA folding, and tRNA stability, although the degree to which a given modification affects an tRNA differs per tRNA analyzed (Phizicky & Alfonzo, 2010; Suzuki, 2014); the discussion below focuses on examples of tRNA modifications impacting mitochondrial function.

Mutations in the pseudouridine synthase 1 (*PUS1*) gene have been well characterized in resulting in Mitochondrial Myopathy and Sideroblastic Anemia (MLASA), a rare autosomal recessive disorder with impaired OXPHOS and sideroblastic anemia (Bykhovskaya et al., 2004; Patton et al., 2005). Pus1 is an enzyme required for pseudouridylation, the most common posttranscriptional modification of structural RNA, for both mitochondrial and cytoplasmic tRNA (Chen & Patton, 1999; Foster et al., 2000; Motorin et al., 1998; Spenkuch et al., 2014). Although there are other pseudouridylation enzymes, Pus1 is the only one currently linked to human disease (Pearce et al., 2017; Phizicky & Alfonzo, 2010). Loss of Pus1 results in decrease of pseudouridylation in both

cytoplasmic and mitochondrial tRNA but only reduces mitochondrial translation (Bykhovskaya et al., 2004; Patton et al., 2005). Despite the lack of a common base modification, most tissues are not affected and the disease phenotype emerges slowly in muscle and erythroid cells (Bykhovskaya et al., 2007). The lack of tRNA pseudouridylation can be compensated for by changes in levels of ribosomal proteins, suggesting that the disease phenotype in affected tissues may be caused by the lack of non-canonical functions of Pus1; microarray data suggests that Pus1 is involved in nucleic acid metabolism, metal ion binding, and iron metabolism (Bykhovskaya et al., 2007).

In humans, bacteria, and yeast, TRMU and Mto1 perform the 5-carboxyl methyl amino methylation ($\text{mnm}^5\text{s}^2\text{U34}$) to stabilize the wobble uridine base in glutamine, glutamate, and lysine mitochondrial tRNAs (Pearce et al., 2013; Umeda et al., 2004; G. Wang et al., 2010). Defects in this tRNA modification result in lowered steady-state levels of these amino acids available for protein synthesis and are linked to Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like episodes (MELAS) and Myoclonic Epilepsy with Ragged-Red Fibers (MERRF) (Kirino et al., 2004; Pearce et al., 2013; Yasukawa et al., 2001). Defects in mitochondrial function caused by loss of Mto1 results in a stress response due to accumulation of unfolded proteins in the cytoplasm associated with reduced import into the mitochondria (Fakruddin et al., 2018). Induction of cytoplasmic stress responses eIF2 α -P reduced overall protein synthesis and increased expression of *ATF4* (*GCN4* in yeast) and *CHOP* (stress-response transcription factor) in mouse cells (Fakruddin et al., 2018).

1.2.4. Mitochondrial tRNA

As observed in nuclear and cytoplasmic tRNA processing, mitochondrial pre-tRNA are processed at the 5' and 3' ends, 3' CCA is added, modified, and aminoacylated; all within the mitochondria by imported nuclear-encoded proteins (Florentz et al., 2003; Rossmannith et al., 1995; Wittenhagen & Kelley, 2003). Mitochondrial tRNA lack of introns and are transcribed as polycistronic primary transcripts, although separate enzymes still cleave the 3' and 5' ends of tRNA (Rossmannith et al., 1995; Turk et al., 2013). Over 400 mutations in the mitochondrial genome are associated with human disease, and more than 60% are associated with the mitochondrial tRNA genes although tRNA only compose of 10% of the mitochondrial genome (Belostotsky et al., 2012; Levinger et al., 2004; Wittenhagen & Kelley, 2003). Although each mt-tRNA has been associated with at least one pathogenic mutation, three mt-tRNAs (tRNA^{Ile} , $\text{tRNA}^{\text{Leu(UUR)}}$, and tRNA^{Lys}) are associated with almost 50 % of the pathogenic mt-tRNA mutations (Turk et al., 2013; Wittenhagen & Kelley, 2003).

Mitochondrial $\text{tRNA}^{\text{Ser(AGY)}}$ and $\text{tRNA}^{\text{Ser(UCN)}}$ have non-classical tRNA cloverleaf secondary structures resulting from a missing D-arm in $\text{tRNA}^{\text{Ser(AGY)}}$ and variations in cloverleaf branch nucleotide number in $\text{tRNA}^{\text{Ser(UCN)}}$ (Florentz et al., 2003; Giege et al., 2012; Helm et al., 2000). Differences in mt-tRNA structure compared to cytoplasmic include: small size of the variable region, smaller D-loop size, variable T-loop sizes, and variable nucleotide GC composition between strands (Helm et al., 2000). Yeast contain more canonical tRNA structures, although still with variable D and T loops and variable GC composition compared to cytoplasmic tRNA (Giege et al., 2012). There is also an overall lack of primary sequence conservation in mitochondrial tRNAs compared to cytoplasmic (Helm et al., 2000; Suzuki et al., 2011). An RNA analysis of yeast mitochondrial tRNA

showed wide variability in the abundance of tRNA molecules, including those on the same polycistronic transcript or with protein coding genes (Turk et al., 2013). The abundance of certain tRNAs likely differ due to the abundance of aliphatic and aromatic amino acids in the strongly hydrophobic mitochondrial-encoded proteins (Florentz et al., 2003).

Mitochondrial aminoacyl synthetases are less efficient at adding amino acids to mitochondrial tRNA than eukaryotic cytosolic or prokaryotic tRNA (Bullard et al., 2000; Jørgensen et al., 2000). In addition, mitochondrial tRNA require different structural elements for amino acylation, such as emphasis on D and T loops compared to the variable arm domains important in cytoplasmic aminoacylation specificity (Florentz et al., 2003; Ueda et al., 1992; Yokogawa et al., 2000). Because mitochondrial tRNA lack the canonical structure and stability observed in the cytoplasm, they are more dependent on base modifications for structural stabilization. This is observed in Pus1 defects resulting in reduced pseudouridines in both cytoplasmic and mitochondrial tRNAs but only impacting mitochondrial translation due to proposed lack of mitochondrial tRNA stability (Bykhovskaya et al., 2004; Helm, 2006; Patton et al., 2005).

1.3. tRNA-NT

tRNA nucleotidyl transferase (tRNA-NT, TRNT1 in mammals, Cca1 in yeast) is the 3' CCA-adding RNA polymerase that catalyzes the addition of two cytosines and one adenine to the 3' end of all nascent tRNA molecules in the nucleus, cytoplasm, and mitochondria (Aebi et al., 1990; Chen et al., 1992; Nagaike et al., 2001). This 3' CCA addition is required for aminoacylation, correct positioning of tRNA at the ribosome, and subsequent protein synthesis and occurs without the assistance of any additional proteins or co-factors (Aebi et al., 1990; Chen et al., 1992; Nagaike et al., 2001; Nissen et al., 2000;

Vörtler & Mörl, 2010). In the nucleus, tRNA 3' CCA addition is required for efficient tRNA nuclear export in vertebrates and yeast (Arts et al., 1998; Lipowsky et al., 1999). Mature tRNA in yeast and mammals are aminoacylated in the nucleus as a proofreading function prior to their export into the cytoplasm (Arts et al., 1998; Lund & Dalhberg, 1998; Sarkar et al., 1999), and defects in 3' tRNA maturation (including CCA addition) or aminoacylation cause tRNA to accumulate in the nucleus (Feng & Hopper, 2002; Sarkar et al., 1999). Additionally, tRNA-NT has been shown to shuttle between the nucleus and cytoplasm and could shuttle the tRNA accompanying its own export (Feng & Hopper, 2002; Uetz et al., 2000).

The addition of 3' CCA is a tightly regulated process with the tRNA-NTs recognizing the acceptor stem and the TΨC stem-loop (the mini-helix) (Li et al., 1996; Lizano et al., 2008; Shi et al., 1998; Tomita et al., 2004). tRNA-NT recognizes both the classical tRNA structure of cytoplasmic tRNAs but also the more variable mitochondrial tRNA structures (Antonellis & Green, 2008; Bujnicki et al., 2003; Helm et al., 2000; Nagaike et al., 2001). Although capable of recognizing various tRNA structures, changes in the substrate tRNA structure can greatly affect CCA adding activity resulting in pathobiologies (Levinger et al., 2004; Tomari et al., 2003).

Several groups have identified mutations in mitochondrial tRNA that result in MELAS due to defective 3' CCA trinucleotide addition (mt-tRNA^{Leu(UUR)} C3303T) (Levinger et al., 2004). Prior to this, it was generally accepted that most cases of MELAS to be due to deficiencies in the Wobble base of the tRNA (Kirino et al., 2004; Yasukawa et al., 2001). The C3303T mutation in mitochondrial tRNA results in an acceptor stem substitution that reduces the efficiency of CCA addition five-fold (Levinger et al., 2004). Additionally, mutations in the TΨC stem-loop of mitochondrial tRNA^{Ile} and tRNA^{Gly} also resulted from

decreased CCA-adding because of resulting structural changes and are found to cause fatal infantile cardiomyopathy and sudden infant death syndrome respectively (Tomari et al., 2003). This lack of CCA-adding to the tRNA is predicted to result in an overall decrease in their aminoacylation and subsequent instability (Tomari et al., 2003). Mutations affecting mt-tRNA that decreased their aminoacylation resulted in increased degradation of that tRNA due to reduced protection by the aminoacyl tRNA synthetase (Belostotsky et al., 2012). Mutations in tRNA-NT may result in decreased CCA trinucleotide addition to tRNA with less conserved secondary structures (especially seen in mitochondrial tRNAs) or those with reduced base modifications, the combination resulting in pathobiologies.

The 3' CCA tRNA terminus is subject to frequent cleavage or degradation and tRNA-NT also functions to maintain or repair previously added CCA sequence, even in *E. coli* where CCA is encoded in the pre-tRNA (Yue et al., 1996; Zhu & Deutscher, 1987). Decreasing tRNA-NT cytoplasmic pools cause defects that are accompanied by an accumulation of end-shortened tRNA in the cytoplasm, highlighting the role of tRNA-NT in cytoplasmic tRNA repair (Wolfe et al., 1996a). Interestingly, with a reduction of tRNA-NT, the amount of end shortening (lack of CCA repair) differs per tRNA species, even within different codons for the same amino acid (Wolfe et al., 1996a).

1.3.2 tRNA-NT structure

The tRNA-NT enzymes belong to the nucleotidyl transferase superfamily of enzymes that add nucleotides in a primer-dependent template-independent manner to RNA, DNA, and protein; other members include the poly (A) polymerases for mRNA modification and terminal deoxynucleotidyl transferase (Holm & Sander, 1995; Martin & Keller, 2007; Shi et al., 1998; Yue et al., 1996). The crystal structure of human tRNA-NT was resolved by

Augustin et al. (2003) and multiple co-crystal structures of tRNA-NT in complex with substrate tRNA have also been resolved (Cho et al., 2006; Liet al., 2002; Toh et al., 2009; Tomita et al., 2004; Tomita et al., 2006; Xiong & Steitz, 2004; Yamashita & Tomita, 2016).

tRNA-NT localization in the cell is regulated by 5' alternative start codons and internal motifs for mitochondrial targeting, but nuclear targeting sequences are currently unknown (Leibovitch et al., 2013b; Nagaike et al., 2001; Schmidt von Braun et al., 2007; Wolfe et al., 1994). tRNA-NTs contain an amino-terminal head, neck, body, and C-terminal tail domain with the single active site responsible for both C and A addition contained in the highly conserved head domain and consisting of five motifs (A to E) (Augustin et al., 2003; Li et al., 2002; Yue et al., 1998) (Figure 3.1). Motifs A through E are highly conserved in all tRNA-NTs: motif A contains the conserved sequences essential for metal-binding catalytic carboxylates required for binding the incoming dNTPs; motif B functions in discriminating between incoming dNTPs; motif C modulates the head and body domains relative to the bound tRNA and contributes to switching specificity between dNTPs; motif D functions as the amino acid template for dNTP binding; and motif E stabilizes the neck domain and is involved in interacting with the extending 3' end of the tRNA and positioning the tRNA for release (Augustin et al., 2003; Betat et al., 2010; Cho et al., 2006; Cho et al., 2007; Ernst et al., 2015; Leibovitch et al., 2018; Li et al., 2002; Lizano et al., 2008; Tomita et al., 2004; 2006; Xiong & Steitz, 2004; Yamashita & Tomita, 2016).

A yeast tRNA-NT mutation, *cca1-1* (ts-352, *cca1-E189K*), was the first characterized tRNA-NT temperature sensitive mutant; the phenotype is due to a lack of protein synthesis at both permissive and non-permissive temperatures (Aebi et al., 1990; Goring et al., 2013; Peltz, et al., 1992). Located within the C motif, this single amino acid change affected the structure in a temperature dependent manner, resulting in 4% enzymatic function at the

permissive temperature and less than 1 % at non-permissive temperatures *in vitro* (Goring et al., 2013; Shan et al., 2008). When *cca1-1* was shifted to the non-permissive temperature, an almost immediate loss of enzyme activity occurred *in vitro*, accompanied by an almost immediate equilibrium of CCA-containing and CCA-absent tRNAs (Aebi et al., 1990; Peltz et al., 1992). This loss of tRNA-NT activity resulted in a 50% drop in protein synthesis within 10 minutes and complete loss within 50 minutes of shifting to the non-permissive temperature (Peltz et al., 1992).

The deleterious effect of the *cca1-1* mutation results in decreased enzymatic activity at all temperatures, but only results in growth defects at high temperatures due to an additional structural change (Goring et al., 2013). Interestingly, a secondary mutation at amino acid in motif A (R64) was able to suppress the *cca1-1* phenotype without affecting the protein structure (Goring et al., 2013). It is hypothesized that E189 and R64 contribute to the organization of the catalytic domain of tRNA-NT. Work by collaborators has also determined that five patient-identified *TRNT1* mutations in the N-terminal head and neck domain (*T154I*, *M158V*, *L166S*, *R190I*, and *I223T*) affect both the structure and enzymatic activity of tRNA-NT *in vitro* (Leibovitch et al., 2018). All mutations had reduced thermostability, and in addition *M158V* and *I223T* had reduced affinity for CTP and *R190I* had reduced ability to add nucleotides to the non-canonical tRNA^{Ser(AGY)} and mini-tRNAs (Leibovitch et al., 2018).

The C-terminal body and tail domains are proposed to interact with the tRNA, evidenced by foot-printing and phosphate modification interference data (Li et al., 2002; Zhu & Deutscher, 1987). Regions in the C-terminal body domain are also involved in subcellular localization by stabilizing the N-terminal import signals (Leibovitch et al., 2013a; von Braun et al., 2007). Mutations in the body domain facing the incoming tRNA molecule resulted in

altered tRNA-binding and the mutated tRNA-NT underwent novel structural changes when tRNA was removed (Leibovitch et al., 2013a). Interestingly, these C-terminal mutations also altered localization between plant mitochondria and plastids, and mutations not interacting with tRNA resulted in an overall decrease in organellar localization with increased presence in the cytoplasm (Leibovitch et al., 2013a).

1.3.3. tRNA-NT CCA-adding activity

Unlike other polymerases, tRNA-NT performs template-independent sequence-specific nucleotide addition, thus requires a specialized protein-tRNA interaction (Vörtler & Mörl, 2010). The tRNA molecules remain attached to the tRNA-NTs throughout the addition of the CCA nucleotides, with successive NTPs added to the growing 3' end of the tRNA. This refolding and reposition of the single active site for consecutive nucleotide addition has been termed the collaborative templating model, described below (Augustin et al., 2003; Cho et al., 2006; Ernst et al., 2015; Shi et al., 1998).

The tRNA acceptor stem and T-loop bind to tRNA-NT the tRNA molecule such that the tRNA acceptor stem directly contacts tRNA-NT C-terminal tail (Augustin et al., 2003; Cho et al., 2003; Li et al., 2002; Shi et al., 1998; Tomita et al., 2004). Once the tRNA is bound, the active site of tRNA-NT coverts from a neutral conformation to a CTP binding state and the C74 is added to the tRNA. The addition of C74 is recognized by a second set of amino acids to orient the new 3'-OH of the ribose for C75 addition (Kuhn et al., 2015; Tomita et al., 2004). The tRNA remains fixed but the new 3' end forces a refolding of the active site to reposition the new 3' end to enable C75 addition (Cho et al., 2006; Kuhn et al., 2015). This collaborative mechanism of both amino acid template and 3' nucleotide interactions contribute to the high nucleotide specificity in the CCA adding and enables

partial CCA repair (Yue et al., 1998). Following the C75 addition, the nucleotide-binding pocket in motif D switches to an ATP binding, altering the nucleotide-binding pocket to incorporate A76 and release the tRNA (Augustin et al., 2003; Cho et al., 2006; Neuenfeldt et al., 2008; Tomita et al., 2006). Mis-incorporation of a nucleotide in place of CTP or ATP results in premature termination of nucleotide addition (Lizano et al., 2008). The tight association of the C-terminal body domain of tRNA-NT with the acceptor stem and T-loop of the tRNA ensure that nucleotide addition ends after CCA (Betat et al., 2004; Tomita et al., 2006).

1.4. Translation regulation

1.4.1. General Amino Acid Control (GAAC)

Internal amino acid sensing is controlled by the General Amino Acid Control (GAAC) in yeast or the Integrated Stress Response (ISR) in mammals through the expression of the transcription factor Gcn4 (Atf4 in mammals) resulting in the induction of amino acid biosynthesis genes and amino acid transporters (Hinnebusch, 2005; Natarajan et al., 2001; Proud, 2005; Zaman et al., 2008). In yeast, phosphorylation of eIF2 α (eIF2 α -P) by Gcn2 decreases translation initiation and promotes the translation of Gcn4, a transcription factor that up-regulates amino acid biosynthesis (Dever et al., 1992; Hinnebusch, 1993; Holcik & Sonenberg, 2005). The eIF2 complex binds with initiator tRNA^{iMet} in a GTP-dependent manner to initiate translation by bringing the initiator tRNA to the ribosome, following which GTP is hydrolyzed to GDP, releasing eIF2. eIF2 α -P is a competitive inhibitor of eIF2B, the subunit responsible for nucleotide exchange of GDP for GTP, thus prevents the recycling of eIF2 complex and subsequent translation (Proud, 2005).

eIF2 α -P reduces the amount of functional ternary complexes for translation initiation, thus the ribosome continues to scan through three upstream inhibitory ORFs of *GCN4* as it waits for the ternary complex (Hinnebusch, 2005). The final ORF on the *GCN4* mRNA contains the protein-coding region, and is thus increased in translation rate under starved conditions. Inhibition of eIF2 α -P (*sui2-S51A* or *gcn2 Δ*) prevents de-repression of *GCN4* (Hinnebusch, 2005). This transcriptional activation (upregulation of transcription factors) versus translational attenuation appears to be the intended evolved consequence in yeast, as mutations that prevent *GCN4* translation have similar phenotype to those that prevent eIF2 α -P; although down-regulation of translation by eIF2 α -P may still have other purposes (Dever, 2002).

1.4.1.1. Gcn2 activation

In yeast, the main induction of GAAC is through uncharged tRNA binding to Gcn2 and subsequent activation resulting in eIF2 α -P; eIF2 α is the only known substrate for Gcn2 kinase activity (Cherkasova & Hinnebusch, 2003; Hinnebusch, 1993; 2005; Wek et al., 1995; Wolfe et al., 1996b; Zaborske et al., 2009). Gcn2 contains a tRNA amino acyl synthetase-like binding domain capable of binding uncharged tRNA resulting in a conformational change which converts it to an active kinase, thus sensing amino acid starvation or defects in tRNA aminoacylation (Dong et al., 2000; Hinnebusch, 2005; Qiu et al., 2001; Wek et al., 1995).

Many different stressors result in uncharged tRNA in addition to amino acid starvation, these include: glucose limitation (Yang et al., 2000), high sodium (Goossens et al., 2001), treatment with rapamycin (Cherkasova & Hinnebusch, 2003; Valenzuela et al., 2001); reviewed in (Hinnebusch, 2005). Glucose limitation result in decreased levels of

cytoplasmic amino acid pools and decreases tRNA amino acylation (Yang et al., 2000). High sodium concentrations affect the dimerization of Gcn2 and making tRNA binding more efficient, this is suppressed with subsequent increase of potassium to balance the Na⁺/K⁺ levels and reduce the sodium stress (Narasimhan et al., 2004). Rapamycin activates Gcn2 because it inhibits TORC1-activated proteins that are responsible for dephosphorylation of Gcn2 thus increasing its tRNA binding ability (Cherkasova & Hinnebusch, 2003; Garcia-Barrio et al., 2002; Valenzuela et al., 2001).

Gcn2 has low activity in nutrient supplied cells, but upon starvation becomes activated by auto-phosphorylation at T882 and T887 (Romano et al., 1998). Snf1, the activator of glucose-repressed genes at low glucose levels (yeast AMPK), also stimulates eIF2 α -P, both by increasing Gcn2 kinase activity by phosphorylating T882 and repressing Glc7 (Cherkasova et al., 2010). Snf1 also activates a large number of genes involved in mitochondrial function in response to nutrient limitation (Young et al., 2003).

Glc7 of the PP1 (Protein Phosphatase 1) and Sit4 complex are phosphatases found to dephosphorylate eIF2 α in yeast (Cherkasova et al., 2010; Wek et al., 1992). Glc7 directly interacts with the eIF2 γ domain to target eIF2 α for dephosphorylation (Rojas et al., 2014). A temperature sensitive allele of *GLC7* resulted in increased eIF2 α -P and over expression of *GLC7* mimics the phenotype of *gcn2* Δ (Wek et al., 1992). When TORC1 is inactivated by rapamycin, Sit4 and PP2A members Pph1 and Pph2 are released from Tap42 and dephosphorylate various TOR substrates, including Gcn2 (Cherkasova & Hinnebusch, 2003). Thus, increases in eIF2 α -P may result from increased Gcn2 activity or decreased Gcn7/Sit4 activity. Deletion of *GCN2* or use of a phosphorylation deficient eIF2 α -P (*sui2-S51A* in yeast) mutant are used to evaluate this pathway, both prevent Gcn4 induction (Dever et al., 1992; Hinnebusch, 2005; Sonenberg & Hinnebusch, 2009). eIF2 α and eIF2B are

dispensable in yeast, with eIF2 γ being the only required subunit as long as adequate tRNA are present (Erickson et al., 2001).

1.4.1.2. Integrated Stress Response (ISR)

In higher eukaryotes, the Integrated Stress Response (ISR) pathways contains three additional kinases in addition to Gcn2: PERK (ER stress), HRI (haem and heavy metal stress), and PKR (dsRNA stress) (Proud, 2005; Sonenberg & Hinnebusch, 2009). These kinases all phosphorylate eIF2 α for translation attenuation and *ATF4* expression, and Gcn2 functions as described in the GAAC to respond to amino acid and nutrient stress. Given this research was done in yeast, I will not go further into the complex ISR pathways, review can be found in (Holcik & Sonenberg, 2005; Proud, 2005; Sonenberg & Hinnebusch, 2009).

1.4.2. eIF4E and eIF4e-BPs

Translation can also be regulated by sequestering eIF4E (encoded by *CDC33* in yeast) by eIF4E binding proteins (4E-BP1 and 4E-BP2 in mammals, *EAP1* and *CAF20* in yeast), thus preventing eIF4E from binding eIF4G that is required for 48S subunit formation (de al Cruz, Iost, Kressler, & Linder, 1997; Dever, Kinzy, & Pavitt, 2016; Pause et al., 1994; Sonenberg & Hinnebusch, 2009). eIF4E binds eIF4G and eIF4A to form the 5' cap binding complex eIF4F, with eIF4G and 4E-BPs competing for the binding of eIF4E, a process that is regulated by the phosphorylation state of the 4E-BPs (Haghighat, Mader, Pause, & Sonenberg, 1995; Pause et al., 1994).

In mammalian cells, phosphorylated 4E-BPs bind eIF4E with increasing phosphorylation on 4E-BPs decreasing binding (Gingras et al., 1998; Sonenberg & Hinnebusch, 2009). In mammals, when nutrients are available, active mTOR

phosphorylates 4E-BP1 to reduce its binding to eIF4E to increase translation, and alternatively, in low amino acid condition reduced phosphorylation on 4E-BP1 allows it to bind eIF4E to reduce translation (Barbet et al., 1996; Gingras et al., 1998; Laplante & Sabatini, 2012; Stanfel et al., 2009).

Yeast 4E-BPs (Eap1 and Caf20) share functional but not structural homology with mammalian 4E-BPs in binding eIF4E with a conserved eIF4E binding motif (Cosentino et al., 2000). Deletion of *EAP1* in yeast results in temperature sensitivity and partial resistance to rapamycin, indicating the possibility of phosphoregulation of Eap1 by the TOR pathway as observed in mammalian cells; deletion of *CAF20* had no impact on rapamycin sensitivity (Cosentino et al., 2000; Gingras et al., 1998; Sonenberg & Hinnebusch, 2009).

Eap1 has also been shown to attenuate TOR-inactivation-dependent Gcn4 activity separate from eIF4E binding, an interesting cross-talk between the GAAC and cap-dependent regulation of transcription (Matsuo et al., 2005). In this case, eIF2 α -P was required for the increase in *GCN4* translation following *EAP1* deletion (Matsuo et al., 2005). The apparent conflict of TOR activating Gcn2 to promote *GCN4* translation while also activating Eap1 to decrease *GCN4* translation is explained by a possible negative-feedback loop to fine tune the cellular response to nutritional stress where mild nutritional stress activates predominantly Gcn2 and severe nutritional stress, while still activating Gcn2, also increases Eap1 activation to reduce *GCN4* in times when other processes such as autophagy will be predominant (Matsuo et al., 2005).

1.4.3. TOR and translation regulation

TOR kinases are master regulators of growth and metabolism in response to nutrient and hormonal cues (González & Hall, 2017; Johnson et al., 2013; Loewith et al., 2002;

Stanfel et al., 2009). Yeast have two TOR paralogs, *TOR1* (not essential, found in TORC1 complex) and *TOR2* (essential, found in TORC1 and TORC2 complexes), but many other eukaryotes have only one (mTOR in humans) (Laplane & Sabatini, 2012). TORC1 is activated by environmental signals such as the presence of amino acids and growth factors, oxygen, and high cellular energy status through AMP/ATP levels indicated by AMP kinase activity (Hardie, 2007; Komeili et al., 2000; Laplane & Sabatini, 2012; Stanfel et al., 2009). This activation leads to stimulation of anabolic pathways leading to the promotion of cell growth in times of nutrient availability. In yeast, TORC1 signaling best responds to the levels of glutamine (Godard et al., 2007; Stracka et al., 2014), but also leucine (Binda et al., 2009), and arginine (Rebsamen et al., 2015). Interestingly, both glutamine and leucine are required for the production of α -ketoglutarate in the Krebs cycle, providing a sensing mechanism linked to the mitochondria (Duran et al., 2012). A review of amino acid sensing and TOR regulation in yeast and mammals can be found in González & Hall, 2017.

TORC1 promotes translation by activating substrates such as ribosomal protein S6 kinase (Sch9 in yeast) to promote growth when nutrients are available and through phosphorylation of 4E-BPs (inhibitory) to promote eIF4E cap-dependent translation (de al Cruz et al., 1997; Hara et al., 1998; Jastrzebski et al., 2007). Phosphorylation of S6 kinase results in increased mRNA biogenesis, translation initiation, and translation elongation through the subsequent activation of multiple effector proteins by the S6 kinase (Jastrzebski et al., 2007; Laplane & Sabatini, 2012). eIF4E and 4E-BP regulation is described in section 1.4.2. In addition, TORC1 binds 5S rRNA and tRNA genomic regions and represses Maf1, a Pol III repressor, thus inducing 5S rRNA and tRNA transcription rapidly following nutrient availability (Kantidakisa et al., 2010) and promotes the expression of rRNA (Mayer et al., 2004).

1.5. Translation homeostasis

Because the vast majority of the proteins functioning in the mitochondria are encoded in the nucleus (1000-1500 nuclear-encoded proteins compared to 8 (yeast) to 13 (mammals) mitochondrial-encoded proteins (Kumar et al., 2002; Lopez et al., 2000)), the maintenance of translation homeostasis is required to prevent the accumulation of pre-proteins and mis-folded proteins while at the same time ensuring adequate protein supply for the mitochondria (Battersby & Richter, 2013; Eisenberg-Bord & Schuldiner, 2017; Raimundo, 2014; Suhm & Ott, 2016; Topf et al., 2016; Yun & Finkel, 2014). Inhibition of cytosolic translation directly results in changes in mitochondrial translation, with some mRNA gaining translational capacity but others losing, independent of nutrient state (Couvillion et al., 2016). In contrast, cytoplasmic translation responded similarly to a nutrient shift whether or not mitochondrial translation was inhibited or mtDNA was absent, indicating that nuclear and mitochondrial translation synchronization is controlled by cytoplasmic signals (Couvillion et al., 2016).

Although mitochondrial translation does not regulate cytoplasmic translation, dysfunctional mitochondria trigger stress response signaling due to changes in ATP, mitochondrial membrane potential, defects in import, and unfolded protein accumulation which regulate cytoplasmic translation to re-establish coordination between cytoplasmic and mitochondrial translation (Battersby & Richter, 2013; Eisenberg-Bord & Schuldiner, 2017; Raimundo, 2014; Suhm & Ott, 2016; Yun & Finkel, 2014). Pathways that sense mitochondrial stress include: the mitochondrial unfolded protein response (mtUPR) (Jovaisaite & Auwerx, 2015; Pellegrino et al., 2013), the mitochondrial precursor over-accumulation stress (mPOS) (Wang et al., 2008a), the accumulation unfolded protein response (amUPR) (Wang & Chen, 2015), mitochondrial compromised protein import

response (mitoCPR) (Weidberg & Amon, 2018), and the retrograde response (RTG) (Trendelewa & Zvyagilskaya, 2018).

1.5.1. Retrograde response (RTG)

One of the best characterized mitochondria-to-nuclear signaling pathway is the Retrograde response (RTG), best characterized in yeast and thought to be NFkB and CREB in metazoans, to ensure glutamate homeostasis by maintaining the first three reactions of the Krebs cycle during mitochondrial dysfunction (Figure 1.1) (Liu & Butow, 2006; Pellegrino et al., 2013; Trendelewa & Zvyagilskaya, 2018; Zaman et al., 2008). Glutamate is the main donor of nitrogen for anabolic biosynthesis processes and its levels are maintained in conditions without functional respiration by the glyoxylate cycle (Zaman et al., 2008). The RTG response activates two sets of reactions, first peroxisomal anaplerotic reactions to counteract mitochondrial dysfunction, and the second increases expression of α -ketoglutarate to supply adequate glutamate for the glyoxylate cycle to supply the Krebs cycle (Liu & Butow, 1999; 2006; Magasanik & Kaiser, 2002). The presence of glutamate or ATP represses the RTG response (Liu & Butow, 1999).

The cytoplasmic Rtg2 senses mitochondrial stress and glutamate to promote the localization of the transcription factors Rtg1/Rtg3 to the nucleus and promote the expression of target genes (Jazwinski, 2014; Liu & Butow, 2006; Sekito et al., 2000). Rtg2 is a cytoplasmic phosphatase that both dephosphorylates Rtg3 for activation and nuclear migration; Rtg1 is not regulated by phosphorylation but is required to keep Rts3 in the nucleus (Liu et al., 2003; Sekito et al., 2000; Sekito et al., 2002; van Heusden & Steensma, 2001). Rtg1/Rtg3 have been proposed to be homologs of the mammalian Myc-Max transcription factor but this pathway is still undefined in mammals (Srinivasan et al., 2010).

In yeast, the RTG response is signaled by decreased ATP concentrations (Zhang et al., 2013), osmotic stress (Pastor et al., 2009), or decrease in mitochondrial membrane potential (Miceli et al., 2012). Both rapamycin and mitochondrial dysfunction have been shown to up-regulate the RTG response (Giannattasio et al., 2005; Harris et al., 2012; Komeili et al., 2000). When cells become respiratory incompetent, gene expression changes to increase expression of proteins required to maintain cellular energy production and prevent amino acid starvation (Liao et al., 1991; Liu & Butow, 1999); many of these genes are regulated by the RTG pathway (Sekito et al., 2002). The exact signals from mitochondrial dysfunction to induce the RTG response has yet to be determined (Eisenberg-Bord & Schuldiner, 2017).

1.5.2. Mitochondrial precursor stress response pathways

Mitochondrial precursor over-accumulation stress (mPOS) is triggered by mitochondrial damage (not limited to import) resulting from over accumulation of precursors and misfolding in the cytoplasm that results in the modulation of cytoplasmic ribosomal biogenesis, mRNA decapping, sequestering the 60S ribosome subunit, and cap-independent translation (Kaukonen et al., 2000; Wang & Chen, 2015; Wang et al., 2008b). All of these functions work together to reduce cytoplasmic protein production, in a proposed effort to maintain mitochondrial and cytoplasmic protein homeostasis. Defects in mitochondrial import, lowering inner membrane potential, or mis-folding of MIM proteins leads to a rapid decrease in cap-dependent and increase in cap-independent cytoplasmic translation, a standard translational stress response (Merrick, 2004; Wang & Chen, 2015).

Figure 1.1.

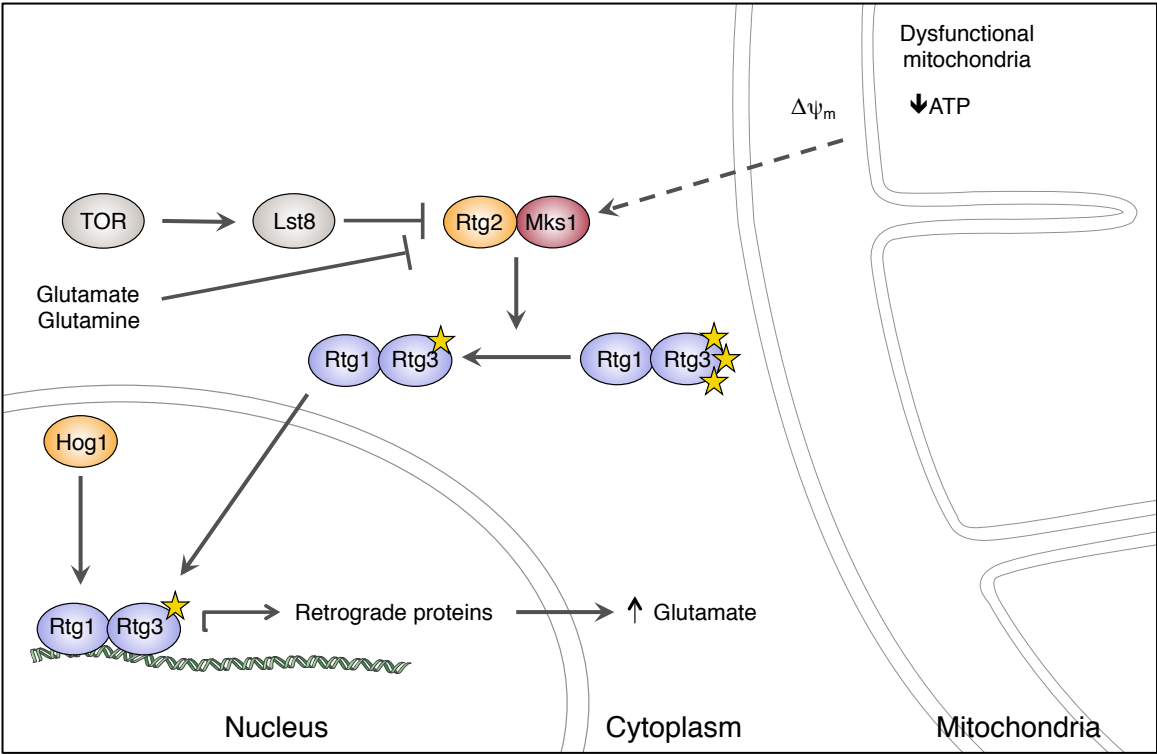


Figure 1.1. The Retrograde (RTG) Response. In yeast, the RTG response maintains glutamate homeostasis by upregulating the first three reactions of the Krebs cycle in times of mitochondrial dysfunction (Pellegrino et al., 2013; Trendeleva & Zvyagilskaya, 2018; Zaman et al., 2008). Decreased ATP is the main signal, occurring due to impaired electron transport or reduced mitochondrial membrane potential (Zhang et al., 2013; Miceli et al., 2012); the presence of glutamate or ATP represses the RTG response (Liu & Butow, 1999). The RTG response is mediated by the positive regulators Rtg1, Rtg2, Rtg3, and Grr1, and negative regulators Mks1, Bmh1, and Bmh2 (Trendeleva & Zvyagilskaya, 2018; Zaman et al., 2008). Rtg2 is a cytoplasmic protein with an ATP-binding domain that binds Mks1 in the presence of ATP, preventing Msk1 from binding Bmh1 and Bmh2. Reduced ATP allows Rtg2 to disassociate from Mks1 and phosphorylate Rtg3, promoting Rtg1/Rtg3 import into the nucleus where they function as transcription factors. Lst8, a member of TORC1, is a negative regulator of the RTG response by controlling amino acid permeases and Rtg3 phosphorylation in both an Rtg2 dependent- and independent- manner (Chen & Kaiser, 2003; Liu et al., 2001; Liu & Butow, 2006). Hog1 induces Rtg1/Rtg3 without a decrease in ATP levels, suggested to maintain low ROS under osmotic stress; osmotic stress such as high salt can increase ROS production (Pastor et al., 2009; Barrow et al., 2004).

The Accumulation Unfolded Protein Response (amUPR) is signaled by the accumulation of mis-targeted protein accumulation in the cytosol resulting in activation of the proteasome and inhibiting protein synthesis; this pathway was found in yeast (Monaghan & Whitmarsh, 2015; Topf et al., 2016; Wrobel et al., 2015). Accumulation precursors results in decreased cytoplasmic translation and increased proteasomal activation; found when proteins were over expressed, altered mitochondrial targeting signals, or impaired mitochondrial import (Merrick, 2004; Wang & Chen, 2015; Wrobel et al., 2015). When mitochondrial protein import and protein homeostasis are impaired, mis-localized cytoplasmic proteins become toxic if not quickly degraded (Chacinska et al., 2009; Wang & Chen, 2015; Wrobel et al., 2015). In yeast, cytoplasmic levels of Hsp60 (mitochondrial chaperone) may play a role in regulating proteasome activity with regard to mitochondrial function (Kalderon et al., 2015). This links mitochondrial biogenesis and function to proteasomal activity and may explain the lifespan extension observed in some mitochondrial mutants in yeast and flies (Wrobel et al., 2015).

A recently identified pathway, mitoCPR, has also been shown to protect the cell from mitochondrial import dysfunction by preventing accumulation of precursors by proteasomal degradation at the mitochondrial surface (Weidberg & Amon, 2018). Interestingly, the pleiotropic drug resistance (PDR) pathway transcription factor *PDR3* was found to mediate the mitoCPR, triggered by mitochondrial import defects (Weidberg & Amon, 2018). It is suggested that Pdr3-mediated gene expression is required for maintaining mitochondrial respiration in times of defective mitochondrial import (Weidberg & Amon, 2018).

The PDR pathway is upregulated in response to drugs, heat shock, and mitochondrial dysfunction to enable tolerance to a wide range of toxic agents and anti-fungal drugs in yeast (Balzi & Goffeau, 1995; Hallstrom & Moye-Rowley, 2000; Prasad & Goffeau, 2012; Shahi

et al., 2010). The pathway contains ATP-binding cassette (ABC) transporters and major facilitator superfamily members that function as transport proteins in the cytoplasmic membrane, metabolic regulators, and transcription factors that up-regulate the transporter proteins (Balzi & Goffeau, 1995; Prasad & Goffeau, 2012). *PDR5* upregulation due to mitochondrial dysfunction is mediated by Pdr3, and is known to provide resistance to cycloheximide, chloramphenicol, and sulfometuron methyl (Balzi & Goffeau, 1995; Hallstrom & Moyer-Rowley, 2000; Moyer-Rowley, 2005). Cells lacking mtDNA (petite, ρ^0) have upregulation of *PDR5* but not all cytoplasmic petite mutations (mutations in nuclear genes resulting in nonfunctional OXPHOS) result in upregulation of the PDR (Hallstrom & Moyer-Rowley, 2000). Deletion of *ATP2* (a nuclear-encoded component of the mitochondrial F1 ATPase, the null is petite and OXPHOS incompetent) only slightly increases *PDR5* induction and does not result in increased resistance to cycloheximide, indicating that loss of OXPHOS does not cause induction of the PDR (Hallstrom & Moyer-Rowley, 2000; Shahi et al., 2010).

1.5.5. Mitochondrial unfolded protein response (mtUPR)

The mtUPR (mitochondrial unfolded protein response) is a protective transcriptional response to increase nuclear production of mitochondrial chaperones and proteases, reduce mitochondrial import, and increase ROS detoxification, while slowing general cytoplasmic protein translation (by eIF2 α -P) and upregulation of stress responses (*GCN4*) to maintain homeostasis for optimal mitochondrial function, found to date in *C. elegans* and mammals, but not currently in yeast (Figure 1.2) (Jovaisaite & Auwerx, 2015; Pellegrino et al., 2013; Shpilka & Haynes, 2017; Topf et al., 2016; Yun & Finkel, 2014; Haynes et al., 2010). Mitochondria degrade 6 to 12% of the mitochondrial proteome constantly to maintain

protein quality control (Augustin et al., 2005). The resulting peptides are exported to the cytoplasm and signal cellular responses sensitive to increased or decreased peptide production (Augustin et al., 2005; Haynes et al., 2010).

The mtUPR is induced by the accumulation of unfolded proteins, oxidative stress, perturbed respiratory chain function, or mtDNA depletion to result in Gcn2-dependent eIF2 α -P to reduce cytoplasmic translation and subsequent mitochondrial protein import (Baker et al., 2012; Michel et al., 2015; Rainbolt et al., 2013). The reduction of cytoplasmic translation reduces the mtUPR by reducing available proteins for mis-folding in the mitochondria, thus protecting from stress (Baker et al., 2012). Counter to this hypothesis, it was found that rapamycin (inhibitor of TORC1, decreases anabolic pathways) up regulates the mtUPR (Houtkooper et al., 2013), although RNAi against specific TOR pathway components was shown to suppress the mtUPR (Baker et al., 2012; Runkel, Baumeister, & Schulze, 2014), highlighting a balance requirement of the translation system in times of mitochondrial and nutrient stress (Runkel et al., 2014).

A homologous yeast mtUPR has not been identified, although there are examples of protease and chaperone upregulation due to mitochondrial stress (Eisenberg-Bord & Schuldiner, 2017; Suhm & Ott, 2016). In yeast, decreased export of degraded mitochondrial peptides signal reduced mitochondrial protein degradation, a condition which would also occur in times of increased mitochondrial function in greater need of nuclear encoded mitochondrial proteins (Arnold et al., 2006). Nuclear gene expression of mitochondrial proteins were upregulated under these conditions, indicating that a mitochondria-to-nucleus signaling pathway involving proteases and chaperones also exists in yeast (Arnold et al., 2006).

Figure 1.2.

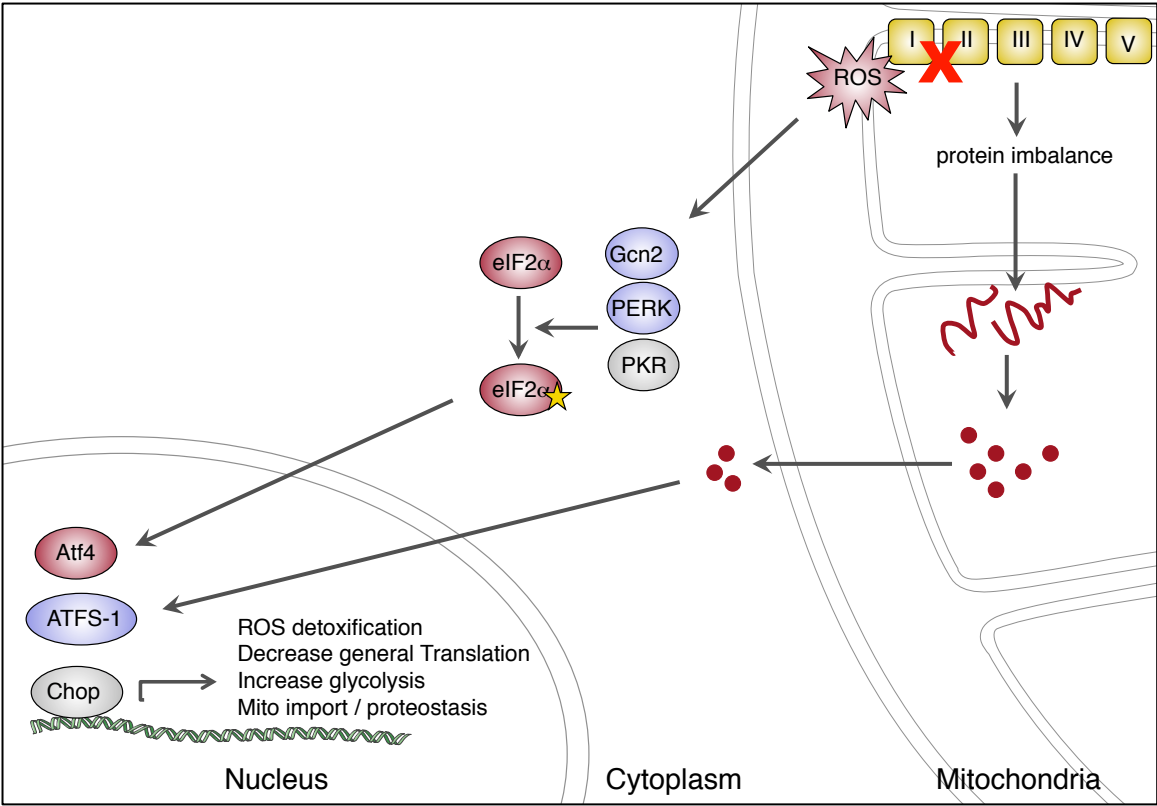


Figure 1.2. Mitochondrial unfolded protein response (mtUPR). Unequal protein production by the cytoplasm and mitochondria results in unfolded mitochondrial proteins waiting for complex assembly, resulting in protein accumulation, impaired electron transport chain, increased ROS, and reduced mitochondrial import (Pellegrino et al., 2013). Unfolded proteins overwhelm the available chaperones and are degraded by proteases, with the peptides exported to the cytoplasm signaling mitochondrial stress (Haynes et al., 2010). Reduced mitochondrial import relocalizes transcription factors such as ATFS-1 to the nucleus (Haynes et al., 2010). Increased ROS from impaired respiratory chain function results in eIF2 α -P and subsequent slowing of cytoplasmic translation and upregulation of Atf4; reduction of cytoplasmic translation reduces the protein load stress on the mitochondria (Baker et al., 2012). Degraded peptides, Atf4, inhibition of the electron transport chain, or loss of mtDNA induce transcription factors such as CHOP and ATFS-1 to upregulate mitochondrial stress proteins including proteases, chaperones, ROS detoxification enzymes, to reduce protein import, and increase glycolysis components (Zhao et al., 2002; Rainbolt et al., 2013; Michel et al., 2015; Fujita et al., 2007; Martinus et al., 1996). MtUPR proteins that are worm specific are blue, mammalian specific are grey, and shared are red; this pathway is not yet identified in yeast.

Another mtUPR-like pathway in yeast was identified in a prohibitin (*phb1Δ phb2Δ*, mitochondrial chaperones) mutant with reduced lifespan and increased levels of mitochondrial stress response proteins (Nijtmans et al., 2000; Schleit et al., 2013). In this case, mitochondrial stress caused by the absence of Phb1 and Phb2 was reduced by caloric restriction (mimic of reduced TOR activity) and reduction of ribosome proteins, both of which cause a reduction of cytoplasmic translation (Schleit et al., 2013). The reduced lifespan caused by deletion of the prohibitins was rescued by deletion of *RAS2*, which reduced mitochondria biogenesis to a point the cell can manage the protein degradation as induced by the retrograde response (Kirchman et al., 2003; Schleit et al., 2013). It was proposed that the suppression of the prohibitin mutant was due to re-balanced protein homeostasis from the cytoplasmic and mitochondrial translation programs for mitochondrial function (Schleit et al., 2013).

A final example in yeast is the expression of a dominant allele of *AAC2* (Adenine nucleotide translocase) that models the mitochondrial degeneration observed in human autosomal dominant Progressive External Ophthalmoplegia (ad PEO) (Wang et al., 2008a; Zhao et al., 2002). This defect was suppressed by reducing cytoplasmic translation using cyclohexamide or ribosomal protein deletions (Wang et al., 2008a), supporting the model that in times of mitochondrial dysfunction a balance between nuclear and mitochondrial production protein is necessary.

1.6. Patient-identified mutations in *TRNT1*

Working with the FORGE consortium (Finding of Rare disease Genes), a whole-exome sequencing strategy was used to identify disease-causing mutations in six patients with probable mitochondrial disease (Wiseman et al., 2013). In Ottawa, a young girl

presented with a mitochondrial disease with symptoms including hearing impairment, sideroblastic anemia, hyperalaninemia, periodic fever, B-cell immunodeficiency, and developmental delay. Whole exome sequencing in patients identified mutations in *TRNT1* which were found to be causative of SIFD (Sideroblastic Anemia With B-Cell Immunodeficiency, Periodic Fevers, And Developmental Delay) (Chakraborty et al., 2014; Wiseman et al., 2013). Two non-conservative missense mutations in the tRNA-NT gene (*TRNT1*, *CCAI* in yeast) were identified in this patient and in the five other patients: seven missense, one frame shift, and three splice site mutations were identified; mutations were biallelic in all patients (Chakraborty et al., 2014). Five of the missense mutations cluster in the well-conserved head and neck domain containing the CCA-adding active site (*T154I*, *M158V*, *L166S*, *R190I*, *I223T*) and the remaining two mutations are in the less conserved C-terminal region (*I326T*, *K416E*) (Betat et al., 2004; Leibovitch et al., 2018; Leibovitch et al., 2013a).

siRNA knockdown of *TRNT1* in control fibroblasts resulted in increased cytotoxicity and apoptosis, consistent with the essential function of this protein in translation (Chakraborty et al., 2014). Patient-identified mutations in fibroblasts were found to have decreased levels of nuclear and mitochondrial encoded OXPHOS proteins (Liwak-Muir et al., 2016). Consistent with this, there was decreased basal and maximal respiratory rates but no change in mitochondrial morphology, membrane potential, or architecture. Additionally, the mutations did not cause defects in *TRNT1* subcellular localization, global translation, or general cellular morphology (Liwak-Muir et al., 2016).

In 2015 another group identified additional patient-identified mutations in *TRNT1* (*A148V* and *Y173F*) resulting in SIFD (Sasarman et al., 2015). Patient muscle cells showed decreased mitochondrial translation of OXPHOS components, but no changes were detected

in patient fibroblasts even though TRNT1 was detected at 10% of wild type levels. Further assessment of patient fibroblasts revealed that while mitochondrial translation rates were slightly reduced, OXPHOS complex formation was not impacted, suggesting that mitochondrial translation could function with decreased TRNT1 levels (Sasarman et al., 2015). Knockdown of TRNT1 by siRNA in one patient's fibroblasts resulted in undetectable levels of the non-canonical mt-tRNA^{Ser(AGY)} and decreased levels of cytoplasmic tRNA^{Lys(UUU)} and cytoplasmic tRNA^{Met} (Sasarman et al., 2015). TRNT1 siRNA resulted in reduced mitochondrial translation of some mtDNA products, and it was suggested that defects in mt-tRNA^{Ser(AGY)} was the cause; mitochondrial encoded ND4L and ATP8 do not have this codon and were unchanged (Sasarman et al., 2015).

Analysis of *TRNT1-R190I* and *I223T* patient fibroblasts found decreased levels of 3' CCA addition to mitochondrial tRNA^{Cys}, tRNA^{His}, and tRNA^{LeuUUR} but no changes in mitochondrial translation; cytoplasmic tRNA were not assessed (Wedatilake et al., 2016). No defects in mitochondrial translation products were detected by western blot or *in vivo* labeling in patient fibroblasts, but assessment of patient respiratory chain enzymes showed decreased levels in muscle and liver cells (Wedatilake et al., 2016).

An additional group studied the patient-identified *TRNT1* mutations *R99W*, *T110I*, *D128G*, and *D163V* and found by sequencing 227 cytoplasmic tRNA that 182 (80 %) were reduced by 1.6-fold on average in patient fibroblasts (Giannelou et al., 2018). Increased IL-1B was also detected in patient tissue, linked to increased ROS production in patient cells (Giannelou et al., 2018). Analysis of the ubiquitin-proteasome system showed increased ubiquitination and impairment of autophagy in patient fibroblasts, suggesting that mutations in *TRNT1* result in impaired ability to up-regulate protein clearance pathways under stress conditions (Giannelou et al., 2018).

Patients with non-syndromic retinitis pigmentosa were also found to contain mutations in *TRNT1* (DeLuca et al., 2016; Hull et al., 2016; Sharma et al., 2017). Analysis of non-syndromic retinitis pigmentosa in zebrafish showed that mutations in *TRNT1* had consistent phenotypes as observed in patients with SIFD, including anemia and sensory organ defects (DeLuca et al., 2016). The defects were proposed to be caused by a lack of 3' CCA addition on cytoplasmic tRNA, with *TRNT1* mutants having 4.3x more transcripts without 3' CCA (DeLuca et al., 2016).

1.7. Homeostasis Hypothesis

Defects in TRNT1 function may result in the observed mitochondrial phenotypes because: (1) defective cytoplasmic tRNA 3'CCA addition impairing cytoplasmic translation and reducing levels of key nuclear-encoded mitochondrial proteins; (2) defective mt-tRNA 3'CCA addition reduces mitochondrial translation and possibly impairs OXPHOS leading to reduced ATP production; and/or (3) the loss of homeostasis between the cytoplasmic and mitochondrial translation systems results in a cellular stress response that contributes to the observed pathobiology. It is likely that all three causes are occurring, but by determining the degree to which they impact cellular function we will further our understanding of SIFD and mitochondrial diseases.

TRNT1/CCAI mutations have been shown to impact cytoplasmic tRNA (hypothesis 1) and mt-tRNA (hypothesis 2) 3'CCA addition and The occurrence of OXPHOS defects in all of these studies suggests a greater impairment of mt-tRNA function leading to dysfunctional mitochondrial translation (Giannelou et al., 2018; Sasarman et al., 2015; Wedatilake et al., 2016). I propose that patient-identified mutations in *TRNT1* and analogous *CCAI* mutations impair mitochondrial protein translation resulting in

dysfunctional mitochondria and an imbalance of mitochondrial and cytoplasmic translation homeostasis (hypothesis 3). Slowing of cytoplasmic translation may re-establish translation homeostasis in times of a high mitochondrial translation requirement but may also be detrimental in times of low mitochondrial translation requirements.

1.8. Redundant Regulation of Cdk1 Tyrosine Dephosphorylation in *Saccharomyces cerevisiae*

In addition to the project described above, another yeast phosphorylation signaling project was undertaken over the course of this degree. The ability to concurrently analyze the phosphorylation of Cdk1 and eIF2 α using similar processes highlights conserved mechanisms in yeast and mammal cell signaling and response to environmental conditions. This was a separate project and was accomplished while the *TRNT1/CCA1* project was in early stages. Unpublished data from Michael Dysart involving this project was summarized in his Honours thesis, Spring 2015.

Errors in the timing of chromosome separation can be catastrophic and lead to chromosome loss or damage, which can lead to the initiation of a cancerous cell. Cyclin-dependent kinase 1 (Cdk1) is a key regulator of chromosome segregation, drives mitotic entry and anaphase onset, and possibly more processes yet defined (Holt et al., 2009; Murray, 2004). Cdk1 is inhibited prior to mitotic entry due to inadequate cell size or altered bud morphogenesis, termed the morphogenesis checkpoint (Lew & Reed, 1995; Sia et al., 1996). While in humans, several cyclin-dependent kinases exist which are thought to regulate different parts of the cell cycle and mitosis (Enders & Maude, 2006), in budding yeast only a single cyclin-dependent kinase is involved in cell cycle regulation, Cdk1

(Russell & Nurse, 1986). The other five budding yeast Cdks are thought to assist in interphase and play a role in transcription (Enserink & Kolodner, 2010).

Cdk1 is regulated by several mechanisms, including the association to the appropriate cyclins (Schwob & Nasmyth, 1993; Surana et al., 1991) and interaction with cyclin dependent kinase inhibitors (Alberghina et al., 2004; Enserink & Kolodner, 2010). A third is the regulation by phosphorylation, activating on conserved T169 by Cyclin dependent kinase activating kinase (Cak1) and inhibitory on Y19 by Wee1 kinase (Swe1 in budding yeast) in yeast (Figure 1.6) (Kaldis et al., 1996; Russell & Nurse, 1987). Reversal of the inhibitory phosphorylation on Y19 is by the Cdc25 (Mih1 in yeast) phosphatase (Kellogg, 2003; Russell et al., 1989). We have recently shown that activating a Swe1-dependant checkpoint or Swe1 overexpression causes an arrest prior to anaphase; however, it is unknown if dephosphorylation of Cdk1 is required to induce anaphase (Liang et al., 2013; Tsuchiya & Lacefield, 2013).

1.8.1. Cyclin Dependent Kinase 1 (Cdk1)

Cdk1 Y19 dephosphorylation is catalyzed by the phosphatase Mih1, the budding yeast homologue of Cdc25 (Enserink & Kolodner, 2010; Russell et al., 1989; Russell & Nurse, 1986). Deletion of the Cdk1 phosphatase, Cdc25, in fission yeast is lethal indicating the essential role of Cdk1 dephosphorylation (Russell & Nurse, 1986). In budding yeast, although Mih1 (Cdc25 yeast homolog) is the only known Cdk1 phosphatase, deletion exhibits only mild defects in mitotic entry and anaphase initiation (Liang et al., 2013; Pal et al., 2008). This mild phenotype led researchers to assume that phosphorylation / dephosphorylation of Cdk1 in budding yeast plays little role in regulating unperturbed mitoses (Gould et al., 1990). However, careful analysis of *mih1Δ* cells has revealed that

dephosphorylation of Cdk1 still occurs, although delayed, and correlates with the initiation of anaphase (Liang et al., 2013; Pal et al., 2008). This result strongly suggests that a redundant phosphatase is needed to fully dephosphorylate Cdk1; the identity of this phosphatase is unknown. Interestingly, although deletion of *CDC25* in fission yeast is lethal, under conditions lacking both *WEE1* and *CDC25*, Cdk1 is still dephosphorylated on Y15, providing additional support for the presence of redundant phosphatases, even in fission yeast (Millar et al., 1992b).

Cdc25 is the only known Cdk1 phosphatase, so demonstrating another phosphatase can also perform this function will alter current models for regulation of cell cycle progression. Understanding the fundamental processes that integrate cellular cues to appropriately time chromosome segregation is central to understanding why chromosome mis-segregation occurs during tumorigenesis and to developing novel anti-mitotic chemotherapeutics that target the regulators of anaphase onset.

1.8.2. Protein Phosphatase 2A (PP2A)

PP2A is a hetero-trimeric complex composed of a catalytic subunit, an A-regulatory or scaffolding subunit and one of two B-regulatory subunits (*CDC55*, the yeast B55, or *RTS1*, the yeast B56) (Healy et al., 1991; Lin & Arndt, 1995; Shu et al., 1997; Stark, 1996). The B-regulatory subunits provide specificity to the two forms of the phosphatase, PP2A^{Cdc55} and PP2A^{Rts1}, and we have shown that PP2A^{Cdc55} is the form that dephosphorylates the APC and Separase, inhibiting anaphase onset (Liang et al., 2013; 2018). Paradoxically, PP2A^{Cdc55} also promotes mitotic entry and anaphase initiation by inhibiting Wee1 (Harvey et al., 2011).

Figure 1.3.

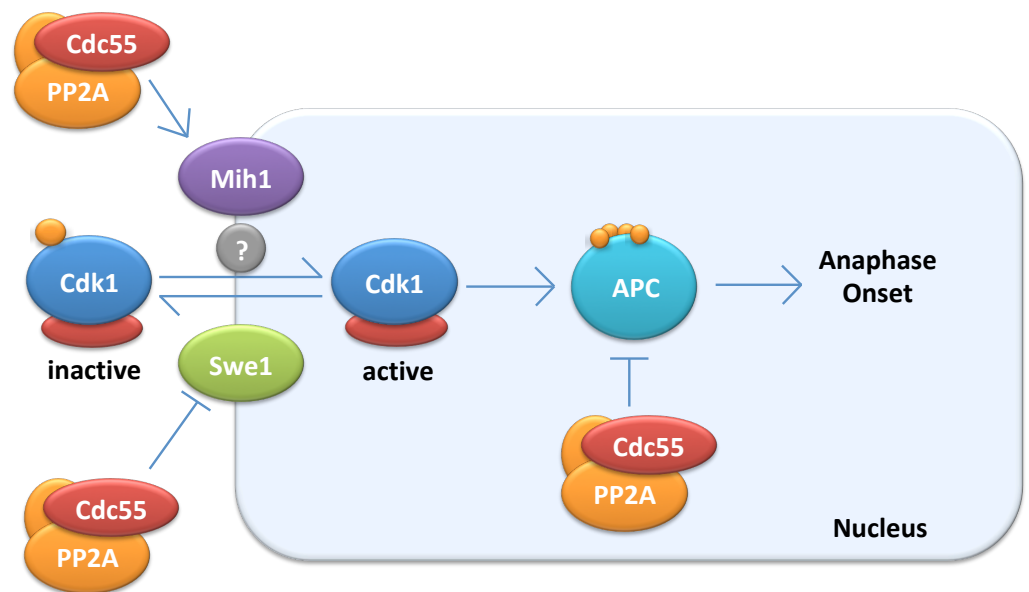


Figure 1.3. A model of Cdk1-Y19 phosphorylation regulation. Active Cdk1 drives entry into mitosis and anaphase onset by phosphorylating and activating the Anaphase promoting complex (orange balls). Swe1 inhibits Cdk1 by inhibitory phosphorylation at Y19 (orange ball) in budding yeast and this inhibitory phosphorylation is removed by the phosphatase Mih1. PP2A^{Cdc55} has multiple roles in this process, while it promotes mitotic progression and anaphase onset by inhibiting Swe1 and activating Mih1, it slows anaphase onset by inhibiting the APC. In budding yeast, although Mih1 is the only known Cdk1 phosphatase, deletion exhibits only mild defects in mitotic entry and anaphase initiation, although dephosphorylation of Cdk1 still occurs, suggesting the presence of another Cdk1 phosphatase.

Recent work has begun to characterize the phosphatases that dephosphorylate Cdk1 substrates and how their inactivation promotes mitosis while their activation assists entry into a subsequent G1 (Enserink & Kolodner, 2010; Mochida et al, 2010; Uhlmann et al., 2011). Protein phosphatase 2A (PP2A) opposes Cdk1 phosphorylation of Wee1 and Cdc25 and was thus believed to be the major phosphatase that antagonizes Cdk1 during mitosis (Castilho et al., 2009; Chen et al., 2007; Harvey et al., 2011; Mochida et al., 2010). Additionally, *mih1Δcdc55Δ* cells are inviable, further supporting the role of PP2A^{Cdc55} as a Cdk1 phosphatase (Pal et al., 2008). However, PP2A^{Cdc55} has opposing functions: while it promotes mitotic progression and anaphase onset by inhibiting Swe1 and activating Mhl1 (Harvey et al., 2011; Pal et al., 2008), it slows anaphase onset by inhibiting the APC and Separase (Liang et al., 2013). This opposing interaction can be explained by negative regulation of Swe1 by PP2A^{Cdc55} and PP2A^{Rts1} (Harvey et al., 2011).

Further complicating the analysis, mitotic PP2A^{Cdc55} and PP2A^{Rts1} activity may also be regulated by phosphorylation (Cayla et al., 1994; Fellner et al., 2003). Although PP2A is capable of tyrosine dephosphorylation *in vitro*, it is believed to function solely as a serine/threonine phosphatase *in vivo* (Foulkes et al., 1983; Janssens & Goris, 2001; Shi, 2009; Tonks, 2013). Two small regulatory proteins (Rrd1 and Rrd2, yeast homologs to mammalian PTPA) were found to regulate the catalytic subunit of PP2A and were proposed to be required PP2A and PP2A-like complexes substrate specificity between tyrosine or serine/threonine (Fellner et al., 2003). Deletion of *RRD2* resulted in a PP2A complex with low catalytic activity to serine/threonine substrates, altered substrate specificity, and metal dependence; Rrd1 was required for PP2A-like complex specificity (Fellner et al., 2003). Later work showed that Rrd2 is required to assemble a PP2A complex with a catalytic

subunit (Pph21 and Pph22), functioning as an activator protein to prevent unregulated catalytic activity (Hombauer et al., 2007).

1.8.3. Alternative phosphatases

In yeast, Ptp1-3 are protein phosphatase type 2C (PP2C) enzymes, with Ptp1 (Pyp3 in fission yeast) having a distinct in sequence and secondary structure compared to Ptp2 and Ptp3 which have homology to each other (Antherton-Fessler et al., 1993; Millar et al., 1992a). Human T-cell Ptp1 and budding yeast Mih1 can complement the fission yeast temperature sensitive *cdc25-22* allele, showing conservation between mammalian cells and various yeast (Gould et al., 1990; Mondesert et al., 1994; Russell et al., 1989). In yeast, neither Ptp2 nor Ptp3 were found to have this ability (Guan et al., 1992). Additionally, under conditions lacking both *wee1* and *cdc25* in fission yeast, Cdk1 is still dephosphorylated on Y15 (Y19 in budding yeast) (Millar et al., 1992b). The *wee1Δ cdc25Δ pyp3Δ* is lethal in fission yeast, suggesting that Ptp1 dephosphorylates the Y15 Cdk1 regulatory site (Millar et al., 1992a). Ptp1 was also shown to dephosphorylate Cdk1 *in vitro* in fission yeast, also shown indirectly in *Drosophila* (Chen et al., 2007; Millar et al., 1992b).

LMW-PTPs (Low Molecular Weight Protein Tyrosine Phosphatases) have been identified in yeast, Stp1 in fission yeast and Ltp1 in budding yeast (Magherini et al., 2004; Modesti et al., 2001; Mondesert et al., 1994). Over expression of *stp1* in fission yeast was able to rescue the *cdc25-22* temperature sensitive lethality, as observed for overexpression of *pyp3* (Mondesert et al., 1994). No assessment of Ltp1 in regards to Cdk1 phosphorylation levels in budding yeast has been done (Caselli et al., 2016).

Strains lacking Ppz1 were shown have roles in the G1/S transition (Clotet et al., 1999) and to accumulate of cells with a 2C DNA content and an increased percentage of

large-budded cells indicative of a G2/M delay or arrest (Niu et al., 2008). Ppz1, a serine/threonine phosphatase, has been shown to have a negative impact on Sit4 regulation and to target Gln3, either likely accounting for its role in cell cycle regulation (Clotet et al., 1999; Hirasaki et al., 2011). Although no phenotypes were observed at the G2/M transition, this was not done in a background lacking *MIH1*, and it could still be a redundant phosphatase for the tyrosine Y19 on budding yeast Cdk1 (Clotet et al., 1999).

1.8.4. Cak1 regulation

Cdk1 is regulated in budding yeast by phosphorylation in two ways, one is the inhibitory phosphorylation at Y19 by the Swe1 kinase, and the other is the activating phosphorylation at T169 by Cak1 (Alberghina et al., 2004; Enserink & Kolodner, 2010; Kaldis et al., 1996; Russell & Nurse, 1987). Cak1 phosphorylates Cdk1 at T169 which is in a large flexible loop that can block the Y19 phosphorylation site when unphosphorylated and stabilizes the cyclin-CDK interaction site (Kaldis et al., 1996; Russo et al., 1996; Thuret et al., 1996). Loss of T169 phosphorylation results in cell cycle arrest due to the inability to activate Cdk1 (Kaldis et al., 1996; Thuret et al., 1996). Cak1 has diverged from other CDKs in that it requires no cyclin nor phosphorylation for activity (Enke et al., 1999; Espinoza et al., 1996; Kaldis et al., 1996; Thuret et al., 1996). Cak1 in budding yeast is proposed to be the homolog to Csk1 in fission yeast but no metazoan homolog has been identified (Espinoza et al., 1996; J. Liu & Kipreos, 2000). T169 can be dephosphorylated by Ptc2 and Ptc3, although the significance of this dephosphorylation is unknown as in wild type cells T169 phosphorylation does not significantly vary over the cell cycle (Cheng et al., 1999; Ross et al., 2000).

Chapter 2: Mutations in *TRNT1* cause congenital sideroblastic anemia with immunodeficiency, fevers, and developmental delay (SIFD)

This chapter includes work describing SIFD (congenital Sideroblastic anemia with Immunodeficiency, Fevers, and Developmental delay) which I produced in collaboration with the Fleming, Holcik, and Chakraborty laboratories for publication in the journal *Blood*. I contributed equally to the work with Klaus Schmitz-Abe, Hapsatou Mamady, Turaya Naas, and Danielle Durie as second author. This included design and performance of the yeast *CCAI* knockout and *TRNT1* rescued strains, western blot analysis, tetrad dissection, and preparation of the draft methods. Data generated produced Figures 1C, S.5, and S.7. As stated on the *Blood* Journal website, using the published article in a thesis and/or dissertation is an acceptable form of author reuse and no copyright permission is required.

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Mutations in *TRNT1*, encoding the CCA-adding enzyme, cause congenital sideroblastic anemia with B cell immunodeficiency, periodic fevers and developmental delay (SIFD)

Running Title: *TRNT1* mutations in SIFD

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Key points

1. SIFD is a syndromic form of congenital sideroblastic anemia associated with immunodeficiency, periodic fevers and developmental delay.
2. SIFD is due to partial loss-of-function mutations in the CCA-adding enzyme TRNT1.

Abstract

Mutations in genes encoding proteins involved in mitochondrial heme synthesis, iron-sulfur cluster biogenesis, and mitochondrial protein synthesis, have previously been implicated in the pathogenesis of the congenital sideroblastic anemias (CSAs). We recently described a syndromic form of CSA associated with B cell immunodeficiency, periodic fevers and developmental delay (SIFD). Here we demonstrate that SIFD is caused by biallelic mutations in *TRNT1*, the gene encoding the CCA-adding enzyme essential for maturation of both nuclear and mitochondrial transfer RNAs (tRNAs). Using budding yeast lacking the *TRNT1* homologue, *CCA1*, we confirm that the patient-associated *TRNT1* mutations result in partial loss-of-function of TRNT1 and lead to metabolic defects in both the mitochondria and cytosol that can account for the phenotypic pleiotropy.

Introduction

The congenital sideroblastic anemias (CSAs) are a heterogeneous group of syndromic and non-syndromic inherited diseases characterized by pathologic iron deposition in the mitochondria of red blood cell precursors in the bone marrow. All identified causative CSA genes encode structural RNAs or proteins that are involved in one of three mitochondrial pathways: heme synthesis, mitochondrial iron-sulfur cluster biogenesis and mitochondrial protein synthesis (Fleming et al., 2011). We recently described a syndromic form of CSA associated with B cell immunodeficiency, periodic fevers and developmental delay (SIFD). Variably severe sensorineural hearing loss, cardiomyopathy, and central nervous system abnormalities also occurred in some patients (Wiseman et al., 2013). SIFD pedigrees indicated an autosomal recessive mode of inheritance. Here, we extend the cohort of patients described with SIFD, and, using whole exome sequencing and a novel method of identity by descent mapping, identify the causative gene as *TRNT1*, a template-independent RNA polymerase required for the maturation of cytosolic and mitochondrial tRNAs.

Methods

Genomic analyses.

We automated the discovery process using a custom build, rule based “Variant Explorer” pipeline using copy number variation (CNV), family linkage as well as population level homozygosity to aid interpretation of the results (K.S.-A. and K.M., unpublished). For the analysis, we analyzed 180 (113 affected) samples from multiplex families or singletons with CSA.

siRNA knockdowns.

Small interfering RNA (siRNA) transfections of fibroblasts were performed using Lipofectamine RNAiMAX according to the protocol provided by the manufacturer (Invitrogen). The cells were transfected TRNT1 siRNA (Hs_TRNT1, Qiagen SI00751464 (#4), SI04142691 (#6), SI04235056 (#7), SI04301857 (#8)), or a non-silencing control siRNA (Dharmacon, 5'-UUCUCCGAACGUGUCACG UdTdT-3). Cells were collected for analysis at 24, 48 or 72 h post-transfection

Immunoblotting.

See Supplementary Methods.

Kinetic measurement of cytotoxicity and Caspase-3/7 activation.

Normal human skin fibroblast cells were transfected with increasing concentrations of siRNA for 24 h in a 96-well plate. Cells were then incubated with either 100 nM YOYO-1 dye (Life Technologies) or 1 mM of Cell PlayerTM reagent (Essen BioScience) and the incubation monitored for 48 h using the INCUCYTETM ZOOM Live-Cell Imaging System as described by the manufacturer (Essen BioScience, MI, USA). The fraction of YOYO-1 and Caspase 3/7 positive cells was measured after treatment with 0.0625% Triton X-100X for the YOYO-1 assay or 1 μ M Vybrant Green DNA (Life Technologies, Invitrogen) for the Caspase 3/7 assay.

Overexpression and purification of native and variant TRNT1 proteins.

HEK293T cells were transfected with either wild-type TRNT1-FLAG plasmid (pTrueORF, Origene, USA) or mutated plasmid DNA in antibiotic-free Opti-MEM with Lipofectamine 2000 (Invitrogen). After 48 hours, lysates were immunoprecipitated with Flag-beads (Sigma) as previously described (Glahn et al., 2013). Prior to assay, protein was quantified using BCA protein assay (Pierce), checked for purity by SDS-PAGE.

CCA-adding enzyme assays.

In vitro transcription of *B. subtilis* tRNA^{Asp} lacking three nucleotides (CCA) at the 3'-terminus was performed using the plasmid G73 (a gift of Dr. Alan M. Weiner, Department of Biochemistry, University of Washington, Seattle, WA, USA) (Kruse et al., 2013), and CCA-adding enzyme activity assayed using either in-gel or glass fiber assays measuring the incorporation of [α^{32} P]-ATP as described in detail in the Supplementary Methods on-line.

Yeast strain construction and plate-based assays.

See Supplementary Methods and Tables

Statistical analyses.

All results are expressed as mean +/- standard error of the mean with a minimum of 3 biological replicates, unless otherwise noted. The Student's t-test was used to determine statistical significance (Graph Pad Prism 5).

Results and Discussion

We performed genome-wide Affymetrix 6.0 SNP analysis on six SIFD probands (Table 1, patients 2, 3, 6, 7, 8, and 10) and ten of their parents. We mapped the disease locus by analyzing genotypes for overlapping regions of identity by descent. We identified a region on chromosome 3p26.1 in which both consanguineous probands (patients 6 and 8) were homozygous for distinct haplotypes (Supplementary Figure 1). Subsequent linkage analysis in another consanguineous Pakistani kindred (patients 1A and 1B, parents, and two unaffected siblings) was also consistent with linkage to 3p26.1, resulting in a cumulative LOD score of 3.38. Affected members of this pedigree shared a partially overlapping, identical homozygous haplotype with proband 8, also Pakistani. On this basis, we were able to narrow the disease interval to a 1.04 Mb, 5cM, region containing four genes, including *TRNT1*. Given its role in mitochondrial tRNA metabolism, *TRNT1* was considered the primary candidate gene. Independently, whole exome sequencing was performed on patient 7. Rare variants in mitochondrial proteins annotated in the MitoCarta (Pagliarini et al., 2000) database were examined, and revealed biallelic, missense variants in *TRNT1*. By Sanger sequencing amplified exons, we found biallelic *TRNT1* mutations in all but one of the other SIFD patients previously described (Table 1) as well as five additional affected individuals from four families (Table 1, patients 11, 12A, 12B, 13, and 14). In patient 4 we identified only one uncommon variant. We did not identify a small deletion or copy number variation in this patient using a custom Nimblegen 720k array spanning the *TRNT1* locus. In aggregate, we identified three frameshift alleles, three splicing variants and seven unique missense *TRNT1* alleles: p.T154I, p.M158V, p.L166S, p.R190I, p.I223T, p.I326T, and p.K416E. Except for p.K416E, each of the missense variants occurs in a highly conserved

residue and received a PolyPhen2 score >0.95, indicating a likely damaging mutation (Adzhubei et al., 2013). Nonetheless, K416 is highly conserved in mammals, suggesting its potential pathogenicity. Five of the missense mutations (p.T154I, p.M158V, p.L166S, p.R190I, and p.I223T) cluster in the active site, and two (p.I326T and p.K416E) are in the less well conserved C-terminal region (Supplementary Figure 2) (Goring et al., 2013). Only one missense variant, p.I223T, occurs in the NHLBI Exome Sequencing Variant Project (<http://evs.gs.washington.edu/EVS/>), and then only at an allele frequency of 0.0077%. We did not identify any uncommon variants in *TRNT1* in 58 other unrelated probands with non-syndromic and phenotypically distinct syndromic CSA (Bergmann et al., 2010).

TRNT1 encodes the human CCA-adding enzyme, an RNA polymerase required for the post-transcriptional, template-independent addition of two cytosines and one adenosine to the 3' end of all tRNA molecules, which is necessary for tRNA aminoacylation (Shi et al., 1998). CCA-adding enzymes are also implicated in tRNA quality control and the stress response (Igarashi et al., 2011; Lizano et al., 2008; Mohan et al., 1999; Czech et al., 2013). *TRNT1* encodes the only human CCA-adding activity and is responsible for the maturation of both cytosolic and mitochondrial tRNAs (Lizano et al., 2008).

Short interfering (si) RNA knockdown of *TRNT1* in wild-type human fibroblasts caused cytotoxicity and apoptosis (Supplementary Figure 3), suggesting that eliminating *TRNT1* function altogether is lethal, while mutations only impairing function could be disease-associated. To determine the functional significance of the disease-associated variants, we examined the effect of the variants in patient-derived skin fibroblasts and in yeast. Only full-length *TRNT1* transcript was detected from control human skin fibroblasts (Supplementary Figure 4a). The c.608+1 G>T and del1054_1056+10 variants resulted in

aberrant splicing (Supplementary Figures 4a, 4b and 4c). The c.1057-7C>G mutation found in three individuals (patients 2, 3, and 9) is strongly predicted by the Human Splicing Finder (Desmet et al., 2009) to result in a new splice acceptor site that, if employed, inserts 6 nucleotides in the cDNA upstream of exon 8, encoding the insertion of sequential threonine and TAG stop codons (p.D352_S353insTX) and premature termination of the protein (Supplementary Figure 4d). Unfortunately, no primary material was available from any of these patients to validate this prediction.

We examined the ability of TRNT1 mutants to complement budding yeast cells harboring a temperature sensitive mutation in *CCA1* (*cca1-1*, originally named ts352) (Goring et al., 2013; Aebi et al., 1990), and in a *CCA1* deletion strain. While expression of the wild-type human TRNT1 in the *cca1-1* strain fully restored growth at a non-permissive temperature (Figure 1a), expression of the mutant TRNT1 alleles provided only partial rescue (Figure 1b). Furthermore, as demonstrated by tetrad dissections, only the wild-type yeast protein and the human p.I326T mutant were able to rescue the lethal deletion of *CCA1* through meiosis in haploid yeast. (Supplementary Figure 5a). Consequently, we rescued the lethal deletion of *CCA1* with wild type and mutant TRNT1 plasmids using a plasmid shuffle assay (Figure 1c and Supplementary Figures 5b & 5c). We found that *TRNT1* with any of the five missense mutations that cluster near the active site of the enzyme all complemented growth to varying degrees as compared to wild-type human TRNT1. The p.R190I and p.I223T variants grew particularly poorly on non-fermentable glycerol medium, indicating a defect in mitochondrial function. Two mutants, p.I326T and p.K416E, not near the active site, complemented the *cca1Δ* strain as efficiently as yeast CCA1 itself, suggesting that they may be milder alleles than the other missense mutations.

While *TRNT1* mRNA levels were comparable between wild-type and mutants, protein levels were variable in wild-type and *cca1-1* yeast (Supplementary Figures 6a & 6b). This was equally true of TRNT1 expression in patient fibroblast cells (Supplementary Figures 6c, 6d, & 6e) suggesting that mutations in *TRNT1* may also affect TRNT1 proteostasis. In support of this hypothesis, we also found that rescued *cca1Δ* strains selected for high expression of *TRNT1* (Supplementary Figures 7a & 7b).

All but one of the missense mutations impaired the ability of TRNT1 to catalyze the formation of the CCA trinucleotide, *in vitro* (Figure 1d and Supplementary Figures, 8a, 8b, & 8c). Most mutant proteins had no detectable activity, whereas the activity of the p.T154I and p.K416E alleles were ~60% and equal to the wild-type, respectively. Due to the limited numbers of patients, we are unable to perform a systematic genotype-phenotype correlation, however, we note that the siblings with the p.K416E allele have a mild phenotype, with predominantly neurological abnormalities, periodic fevers and infections, but minimal anemia and longer survival. Similarly, patient 7 carrying the p.T154I variant, is neither transfusion nor intravenous immunoglobulin dependent. Patient 10 carrying the p.I326T allele, also has a variant phenotype having few neurological issues and not requiring intravenous immunoglobulins. Altogether, these genetic and functional assays indicate that all SIFD patients all have *TRNT1* mutations and an individual patient may present with a range of clinical severity of the constituent phenotypes depending upon the degree of CCA-adding enzyme loss-of-function.

Conflict of interest:

The authors declare no relevant conflict of interest

Acknowledgements:

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Author Contributions

P.K.C., K.M., A.D.R., M.H., and M.D.F. designed and supervised the study. R.F.W., D.H.W., A.M., S.J., P.C., C.P., M.M.H, P.J.G., R.J.K, C.K., I.T., A.A.T., L.M., S.H., D.K.B., S.S.B., R.M.L., C.P.T., J.M., C.M, V.B., M.T.G., P.K.C., and M.D.F. characterized SIFD syndrome and collected clinical data and patient samples. K.S-A. was supervised by K.M. and developed the informatics pipeline and identified the causative genetic defect by way of linkage studies. D.R.C., and A.L. performed mutation analysis in CSA patients. P.K.C, and M.T.G. identified the causative genetic defect post exomic sequencing. E.K.K., supervised by A.D.R., M.H., and P.K.C., designed and performed the yeast CCA knockout and rescue experiments. T.N., supervised by M.H. and P.K.C. designed and performed the siRNA knockdown experiments. H.M., supervised by A.D.R., M.H., and P.K.C., designed and performed the adenylation assays. D.D., supervised by A.D.R., M.H., and P.K.C., performed the temperature sensitive yeast rescue experiments with advice from A.D.R. and P.B.M.J. A.K.S. created the figure depicting the locations of the missense mutations on the TRNT1 X-ray crystal structure. E.K.K., H.M., T.N., and D.D. prepared the draft methods. P.K.C., A.D.R., M.H., and M.D.F. prepared the final manuscript.

Table 1: SIFD patients and *TRNT1* mutations

Patient	Consanguinity	Ethnicity	Allele 1				Allele 2			
1A	Yes	South Asian (Pakistani)	c.569G>T	p.R190I	exon 5	missense	c.569G>T	p.R190I	exon 5	missense
1B	Yes	South Asian (Pakistani)	c.569G>T	p.R190I	exon 5	missense	c.569G>T	p.R190I	exon 5	missense
2	No	Caucasian	c.668T>C	p.I223T	exon 6	missense	c.1057-7C>G	NA	ivs7	splicing
3	No	Caucasian	c.668T>C	p.I223T	exon 6	missense	c.1057-7C>G	NA	ivs7	splicing
4	No	Caucasian	c.668T>C	p.I223T	exon 6	missense	No mutation / deletion detected.			
5	No	Caucasian	c.218_219ins22	NA	exon 3	frameshift	c.668T>C	p.I223T	exon 6	missense
6	Yes	Caucasian (Hispanic)	c.668T>C	p.I223T	exon 6	missense	c.668T>C	p.I223T	exon 6	missense
7	No	Caucasian	c.497T>C	p.L166S	exon 5	missense	c.461C>T	p.T154I	exon 4	missense
8	Yes	South Asian (Pakistani)	c.569G>T	p.R190I	exon 5	missense	c.569G>T	p.R190I	exon 5	missense
9	No	Caucasian	c.668T>C	p.I223T	exon 6	missense	c.1057-7C>G	NA	ivs7	splicing
10	No	Caucasian	c.977T>C	p.I326T	exon 7	missense	c.472A>G	p.M158V	exon 4	missense
11	No	Caucasian	c.608+1 G>T	NA	ivs5	splicing	c.461C>T	p.T154I	exon 4	missense
12A	No	Caucasian	c.1246A>G	p.K416E	exon 8	missense	c. del1054_1056+10	NA	exon 7	splicing
12B	No	Caucasian	c.1246A>G	p.K416E	exon 8	missense	c. del1054_1056+10	NA	exon 7	splicing
13	No	Afro-Caribbean	c.668T>C	p.I223T	exon 6	missense	c.1142insATGT	p.W381fs	exon 8	frameshift
14	No	Caucasian	c.668T>C	p.I223T	exon 6	missense	c.1252_1253insA	S418fs	exon 8	frameshift

Sibling pairs are denoted by A&B. Patient 2 had a similarly affected sibling, however DNA was not available for study. cDNA numbering is based on NM_182916.2. Protein numbering is based on NP_886552.2. NA, not applicable.

Figure 1

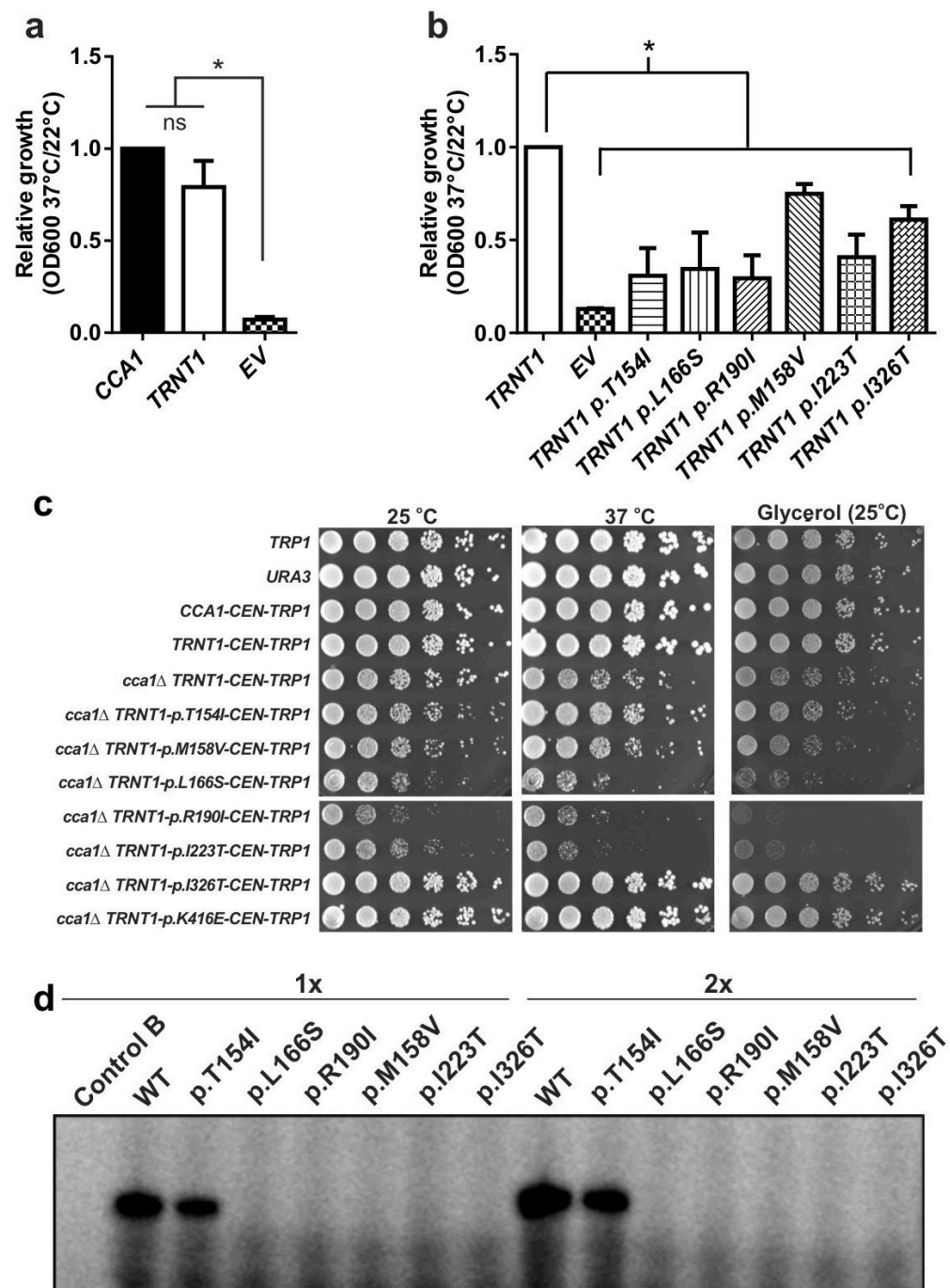


Figure 1. Mutations in *TRNT1* in SIFD patients are loss of function alleles.

(a) Yeast *cca1-1* cells were transformed with centromeric plasmids encoding yeast *CCA1*, human *TRNT1* or an empty vector control (EV) and grown at permissive (22°C) or non-permissive (37°C) temperature overnight in selective media. The OD₆₀₀ value for the empty vector was subtracted from each value and the ratio of OD₆₀₀ at 37°C/22°C was calculated and normalized so that the relative growth rate of cells harbouring a *CCA1* plasmid is 1 (mean +/- SEM, with minimum n=5). The ability of human *TRNT1* to rescue growth at the non-permissive temperature was not statistically different from *CCA1* as determined by Student's t-test.

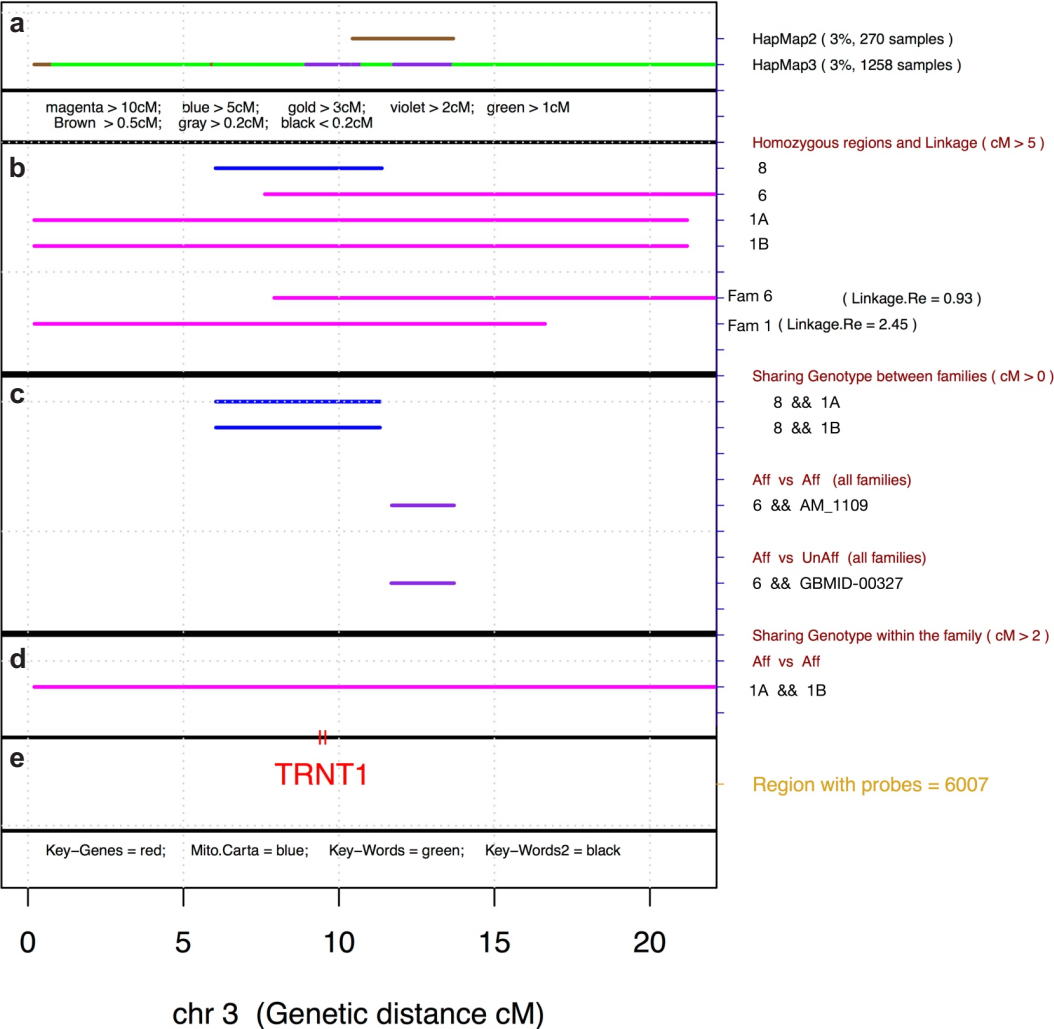
(b) Patient *TRNT1* mutants partially rescue growth impairment of *cca1-1* cells. *cca1-1* cells were transformed with empty vector (EV), wild-type *TRNT1* or *TRNT1* mutants (p.T154I, p.L166S, p.R190I, p.M158V, p.I223T, and p.I326T) and grown as in (a). The OD₆₀₀ value for the empty vector control was subtracted from each value and the ratio of OD₆₀₀ at 37°C/22°C was calculated and normalized so that the relative growth rate of cells harboring a *TRNT1* plasmid is 1 (mean +/- SEM, with minimum n=3; Student's t-test *P≤0.05).

(c) Complemented *cca1Δ* strains that contain only the *TRNT-CEN-TRP1* plasmid were isolated from SC-trp + 5-FOA plates (Supplementary Figure 5b) Ten-fold serial dilutions of 10⁶ cells of the indicated genotypes were spotted on YEP + 2% dextrose or 3% glycerol plates at the indicated temperatures. Wild-type and most *TRNT1* mutants have growth defects and temperature sensitivity, with the exception of *TRNT1-p.K416E* and *TRNT-p.I326T* that are indistinguishable from *CCA1* cells.

(d) Two different concentrations of purified *TRNT1*-FLAG protein with patient specific mutations were incubated with [³²P]-ATP and tRNA^{Asp} lacking the terminal CCA sequence at 37°C for 5 min as described in Methods and the reactions were terminated by addition of loading dye and resolved on a denaturing 5% polyacrylamide/8M urea gel, dried, and exposed to a phosphorimager screen. Other than the T154I variant, all of the mutants have negligible activity in this assay.

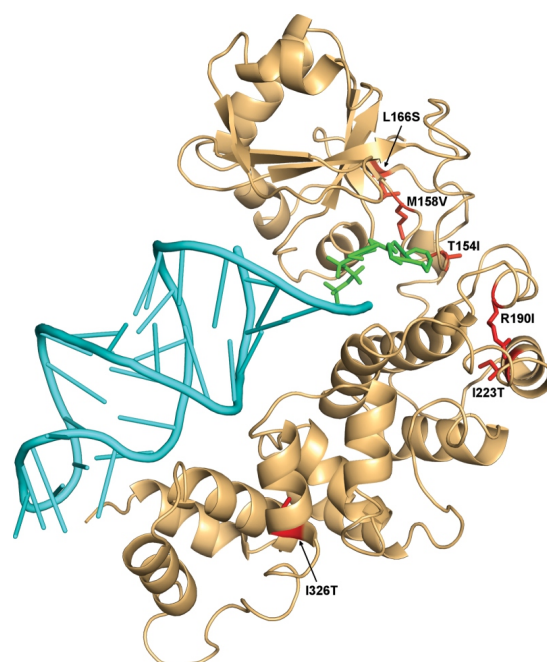
Supplementary Material

Supplementary Figure 1



Supplementary Figure 1. Variant Explorer pipeline graphical output. The diagram represents the graphic output of the analysis of chromosome 3p from 180 congenital sideroblastic anemia (CSA) samples (113 affected individuals and 67 relatives). Patients 6, 8, 1A & 1B carry *TRNT1* mutations as described in the text. Individuals AM_1109 & GBMID-00327 do not carry *TRNT1* mutations, but share haplotypes with one or more of the affected individuals, (e.g. 6 & AM_1109 share a short haplotype to the telomeric to *TRNT1*). Individual panels represent the output from the following analyses: **(a)** homozygosity observed in HapMap **(b)** homozygous regions and linkage in families. **(c)** genotype sharing between families. **(d)** genotype sharing within families. **(e)** gene position and names of all genes considered relevant to the phenotype, including known CSA genes, MitoCarta genes, as well as genes participating in known pathways involved in CSA.

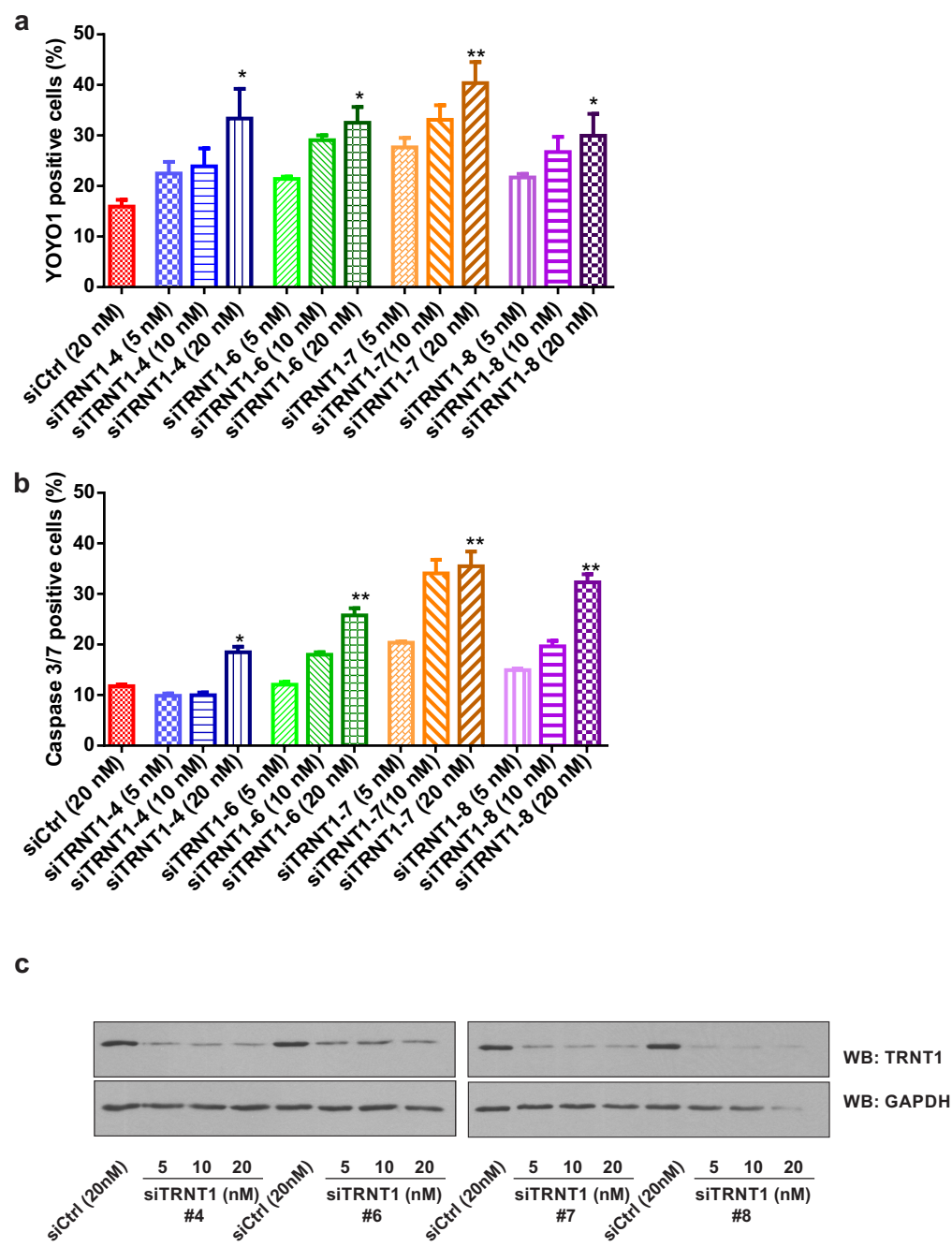
Supplementary Figure 2



Supplementary Figure 2. SIFD patient mutations mapped to TRNT1 crystal structure.

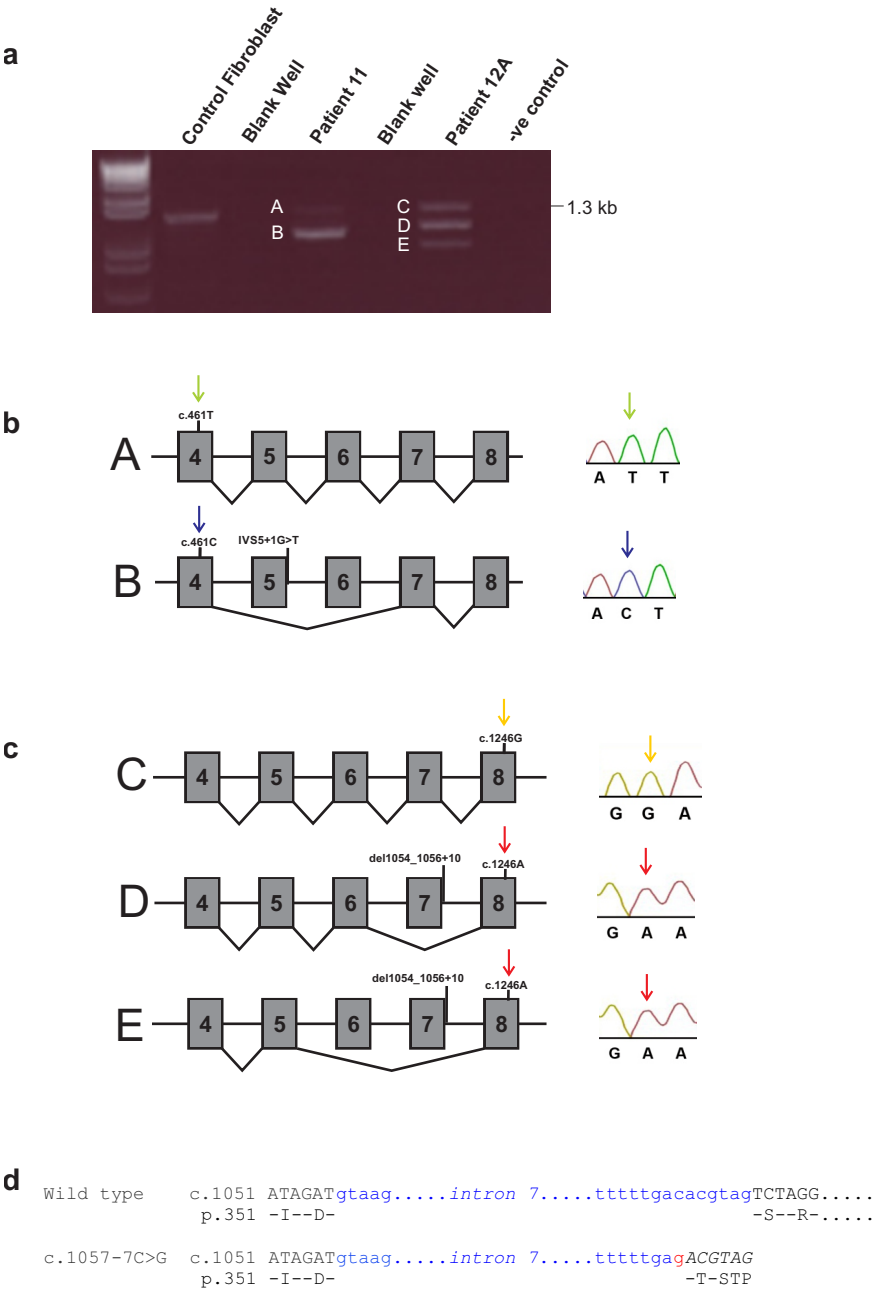
The figure depicts a ribbon diagram of the human TRNT1 structure and its predicted interaction with its substrates, tRNA (cyan) and AMPcPP (green). The relative positioning of the substrates with respect to TRNT1 is based on the structural superimposition of human TRNT1 on bacterial poly-A polymerase (PDB IDs: 1OU5 (Adzhubei et al., 2013) and 1VFG (Goring et al., 2013), respectively). The structures superimpose with a RMSD of 2.79Å over 261 equivalent residues. The rotational matrix for the superimposition was generated using EBI-PDBeFold (Bergmann et al., 2010) and the structure was rotated using LSQKAB (Shi et al., 1998). The p.K416E mutation is not shown as it falls outside of the resolved crystallographic structure.

Supplementary Figure 3



Supplementary Figure 3. *TRNT1* knockdown-induced cytotoxicity and caspase activation. Normal human skin fibroblasts were transfected with increasing concentration of four different siRNAs (Qiagen SI00751464 (#4), SI04142691 (#6), SI04235056 (#7), SI04301857 (#8)) for 24 h and subsequently assayed with YOYO-1 or Cell PlayerTM reagents. The number of **(a)** YOYO-1 positive (dead) and **(b)** Caspase-3/7-positive cells was determined using the INCUCYTE kinetic imaging system for 48 h. Bars represent mean +/- SEM (n=4). *TRNT1* knockdown at 20 nM was found to be statistically different from siCtrl as determined by Student's t-Test (* < 0.05; ** < 0.01). **(c).** The efficiency of *TRNT1* knockdown for each siRNA was confirmed by western blotting at 72 h.

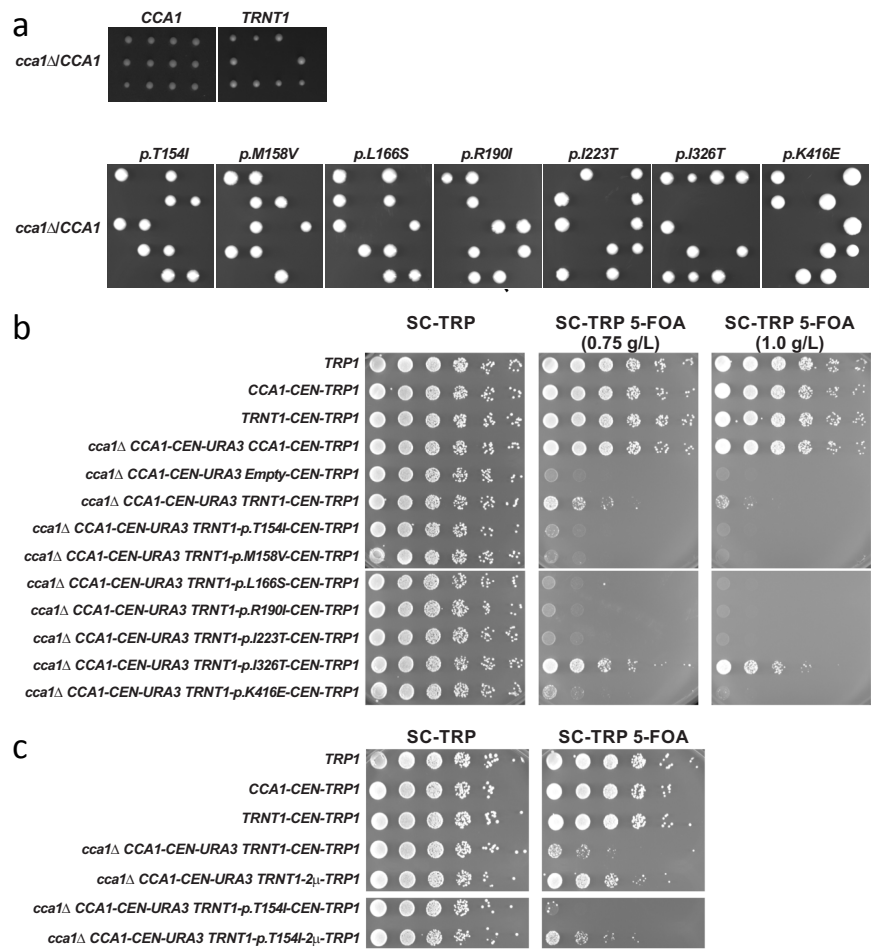
Supplementary Figure 4



Supplementary Figure 4. *TRNT1* splicing mutation in Patient 11 and 12A fibroblasts.

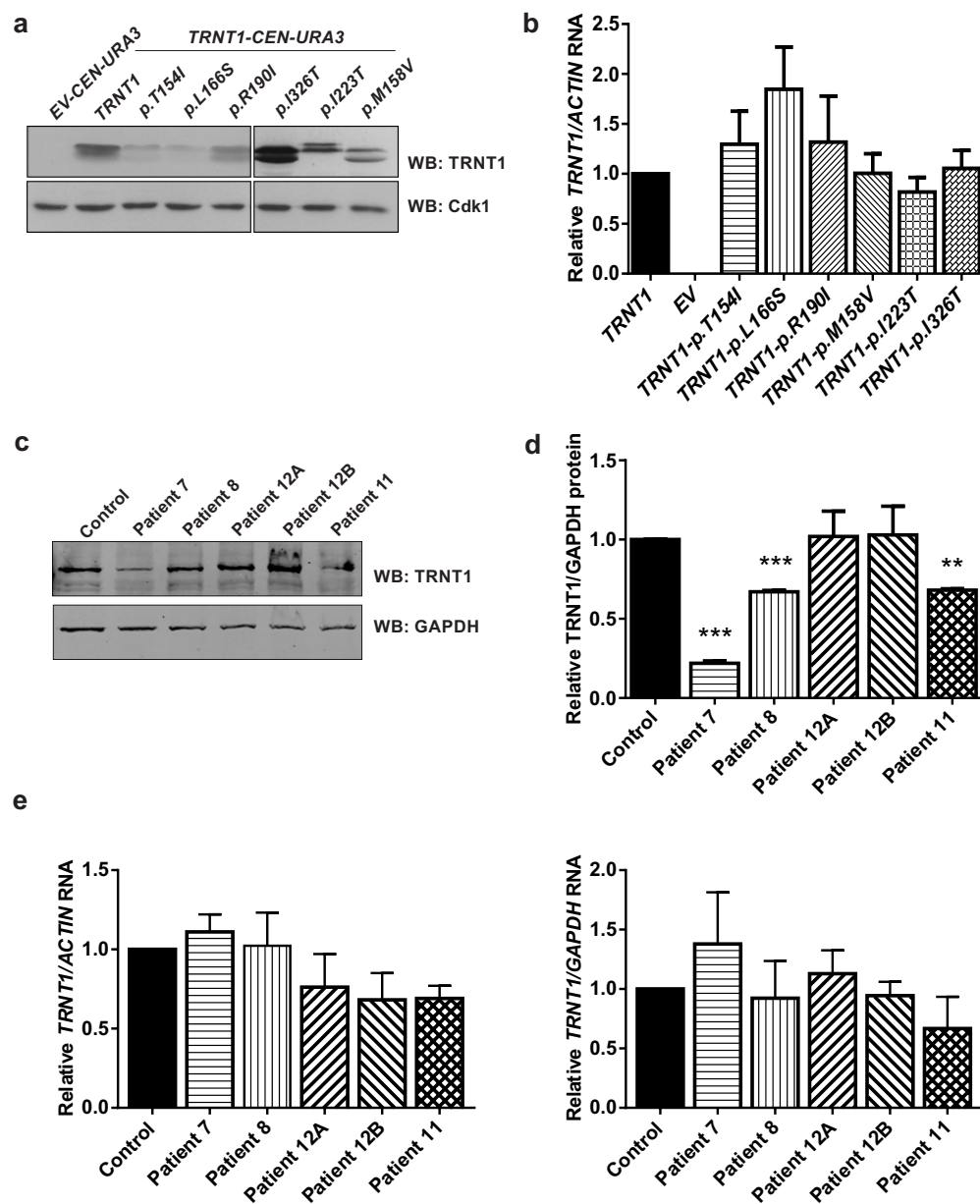
(a) RT-PCR was performed to amplify *TRNT1* using RNA from normal and patient fibroblasts. PCR products were separated on a 1.5% agarose gel and visualized under ultraviolet light. **(a)** A single PCR product representing full-length *TRNT1* was amplified from control fibroblasts. Full-length transcript (band A), and a shorter transcript (band B) were amplified from Patient 11 fibroblasts (compound heterozygote for IVS5+1 G>T and c.461C>T mutations). Full-length transcript (band C), and two shorter transcripts (bands D and E) were amplified from Patient 12A fibroblasts (compound heterozygote for c.1246A>G and del1054_1056+10 mutations). **(b)** The full-length transcript from Patient 11 fibroblasts (band A) arises from the c.461C>T allele, while the shorter transcript only arises from the c.608+1 G>T allele and results from skipping exons 5 and 6. **(c)** The full-length transcript from Patient 12A fibroblasts (band C) arises from the c.1246A>G allele, while the middle (band D – skipping exon 7) and lower (band E – skipping exons 6 and 7) transcripts arise from the del1054_1056+10 allele. **(d)** The c.1057-7C>G allele is predicted by Human Splicing Finder (<http://www.umd.be/HSF/>) to generate a new splice acceptor site and to result in the insertion of a threonine residue followed by a stop codon (p.D352_S353insTX).

Supplementary Figure 5



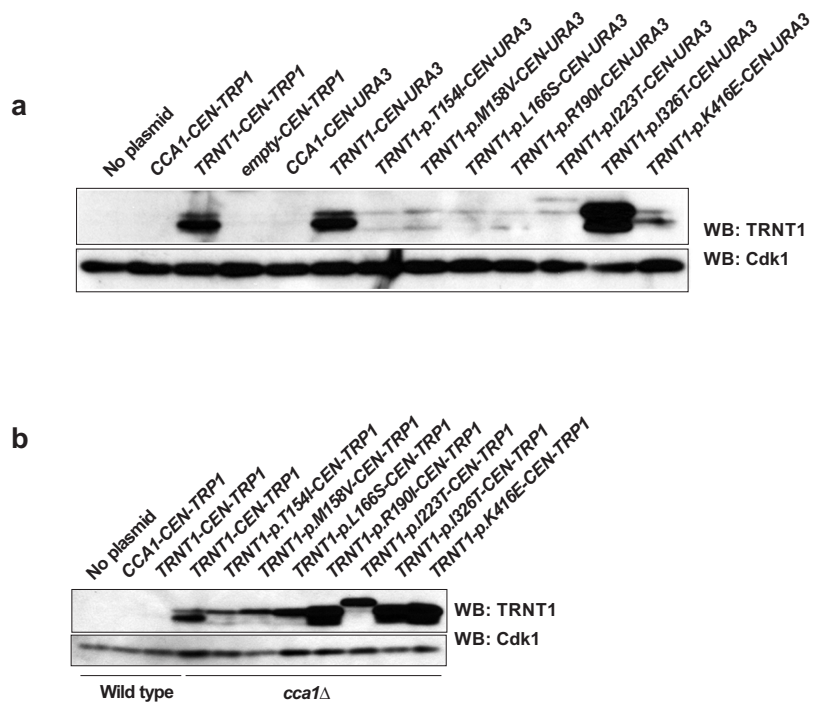
Supplementary Figure 5. *TRNT1* mutations in SIFD patients variably complement *CCA1* deficient yeast. (a) The *cca1Δ* /*CCA1* diploid yeast knockout strain was transformed with a *CEN/ARS* plasmid encoding *CCA1*, *TRNT1* or *TRNT1* mutants and grown on sporulation media (2% potassium acetate) plates at room temperature for 3 days. Tetrads were dissected onto YPD plates and replica plated onto selective media to analyze meiotic segregation. Only the wild type yeast and human genes and the human p.I326T mutant support growth (b) *cca1Δ* cells surviving with only wild-type or mutant *TRNT1* were selected by spotting ten-fold serial dilutions of 10^6 cells of the indicated genotypes onto SC-trp plates in the absence or presence of 5-Fluoroorotic acid (5-FOA). 5-FOA selects against cells expressing *URA3* that is present on a sheltering *CCA1-CEN-URA3* plasmid. (c) *cca1Δ* yeast were transformed with human *TRNT1* from a *CEN/ARS* plasmid, occurring in 1 to 5 copies per cell, or a high-copy 2μ plasmid. Expressing *TRNT1* from the 2μ plasmid improves survival compared to the *CEN/ARS* plasmid, suggesting a strong selection for many *CEN/ARS* plasmids and/or high protein expression in each cell (see also Supplementary Figure 6).

Supplementary Figure 6



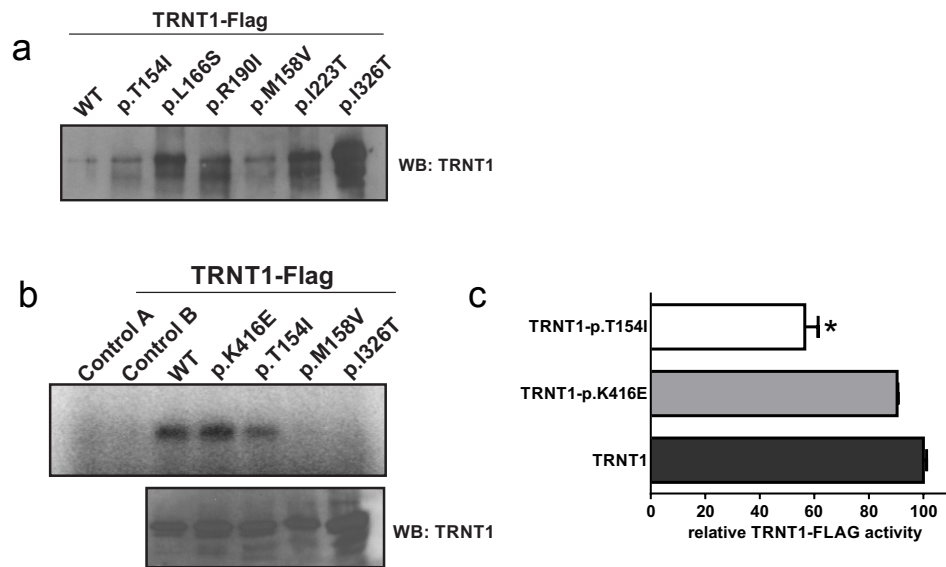
Supplementary Figure 6. Discordant expression of TRNT1 protein and mRNA in patients and yeast. (a) *cca1-1* yeast strain transformed with either an empty vector (EV), wild-type *TRNT1* or *TRNT1* mutants (p.*T154I*, p.*L166S*, p.*R190I*, p.*M158V*, p.*I223T* and p.*I326T*) were grown at 22°C overnight and protein lysates were resolved on 10% SDS-PAGE, transferred onto nitrocellulose membrane and incubated with indicated antibodies. The p.*K416E* was not expressed in the *cca1-1* yeast strain as the sib pair with this mutation was identified after these experiments had been completed. **(b)** Total RNA was isolated from the yeast strains from (a) and RT-qPCR analysis was performed using *TRNT1*- or *ACTIN* specific primers. Graph represents the *TRNT1* (relative to *ACTIN*) mRNA levels, and are normalized so that wild-type *TRNT1* (relative to *ACTIN*) mRNA level is equal to 1 (mean +/- SEM, minimum n=3). **(c)** Immunoblot analysis of human TRNT1 protein from control and patient fibroblasts. Cell lysates of total protein (40 µg) of each sample were resolved by SDS-PAGE, blotted onto PVDF membranes and probed with a rabbit polyclonal anti-TRNT1 antibody. The immune complexes were detected with anti-rabbit Alexa700-conjugated secondary antibody. **(d)** band intensity was quantified using the Odyssey imaging software and plotted relative to GAPDH expression. Error baars represent the mean +/- SEM; n=4; *** p<0.001; ** p<0.01, Student's t-test. **(e)** RT-qPCR analysis of patient cell lines show no difference in the expression of *TRNT1* relative to actin and *GAPDH* mRNA compared to control fibroblasts.

Supplementary Figure 7



Supplementary Figure 7. Western blot analysis of wild-type and variant TRNT1 protein in yeast cells where it is not needed for viability (*CCA1*) and where it is required (*cca1Δ*). **(a)** In *CCA1* cells, the levels of TRNT1 differ greatly between the wild-type and mutants. **(b)** The rescued strains (from Figure 1c) contain similar levels of TRNT1 protein suggesting high expression is needed for complementation of *cca1Δ*.

Supplementary Figure 8



Supplementary Figure 8. Nucleotide addition is defective in TRNT1 mutants. (a) The amount of purified protein present in each reaction in Figure 1d was assessed by western blot. (b) Purified TRNT1-FLAG was incubated with tRNA^{Asp} lacking the terminal CCA sequence at 37°C for 5 min as described in the Methods, the reactions were terminated by addition of loading dye, and resolved on a denaturing 5% polyacrylamide/8M urea gel, dried, and exposed to a Phosphoimager screen. (Control A was prepared from empty plasmid transfected cells; control B is wild-type TRNT1 boiled prior to adding in the reaction). Western blot (WB) indicates the amount of protein added to each assay. (c) 20 μ M of tRNA^{Asp} lacking the terminal CCA sequence was incubated with 50 ng of purified TRNT1 (wild-type, p.T154I or p.K416E) in the presence of [α^{32} P]-ATP at 37°C for 90 sec and processed as described in the Methods. Specific enzymatic activity of each mutant is shown relative to wild-type TRNT1. Error bars represent mean $\pm$ SEM; n=3, p < 0.05.

Supplementary Table 1: Clinical features of SIFD patients with *TRNT1* mutations

Patient Number	DEMOGRAPHICS		INTERVENTIONS				OUTCOME		
	Country of birth	Year of Birth	Regular Transfusions	IVIg Replacement	Bone Marrow Transplantation	Alive/Dead	Age at Death	Terminal Event/Cause of Death	
1A	Wales	1996	Yes	Yes	No	Dead	14 years	Sepsis with multiorgan failure and toxic epidermal necrolysis (attributed to a cephalosporin)	
1B	Wales	2005	No	Yes	Yes (aged 7 years)	Alive (8 years)	n/a	n/a	
2A	USA	1991	Yes	Yes	No	Dead	61 months	Multiorgan failure	
2B	USA	1985	Yes	Yes	No	Dead	4 years	Admission with seizures, hypokalemia and fevers; rapid onset multiorgan failure; adrenal hemorrhage on autopsy	
3	USA	1998	Yes	?	No	Dead	56 months	Arrived in shock with severe hypoglycemia; died within hours in multiorgan failure	
4	Portugal	2003	Yes	Yes	No	Dead	25 months	Cardiac failure secondary to cardiomyopathy (not related to iron overload)	
5	France	2003	Yes	Yes	No	Dead	16 months	Multiorgan failure (pneumonitis; cardiac failure)	
6	USA	1993	Yes	Yes	No	Alive (20 years)	n/a	n/a	
7	Canada	2007	No	No	No	Alive (6 years)	n/a	n/a	
8	Canada	2007	Yes	Yes	No	Dead	28 months	Cardiac failure secondary to cardiomyopathy (not related to iron overload)	
9	England	2009	Yes*	Yes*	Yes (aged 9 months)	Alive (4 years)	n/a	n/a	
10	USA	2009	Yes	No	No	Alive (4 years)	n/a	n/a	
11	Canada	2012	Yes	Yes	Yes (aged 9 months)	Dead	10 months	Pulmonary hemorrhage post bone marrow transplantation	
12A	Wales	1998	No	Yes	No	Alive (15 years)	n/a	n/a	
12B	Wales	1995	No	Yes	No	Alive (18 years)	n/a	n/a	
13	England	2013	Yes	Yes	No	Alive (12 months)	n/a	n/a	
14	Belgium	2014	Yes	Yes	No	Alive (6 months)	n/a	n/a	

Supplementary Table 2 - Yeast strain and plasmid list

All strains except ts352 were created in W303-1a: *ura3-1 leu2-2, 112 trp1-1 his3-11 ade2-1 can1-100*

Yeast strains

Figure 1a and b

Strain #	Mating type	Relevant genotype
ts352	<i>MATα</i>	<i>cca1-1^a ade2-101 his3Δ200 ura3-52 tyr1 (S288C)</i>

^a the mutation in *cca1-1* causes a p.E189K substitution

This strain was transformed with the indicated *CEN-URA3* plasmid.

Figure 1c

ADR6511	<i>MATa</i>	<i>cca1Δ::HYG^R CCA1-CEN-URA3</i>
ADR410	<i>MATa</i>	<i>TRP1</i>
ADR6836	<i>MATα</i>	<i>CCA1-CEN-TRP1</i>
ADR6834	<i>MATα</i>	<i>TRNT1-CEN-TRP1</i>
ADR6840	<i>MATa</i>	<i>cca1Δ::HYG^R CCA1-CEN-URA3 CCA1-CEN-TRP1</i>
ADR6838	<i>MATa</i>	<i>cca1Δ::HYG^R TRNT1-CEN-URA3 empty-CEN-TRP1</i>
ADR6842	<i>MATa</i>	<i>cca1Δ::HYG^R CCA1-CEN-URA3 TRNT1-CEN-TRP1</i>
ADR6844	<i>MATa</i>	<i>cca1Δ::HYG^R CCA1-CEN-URA3 TRNT1-p.T154I-CEN-TRP1</i>
ADR6846	<i>MATa</i>	<i>cca1Δ::HYG^R CCA1-CEN-URA3 TRNT1-p.M158V-CEN-TRP1</i>
ADR6848	<i>MATa</i>	<i>cca1Δ::HYG^R CCA1-CEN-URA3 TRNT1-p.L166S-CEN-TRP1</i>

ADR6850	<i>MATa</i>	<i>cca1Δ::HYG^R CCA1-CEN-URA3 TRNT1-p.R190I-CEN-TRP1</i>
ADR6852	<i>MATa</i>	<i>cca1Δ::HYG^R CCA1-CEN-URA3 TRNT1-p.I223T-CEN-TRP1</i>
ADR6854	<i>MATa</i>	<i>cca1Δ::HYG^R CCA1-CEN-URA3 TRNT1-p.I326T-CEN-TRP1</i>
ADR7952	<i>MATa</i>	<i>cca1Δ::HYG^R CCA1-CEN-URA3 TRNT1-p.K416E-CEN-TRP1</i>

Supplementary Figure 5a

ADR941	<i>MATa/α</i>	diploid wild type W303-1a (<i>CCA1/CCA1</i>)
ADR7020	<i>MATa/α</i>	<i>cca1Δ::HYG^R/CCA1</i>

ADR7020 was transformed with the indicated *CEN-TRP1*, with the exception of *TRNT1-CEN-URA3* and *CCA1-CEN-URA3* plasmids. Crosses not shown have confirmed that all *TRNT1-mutant-CEN-URA3* plasmids behave identically to *TRNT1-mutant-CEN-TRP1* plasmids.

Supplementary Figure 5b

ADR410	<i>MATa</i>	<i>TRP1</i>
ADR6836	<i>MATα</i>	<i>CCA1-CEN-TRP1</i>
ADR6834	<i>MATα</i>	<i>TRNT1-CEN-TRP1</i>
ADR6842	<i>MATa</i>	<i>cca1Δ::HYG^R CCA1-CEN-URA3 TRNT1-CEN-TRP1</i>
ADR7808	<i>MATa</i>	<i>cca1Δ::HYG^R CCA1-CEN-URA3 TRNT1-2μ-TRP1</i>
ADR6844	<i>MATa</i>	<i>cca1Δ::HYG^R CCA1-CEN-URA3 TRNT1-p.T154I-CEN-TRP1</i>
ADR7812	<i>MATa</i>	<i>cca1Δ::HYG^R CCA1-CEN-URA3 TRNT1- p.T154I-2μ -TRP1</i>

Supplementary Figure 5c

ADR410	<i>MATa</i>	<i>TRP1</i>
ADR408	<i>MATa</i>	<i>URA3</i>
ADR6836	<i>MATα</i>	<i>CCA1-CEN-URA3</i>
ADR6834	<i>MATα</i>	<i>TRNT1-CEN-TRP1</i>
ADR6791	<i>MATa</i>	<i>cca1Δ::HYG^R TRNT1-CEN-TRP1</i>
ADR6793	<i>MATa</i>	<i>cca1Δ::HYG^R TRNT1-p.T154I-CEN-TRP1</i>
ADR6933	<i>MATa</i>	<i>cca1Δ::HYG^R TRNT1-p.M158V-CEN-TRP1</i>
ADR6935	<i>MATa</i>	<i>cca1Δ::HYG^R TRNT1-p.L166S-CEN-TRP1</i>
ADR6937	<i>MATa</i>	<i>cca1Δ::HYG^R TRNT1-p.R190I-CEN-TRP1</i>
ADR6939	<i>MATa</i>	<i>cca1Δ::HYG^R TRNT1-p.I223T-CEN-TRP1</i>
ADR6941	<i>MATa</i>	<i>cca1Δ::HYG^R TRNT1-p.I326T-CEN-TRP1</i>
ADR7953	<i>MATa</i>	<i>cca1Δ::HYG^R TRNT1-p.K416E-CEN-TRP1</i>

Supplementary Figures 6a and 6b

ts352	<i>MATα</i>	<i>cca1-E189K ade2-101 his3Δ200 ura3-52 tyr1 (S288C)</i>
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This strain was transformed with the indicated *CEN-URA3* plasmids

Supplementary Figure 7a

ADR410	<i>MATa</i>	<i>TRP1</i>
ADR6836	<i>MATa</i>	<i>CCA1-CEN-TRP1</i>

ADR6834	<i>MATα</i>	<i>TRNT1-CEN-TRP1</i>
ADR6838	<i>MATa</i>	<i>cca1Δ::HYG^R empty-CEN-TRP1</i>
ADR6840	<i>MATa</i>	<i>cca1Δ::HYG^R CCA1-CEN-URA3 CCA1-CEN-TRP1</i>
ADR6838	<i>MATa</i>	<i>cca1Δ::HYG^R TRNT1-CEN-URA3 empty-CEN-TRP1</i>
ADR6842	<i>MATa</i>	<i>cca1Δ::HYG^R CCA1-CEN-URA3 TRNT1-CEN-TRP1</i>
ADR6844	<i>MATa</i>	<i>cca1Δ::HYG^R CCA1-CEN-URA3 TRNT1-p.T154I-CEN-TRP1</i>
ADR6846	<i>MATa</i>	<i>cca1Δ::HYG^R CCA1-CEN-URA3 TRNT1-p.M158V-CEN-TRP1</i>
ADR6848	<i>MATa</i>	<i>cca1Δ::HYG^R CCA1-CEN-URA3 TRNT1-p.L166S-CEN-TRP1</i>
ADR6850	<i>MATa</i>	<i>cca1Δ::HYG^R CCA1-CEN-URA3 TRNT1-p.R190I-CEN-TRP1</i>
ADR6852	<i>MATa</i>	<i>cca1Δ::HYG^R CCA1-CEN-URA3 TRNT1-p.I223T-CEN-TRP1</i>
ADR6854	<i>MATa</i>	<i>cca1Δ::HYG^R CCA1-CEN-URA3 TRNT1-p.I326T-CEN-TRP1</i>
ADR7952	<i>MATa</i>	<i>cca1Δ::HYG^R CCA1-CEN-URA3 TRNT1-p.K416E-CEN-TRP1</i>

Supplementary Figure 7b

ADR410	<i>MATa</i>	<i>TRP1</i>
ADR6836	<i>MATα</i>	<i>CCA1-CEN-TRP1</i>
ADR6834	<i>MATα</i>	<i>TRNT1-CEN-TRP1</i>
ADR6791	<i>MATa</i>	<i>cca1Δ::HYG^R TRNT1-CEN-TRP1</i>
ADR6793	<i>MATa</i>	<i>cca1Δ::HYG^R TRNT1-p.T154I-CEN-TRP1</i>
ADR6933	<i>MATa</i>	<i>cca1Δ::HYG^R TRNT1-p.M158V-CEN-TRP1</i>
ADR6935	<i>MATa</i>	<i>cca1Δ::HYG^R TRNT1-p.L166S-CEN-TRP1</i>

ADR6937	<i>MATa</i>	<i>cca1Δ::HYG^R TRNT1-p.R190I-CEN-TRP1</i>
ADR6939	<i>MATa</i>	<i>cca1Δ::HYG^R TRNT1-p.I223T-CEN-TRP1</i>
ADR6941	<i>MATa</i>	<i>cca1Δ::HYG^R TRNT1-p.I326T-CEN-TRP1</i>
ADR7953	<i>MATa</i>	<i>cca1Δ::HYG^R TRNT1-p.K416D-CEN-TRP1</i>

Plasmids

Plasmid	Genotype
SCAW1-2	<i>pADH1-CCA1-CEN-URA3</i>
A163-3	<i>pADH1-CEN-URA3</i> (empty plasmid)
pAR1159	<i>pADH1-TRNT1-2μ-TRP1</i>
pAR1160	<i>pADH1-TRNT1-p.T154I-2μ-TRP1</i>
pAR1190	<i>pADH1-TRNT1-p.K416D-CEN-TRP1</i>
pAR1191	<i>pADH1-TRNT1-CEN-URA3</i>
pAR1192	<i>pADH1-TRNT1-p.T154I-CEN-URA3</i>
pAR1193	<i>pADH1-TRNT1-p.M158V-CEN-URA3</i>
pAR1194	<i>pADH1-TRNT1-p.L166S-CEN-URA3</i>
pAR1195	<i>pADH1-TRNT1-p.R190I-CEN-URA3</i>
pAR1196	<i>pADH1-TRNT1-p.I223T-CEN-URA3</i>
pAR1197	<i>pADH1-TRNT1-p.I326T-CEN-URA3</i>
pAR1198	<i>pADH1-CEN-TRP1</i> (empty plasmid)
pAR1199	<i>pADH1-CCA1-CEN-TRP1</i>
pAR1200	<i>pADH1-TRNT1-CEN-TRP1</i>

pAR1201	<i>pADH1-TRNT1-p.T154I-CEN-TRP1</i>
pAR1202	<i>pADH1-TRNT1-p.M158V-CEN-TRP1</i>
pAR1203	<i>pADH1-TRNT1-p.L166S-CEN-TRP1</i>
pAR1204	<i>pADH1-TRNT1-p.R190I-CEN-TRP1</i>
pAR1205	<i>pADH1-TRNT1-p.I223T-CEN-TRP1</i>
pAR1206	<i>pADH1-TRNT1-p.I326T-CEN-TRP1</i>

Supplementary Methods

Variant Explorer pipeline

We automated the discovery process using a custom build, rule based “Variant Explorer” pipeline using copy number variation (CNV), family linkage as well as population level homozygosity to aid interpretation of the results. Furthermore we automate the identification of overlaps with a database of candidate genes likely to play a role in CSA as well as external phenotype relevant databases like MitoCarta.

For the specific analysis we analyzed 180 (113 affected) samples from multiplex families or singletons satisfying a narrow definition of CSA. No plausible causative CNVs were identified. We observed homozygosity overlaps in 3 regions (5 cM or more) in chr 3, 4 & 22 but only one of the three regions showed overlap for more than two families. Furthermore it was the only region where we observed haplotype overlap (5.11cM) across families. Finally it was the only region highlighting a plausible causative gene.

We automate quality control, analysis and result visualization as part of the Variant Explorer pipeline. Automation is crucial as it minimizes the error inherent in manual curation of large data sets and greatly speeds up interpretation of the results. For QC we eliminate samples with low contrast_QC (Birdsuite and Affymetrix Genotyping Console) and for CNV analysis samples with high MAPD values (3 CNV algorithms). Within pedigrees we test consistency of the data with stated gender and we incorporate a rigorous relationship family test for each family in our database. Visualization of supporting evidence, linkage, homozygosity as well as gene content is used for manual inspection. As an example, Supplementary Figure 1 shows supporting evidence for the TRNT1 region.

siRNA knockdowns

Patient and control fibroblasts were maintained at 37°C, 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Burlington, ON, Canada) supplemented with 10% foetal calf serum (FCS), 1% glutamine, 100 mg/ml streptomycin and 100 U/ml penicillin. Small interfering RNA (siRNA) transfections were performed using Lipofectamine RNAiMAX according to the protocol provided by the manufacturer (Invitrogen). Briefly, cells were seeded at a density of 7500 cells/well in 6-well plates or 5000 cells/well in 96-well plates. The cells were transfected 24 h later with 5, 10 or 20 nM of TRNT1 siRNA (Hs_TRNT1, Qiagen SI00751464 (#4), SI04142691 (#6), SI04235056 (#7), SI04301857 (#8)), or a non-silencing control siRNA (Dharmacon, 5'-UUCUCCGAACGUGU CACGUdTdT-3). Cells were collected for analysis 24, 48 or 72 h post-transfection

Kinetic measurement of cytotoxicity and Caspase-3/7 activation

Normal human skin fibroblast cells were transfected with increasing concentration of siRNA for 24 h in a 96-well plate. Cells were then incubated with either 100 nM YOYO-1 dye (Life Technologies) or 1 µM of Cell PlayerTM reagent (Essen BioScience) and incubation monitored for 48 h using the INCUCYTETM ZOOM Live-Cell Imaging System as described by the manufacturer (Essen BioScience, MI, USA). The number of YOYO-1 and Caspase 3/7 positive cells was divided by the total number of cells at endpoint (measured after treatment with 0.0625% Triton X-100X for YOYO-1 assay and treatment with 1 µM Vybrant Green DNA (Life Technologies, Invitrogen) for Caspase -3, -7 assay).

Immunoblotting

TRNT1, GAPDH and Cdk1 protein levels were assayed by western blot. Patient fibroblast cells were lysed in RIPA buffer (Faye et al., 2013) and protein quantity was assayed with the BCA Protein Assay (Bio-Rad). Yeast immunoblotting methods have been previously described (Rudner et al., 2000; Rudner & Murray, 2000). Previously-frozen yeast cell pellets were lysed in lysis buffer (20 mM HEPES pH7.9, KOH, 1 mM DTT, 1 µg/mL each of pepstatin, leupeptin and aprotinin (Sigma)). Equal quantities of total protein were loaded on a 10% polyacrylamide gel and separated by SDS-PAGE. Membranes were blocked in 4% milk and 5% glycerol or in Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE) for 1 hr at room temperature. Membranes were then probed overnight at 4°C with primary antibodies, washed, then probed for 1 hr at room temperature with secondary antibodies. The following primary antibodies were used: rabbit anti-TRNT1 (Novus Biologicals, Oakville, ON, Canada) at 1:1000-1:2000 dilution, mouse anti-GAPDH (BD Biosciences, Mississauga, ON, Canada) at 1:50000 dilution and rabbit anti-Cdk1 (Novus Biologicals, Oakville, ON, Canada) at 1:1000 dilution. The following secondary antibodies were used: rabbit Alexa⁷⁰⁰-conjugated or mouse Alexa⁸⁰⁰-conjugated secondary antibodies (Invitrogen, Burlington, ON, Canada) were used at a dilution of 1:3000, anti-rabbit and anti-mouse IgG HRP-conjugated secondary antibodies (Cell Signaling) were used at 1:5,000 and 1:10,000, respectively. Membranes were either scanned and analyzed with the associated software using the Odyssey infrared imaging system (Li-Cor Biosciences) or incubated with ECL or ECL Prime Luminescent Reagents (GE Healthcare) and exposed to film. Films were scanned at a resolution of 600 d.p.i. and densitometry analysis was performed using Odyssey software. Figures were generated in Adobe Photoshop; blots were cropped and exposure was adjusted evenly across all blots.

Overexpression and purification of native and variant TRNT1.

HEK293T cells were transfected with either wild-type TRNT1-FLAG plasmid (pTrueORF, Origene, USA) or mutated plasmid DNA (TRNT1-p.T154I-FLAG, TRNT1-p.L166S- FLAG, TRNT1-p.R190I-FLAG, TRNT1-p.M158V-FLAG, TRNT1-p.I223T-FLAG, TRNT1- p.I326T-FLAG, and TRNT1-p.K416E-FLAG), in antibiotic-free Opti-MEM with Lipofectamine 2000 (Invitrogen). After 4 hours incubation at 37°C, a complete medium (DMEM) supplemented with 10% FCS, penicillin, streptomycin, and glutamine (all from Invitrogen) was added and the cells were maintained at 37°C for 48 hours. The cells were trypsinized and washed in PBS at 300 x g for 5 min then resuspended in 1 ml of immunoprecipitation lysis buffer supplemented with PMSF (Liwak et al., 2012). The Flag-IP was performed as previously described (Liwak et al., 2012) with modifications. The lysates were sonicated for 10 sec and centrifuged at 10 000 x g for 20 min. The supernatant was incubated at 4°C overnight with 40 µl of Flag-beads (Sigma). The Flag- beads bound to TRNT1-FLAG were then washed by centrifugation and the TRNT1-FLAG was eluted following the manufacturer's instructions. Before assaying the TRNT1-FLAG for activity, the protein was equilibrated in 100 mM glycine buffer pH 9, quantified using BCA protein assay (Pierce), checked for purity by SDS-PAGE and kept in 10% glycerol at -80°C until use.

Preparation of tRNA substrate.

In vitro transcription of *B. subtilis* tRNA^{Asp} lacking three nucleotides (CCA) at the 3'- terminus was performed using the plasmid G73 (Leibovitch et al., 2013), a generous gift of Dr. Alan M. Weiner (Department of Biochemistry, University of Washington, Seattle, WA, USA). The *in vitro* run-off transcription assay was carried out using 50 µg/ml of *FokI*-

digested G73 plasmid and the T7 Megashortscript reagents as described by the supplier (Ambion, Life Technologies, Burlington, ON, Canada). After a phenol: chloroform extraction, the transcripts were concentrated by ethanol precipitation and stored at -80°C.

Adenylation assays

Gel-based assay. The detection of CCA addition activity was performed for the TRNT1- FLAG native and variant proteins. The reactions were performed at 37°C in the presence of [α^{32} P]-ATP (3000 Ci/mmol, 10 mCi/ml, Perkin Elmer, Boston, MA, USA) and 20 μ M tRNA. A saturated concentration of 20 μ M of tRNA^{Asp} lacking CCA at the 3'terminus was incubated at 37°C for 5 min with 1 or 2 μ g of purified TRNT1-FLAG in a 10 μ l reaction containing 0.4 mM CTP, 10 mM MgCl₂, 100 mM glycine buffer pH 9.0 and 0.33 μ M of [α^{32} P]-ATP. The reactions were terminated by addition of 10 μ l loading dye (Peattie et al., 1979) containing 10 M urea and resolved on a denaturing 5% polyacrylamide/8M Urea gel. The gel was dried and exposed for 4 hours to a phosphorscreen (Molecular Probes). The bands were scanned using the Typhoon phosphorimager system (GE Healthcare) and the bands were quantified using the Odyssey software (Li-Cor Biosciences, Lincoln, NE).

Glass fibre-based assay. Enzyme activity assays were modified from the method previously described (Shan et al., 2008). A saturated concentration of 20 μ M of tRNA^{Asp} lacking CCA at the 3'terminus was incubated at 37°C for 90 sec with 50 ng of purified TRNT1-FLAG (native and variants p.T154I or p.K416E) in a 10 μ l reaction containing 0.4 mM CTP, 10 mM MgCl₂, 100 mM Glycine buffer pH 9.0 and 2 mM of ATP and [α^{32} P]-ATP (final concentration 1.49 mCi/ml). The reactions were terminated by addition of 10 μ l

2M HCl and samples were collected on GF/C glass microfiber filters (Whatman, GE Healthcare, UK). The filters were then washed with 10 ml 1M HCl followed by 1 ml 99% ethanol to remove any unincorporated [$\alpha^{32}\text{P}$]-ATP. After the washes, the filters were air-dried, cut accordingly and placed into wells of 96-well sample plates (1450-MicroBeta, Perkin Elmer). The radioactivity in the samples was measured in 200 μl of liquid scintillation fluid (OptiPhase supermix, Perkin Elmer) in a Wallac 1450- MicroBeta Trilux scintillation counter (Perkin Elmer). The specific activity of the [$\alpha^{32}\text{P}$]-ATP was used to convert the radioactivity of the tRNA-bound to the filters into moles of incorporated nucleotide. The specific radioactivity of the ATP in terms of d.p.m. per nanomole of ATP was determined by counting in triplicate 1 μl aliquots of the stock of 1 mM ATP. The statistical analyses of data for the incorporation of [$\alpha^{32}\text{P}$]-ATP into tRNA were performed with GraphPad Prism 5 software. Each experiment was carried out at least 3 times. The statistical significance was defined as $P < 0.05$ unless otherwise indicated. Two separate controls (lysate prepared from cells transfected with empty plasmid, control A, and boiled TRNT1 enzyme, control B) were used to account for non-specific interactions with the purified protein.

Yeast plasmid construction

The empty vector (EV, A163-2 (Goring et al., 2013) and *CCA1* (SCAW1-2) yeast expression plasmids containing the *URA3* marker and driven by the *ADHI* promoter were constructed by P. Hanic- Joyce (Concordia University) and used as the backbone for expression of TRNT1 in yeast. The *TRNT1-CEN-URA3* plasmid was created by amplifying HeLa cells *TRNT1* cDNA and cloning into the plasmid A163-2 (Goring et al., 2013). Plasmids were confirmed by sequencing (Applied Biosystems 3730 DNA Analyzer and MJ

Research Tetrad Thermocyclers, StemCore Laboratories, Ottawa Hospital Research Institute).

The *TRNT1-mutant-CEN-URA3* plasmids were generated using site-directed mutagenesis on *TRNT1-CEN-URA3* with primers (Appendix) containing each of the six *TRNT1* missense mutations: c.461C>T (p.T154I), c.472A>G(p.M158V), c.497T>C(p.L166S), c.569G>T(p.R190I), c.668T>C(p.I223T), c.977T>C(p.I326T), c.1246A>G(p.K416E). The *TRNT1-mutant-CEN-TRP1* plasmids were generated by cloning the SacI/KpnI fragment from the *TRNT1-mutant-URA3* plasmids into *pRS314 (TRP1)* plasmid. The *pADHI-CCAI* fragment from *CCAI-CEN-URA3* (SCAW1-2) was cloned into *pRS314* to generate the *pADHI-CCAI- CEN-TRP1* plasmid. Plasmids were confirmed by sequencing (StemCore Laboratories). These plasmids were used to transform the *cca1Δ* strain (ADR6511) for the plasmid shuffling experiments.

Yeast strain construction

The previously described temperature-sensitive strain *cca1-1* (Aebi et al., 1990) (previously called *ts-352*) was a gift from Dr. Anita Hopper (Ohio State University). Supplemental Table I lists the yeast strains used in this work. All strains, other than *cca1-1*, are derivatives of the W303 strain background (W303-1a). All deletions and replacements were confirmed by PCR and/or sequencing (StemCore Laboratories). The sequences of all primers used in this study are available upon request. The bacterial strains DH5α and TG1 were used for amplification of DNA. *CCAI/cca1Δ::HYG^R* diploid yeast knockout strain was created using PCR-targeted recombination. Using gene specific primers, the gene was replaced with a hygromycin resistance cassette (*HYG^R*) amplified from *pAG32-hphMX4* (Goldstein & McCusker, 1999).

The *cca1Δ:: HYG^R CCA1-CEN-URA3* sheltering strain was created by transforming *CCA1-CEN-URA3* into the *CCA1/cca1Δ:: HYG^R* diploid (ADR7020), inducing meiosis, dissecting tetrads, and selecting for haploids containing both the *HYG^R* marker for the knockout and the *URA3* marker for the sheltering plasmid. All hygromycin-resistant strains were also prototrophic for uracil, confirming deletion of *CCA1* as lethal. Plasmid shuffling strains containing *cca1Δ:: HYG^R CCA1-CEN-URA3* and *TRNT1-CEN-TRP1* plasmids were created by transforming *TRNT1-CEN-TRP1* plasmids into the *cca1Δ:: HYG^R CCA1-CEN-URA3* sheltering strain and selecting for the presence of hygromycin, uracil, and tryptophan requirements.

Viable *cca1Δ:: HYG^R TRNT1-CEN-TRP1* cells were found following spotting serial dilutions of the plasmid shuffling strains containing *cca1Δ:: HYG^R CCA1-CEN-URA3* *TRNT1- CEN-TRP1* onto media containing 5- Fluoroorotic acid (5-FOA), these are referred to as rescued strains. The presence of 5-FOA selects against cells that are *URA3* as the activity of the Ura3 protein results in the conversion of 5-FOA to 5-fluorouracil, a toxic compound. The rescued strains only contain *TRNT1-CEN-TRP1* plasmids and this was confirmed by these strains inability to grow on 5-fluoroanthranilic acid (5-FAA) medium (data not shown), which selects against cells that are *TRP1*. FAA, like 5-FOA, is converted into a toxic compound, 5- fluorotryptophan (Toyn et al., 2000). The identity of plasmid-bourne *TRNT1* in these strains was confirmed by sequencing (StemCore Laboratories).

Yeast growth assays

Exponentially growing cells were grown to a concentration of 1.85×10^7 cells/ml in complete medium (YPD) or synthetic complete medium minus tryptophan (SC-TRP). Cells were concentrated to 1.85×10^8 cells/ml and ten-fold serial dilutions were spotted onto YEP

dextrose (2%), YEP glycerol (3%), SC-TRP FOA or SC-TRP 5-FAA plates. 5-Fluoroorotic acid (5-FOA) selects against *URA3* cells, in this case the sheltering *CCA1-CEN-URA3* plasmid. 5-Fluoroanthranilic acid (5-FAA) selects against *TRP1* cells, in this case the *TRNT1* wild-type or mutant genes on the *TRNT1-CEN-TRP1* plasmids. Strains which are wild-type *TRP1* or *URA3* cells were controls for these inhibitors. Plates were grown for two to three days at 25°C or as indicated. *cca1-1* yeast, transformed with *TRNT1-CEN-URA3* plasmids, were grown in YPD at 22°C to exponential growth phase. 6.5×10^5 cells were re-cultured in synthetic complete medium minus uracil (SC-URA) and were grown at the permissive (22°C) or non-permissive (37°C) temperatures overnight. OD₆₀₀ was determined for each sample; the empty vector control was subtracted from each value and the ratio at 37°C /22°C was calculated.

Sporulation and tetrad dissection analysis

The *cca1Δ::HYG^R/CCA1* diploid yeast knockout strain was grown on sporulation medium (2% potassium acetate) plates at room temperature for 3 days. Tetrads were dissected onto YPD plates and replica plated onto selective media to analyze meiotic segregation as required.

Quantitative real-time PCR

Liquid cultures grown for the *cca1-1* Yeast Growth Assay were prepared in parallel and used for RT-qPCR. Cell pellets were flash frozen and stored at -80°C until use. RNA was extracted as previously described (Collart & Oliviero, 2001) and quantified using a Nanodrop 2000 Spectrophotometer (Thermo Scientific). 600 ng of total RNA was reverse transcribed (qScript™ cDNA Supermix, Quanta Biosciences) to obtain cDNA (25°C x 5

min, 42°C x 30 min, 85°C x 5 min and hold at 4°C). For quantitative real-time PCR, *TRNT1* primers (PrimeTime™ Std qPCR Assay, IDT) and yeast *Actin* primers (Appendix) were used with SYBR Green mix (QIAGEN) and samples were tested in triplicate with the following cycling conditions: 95°C x 2 min, 95°C x 10 sec, 56°C x 30 sec, 72°C x 10 sec for 40 cycles (Mastercycler® ep realplex, Eppendorf). RNA from patients fibroblasts was extracted using RNeasy® RT (Molecular Research Center, Inc.). Reverse transcription and quantitative PCR was performed as described above using GAPDH and ACTIN primers (Appendix) and the same TRNT1 conditions. cDNA from patient 11 and 12A was used to perform standard KOD PCR as described above to amplify *TRNT1* (primers described in the Appendix). Bands were excised and gel-purified (QIAquick Gel Extraction Kit, QIAGEN) before sending for sequencing (StemCore Laboratories).

Statistical analysis

All data are expressed as mean +/- standard error mean with a minimum of 3 biological replicates, unless otherwise notes. Biological replicates for RT-qPCR were performed in triplicate. Student's t-test was used to determine statistical significance (Graph Pad Prism5).

Appendix

TRNT1 Site-Directed Mutagenesis Primers

Mutation	Primers (5'→3')
c.461 (ACT>ATT, T154I)	FP: GATGCGGAACGCAGAGATCTCATTATAAATTCTATGTTTTTAGGT RP: AACCTAAAAACATAGAATTTATAATGAGATCTCTGCGTCCGCATC
c. 497 (TTA>TCA, L166S)	FP: TATGTTTTAGGTTTTGATGGCACTTCATTTGACTACTTTAATGGTTATGAAG RP: CTCATAACCATTAAAGTAGTCAAATGAAGTGCCATCAAAACCTAAAAACATA
c.569 (AGA>ATA, R154I)	FP : GATTTGTTGGACATGCTAAACAGATAATACAAGAGGATTATCTTAGAAT RP : ATTCTAAGATAATCCTCTTGTATTATCTGTTTAGCATGTCCAACAAATC
c.472 (ATG>GTG, M158V)	FP: GCAGAGATCTCACTATAAATTCTGTGTTTTAGGTTTTGATGGCAC RP : GTGCCATCAAAACCTAAAAACACAGAATTTATAGTGAGATCTCTGC
c.668 (ATT>ACT, I223T)	FP: CCTGAGACTTTGGAAGCAACTGCAGAAAATGCAAAAGGC RP : GCCTTTTGCAATTTCTGCAGTTGCTTCCAAGTCTCAGG
c.977 (ATA>ACA, I326T)	FP: CCTTGGCTTATTTACAGTTAAAAATAGG RP: CCTATTTTAACTGTAAATAAGCCAAGG
c.1246 (AAA>GAA, K416E)	FP: AACAGTTGCGAGAACAGTGGGAAAAAAGTGGTTACCAAATG RP: CATTTGGTAACCACTTTTTTCCCACTGTTCTCGCAACTGTT

Standard primers for PCR and sequencing TRNT1

FP: 5'-ATCCGGATCCATGCTGAGGTGCCTGTATCATTG-3'

RP: 5'-ATCCGTCGACTTAGGTCTTCTTTATGTAACCTCAGAAG-3'

qPCR Primers

TRNT1:

Hs.PT.53a.4489325.g (PrimeTime™ Std qPCR Assay, IDT)

(NM_182916; exon location 2-2; detects all variants)

Primer 1: 5'-CCTTCTGTGAAAAGTGAAGTGA-3'

Primer 2: 5'-GATGCTGAGGTGCCTGTATC-3'

GAPDH:

Primer 1: 5'-ACAGTCAGCCGCATCTTCTT-3'

Primer 2: 5'-ACGACCAAATCCGTTGACTC-3'

ACTIN (human):

Primer 1: 5'-CTGGAACGGTGAAGGTGACA-3'

Primer 2: 5'-AAGGGACTTCCTGTAACAATGCA-3'

ACTIN (yeast):

499- JH301: 5'-GCCTTCTACGTTTCCATCCA-3'

500- JH302: 5'-GGCCAAATCGATTCTCAAAA-3'

Chapter 3: Translation homeostasis contributes to SIFD pathobiology in yeast

This chapter comprises the bulk of my research data toward this doctorate degree and is in current preparation for publication. I generated the data presented here with assistance in the following areas: the initial diploid and *CCA1* null strains were generated by Dr. Adam Rudner; empty vectors for *TRNT1* expression were provided by Dr. P. Hanic-Joyce (Concordia University); *TRNT1-T154I*, *M158V*, *L166S*, *R190I*, *I223T*, and *I326T* mutations were generated on plasmid by Dr. Turaya Naas and Danielle Durie (CHEO Research Institute); *TRNT1 R99W* and *K107R* were provided by Dr. Matthew Leibovitch and Dr. Paul Joyce (Concordia University); *sui2-S51A* mutant was made by Dr. Adam Rudner; the Cca1 and Sui2 antibodies were made by Dr. Adam Rudner, Alick Wang, and Camille Fortinez; and polysome analysis was done by Thet Naing (Dr. Martin Holcik laboratory, CHEO Research Institute) and Laura Gillis (Dr. Stephen Lewis Laboratory, Dalhousie University, Moncton, NB). Unpublished data associated with this work is in Appendix 1 of this thesis.

Translation homeostasis contributes to SIFD pathobiology in yeast

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3.1. Abstract

tRNA nucleotidyl transferase (TRNT1 in mammals, Cca1 in yeast) is the RNA polymerase that adds the 3' CCA nucleotides to all tRNA and is required for translation, tRNA quality control, and protection of the translation machinery (Aebi et al., 1990; Vörtler & Mörl, 2010; Wellner et al., 2018). Mutations in *TRNT1* cause SIFD, Sideroblastic Anemia With B-Cell Immunodeficiency, Periodic Fevers, And Developmental Delay, a rare mitochondrial disease (Chakraborty et al., 2014). Yeast surviving on human patient-identified *TRNT1* mutant genes, an analogous yeast *CCA1* mutant, and a degra-tagged *CCA1* have impaired mitochondrial and cytoplasmic translation. In addition to OXPHOS defects previously reported (Liwak-Muir et al., 2016; Sasarman et al., 2012; Wedatilake et al., 2016), I show that defects in TRNT1/Cca1 induce the eIF2 α -P translation stress response and this induction occurs when mitochondrial translation was required; in yeast, mitochondrial translation is only required under respiratory, non-fermentation, conditions. This stress response signaling is proposed to slow cytoplasmic translation and be suppressive to maintain homeostasis when mitochondrial translation is impaired. In contrast, the eIF2 α -P signaling was found to be synthetic under non-respiring conditions when yeast mitochondrial translation was not required, suggesting that inappropriate signaling may contribute to the pathobiology of SIFD. This data suggests that impairment of TRNT1/Cca1 function results in an imbalance of translation homeostasis and that altering the balance between mitochondrial and cytoplasmic translation may be an effective therapy for SIFD.

3.2. Introduction

TRNT1 is the human CCA-adding RNA polymerase that catalyzes the addition of two cytosines and one adenine to the 3' end of all nascent tRNA molecules in the nucleus, cytoplasm, and mitochondria (Aebi et al., 1990; Chen et al., 1992; Nagaike et al., 2001). The 3' CCA addition is required for mitochondrial and cytoplasmic tRNA aminoacylation, tRNA nuclear export, binding of aminoacyl tRNA synthetases, peptide bond formation at the ribosome, and subsequent protein synthesis; CCA addition occurs without the assistance of any additional proteins or co-factors (Aebi et al., 1990; Chen et al., 1992; Feng & Hopper, 2002; Nagaike et al., 2001; Nissen et al., 2000; Vörtler & Mörl, 2010; Wellner et al., 2018). The 3' CCA tRNA terminus is subject to frequent cleavage or degradation and TRNT1 also functions to repair the CCA sequence (Nagaike et al., 2001; Vörtler & Mörl, 2010; Wolfe et al., 1996).

Mutations in *TRNT1* were identified in patients and found to be causative of SIFD (Sideroblastic Anemia With B-Cell Immunodeficiency, Periodic Fevers, And Developmental Delay) (Chakraborty et al., 2014; Wiseman et al., 2013), a spectrum of phenotypes that resemble many inherited mitochondrial disorders (Calvo & Mootha, 2010; DiMauro et al., 2006; Soumalainen et al., 1995). The mutations were shown to result in functional TRNT1 protein given their ability to rescue a yeast null, but the rescued yeast had impaired growth on a non-fermentable carbon source, a condition that requires mitochondrial translation for oxidative phosphorylation (OXPHOS). This suggests that mutations in *TRNT1* result in reduced mitochondrial function (Chakraborty et al., 2014).

Further work showed that patient fibroblasts and myoblasts with mutations in *TRNT1* had decreased levels of OXPHOS proteins, both nuclear and mitochondrially encoded (Liwak-Muir et al., 2016; Sasarman et al., 2015; Wedatilake et al., 2016). tRNA analysis in

an siRNA knockdown showed greatly reduced levels of 3' CCA addition to mt-tRNA^{Ser(AGY)} and slightly decreased levels of cytoplasmic tRNA^{Lys(UUU)} and cytoplasmic tRNA^{Met} (Sasarman et al., 2015). The mt-tRNA^{Ser(AGY)} has a non-canonical tRNA structure which was proposed to impair mutant *TRNT1* 3'CCA addition and result in tRNA degradation. Another analysis of patient cells with *TRNT1* mutations found decreased levels (1 to 2% of total) of 3' CCA addition to mt-tRNA^{Cys}, tRNA^{His}, and tRNA^{LeuUUR} in fibroblast cells but no changes in mitochondrial translation (Wedatilake et al., 2016). Furthermore, sequencing of cytoplasmic tRNA in patient fibroblasts found 80% of tRNA had reduced levels, on average 1.6-fold less than in control cells (Giannelou et al., 2018). This data shows a decrease of 3'CCA on both cytoplasmic and mt-tRNA resulting from mutations in *TRNT1*, although the presence of impaired OXPHOS suggests the impaired mt-tRNA is important to the cause of SIFD pathobiologies.

Mitochondrial diseases are a large group of inborn errors in mitochondrial energy metabolism that are both clinically and genetically heterogeneous. The vast majority of proteins functioning in the mitochondrial are nuclear encoded, with the nuclear DNA providing 1000 to 1500 proteins compared to 13 encoded on the mtDNA (Goldstein et al., 1999). After mtDNA mutations or mitochondrial depletion, mutations in the 200 to 300 nuclear-encoded proteins required for mitochondrial translation are a major cause of respiratory chain deficiencies found in patients (Koopman et al., 2012). Approximately 70% of the more than 150 nuclear gene mutations resulting in mitochondrial disease have homologous proteins in yeast (Koopman et al., 2012). Yeast can function without OXPHOS and thus without mitochondrial translation, using fermentation as a source of ATP. Therefore, with pathway conservation and translation requirement flexibility, yeast is a good model to study impaired mitochondrial translation resulting in human disease.

Yeast contain 275 nuclear encoded cytoplasmic tRNA genes for the 21 isodecoders needed for translation, with more than 1% of the genome is used to process pre-tRNA into mature functioning molecules (Hopper & Phizicky, 2003). In contrast, yeast contain only 24 mitochondrial tRNA genes, all of which are encoded on the mtDNA and processed in the mitochondria by nuclear encoded proteins (Richter et al., 2015; Turk et al., 2013). Mitochondrial tRNA also differ from cytoplasmic tRNA in their non-classical tRNA cloverleaf secondary structures, lack of sequence conservation, and have wide variability in abundance (Florentz et al., 2003; Helm et al., 2000; Suzuki et al., 2011; Turk et al., 2013). Of the over 400 mutations in the human mitochondrial genome associated with disease, more than 60% are associated with the mitochondrial tRNA genes while tRNA only compose 10% of the mitochondrial genome, highlighting the importance of the mitochondrial tRNA pool to mitochondrial function (Abbott et al., 2014; Belostotsky et al., 2012; Levinger et al., 2004). Therefore, defects in tRNA processing may impact mitochondrial tRNA pools greater than cytoplasmic due to these differences.

Impacts on mitochondrial translation due to deficient 3'CCA tRNA addition have been shown to occur to individual mutated mt-tRNAs (Levinger et al., 2004; Tomari et al., 2003). Mutations identified in mt-tRNA^{Leu(UUR)}, mt-tRNA^{Ile}, and mt-tRNA^{Gly} result in reduced 3' CCA addition causing Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like episodes (MELAS), fatal infantile cardiomyopathy, and sudden infant death syndrome respectively (Levinger et al., 2004; Tomari et al., 2003). This lack of 3' CCA-adding to mt-tRNA is predicted to decrease tRNA aminoacylation and increase tRNA instability leading to increased degradation (Tomari et al., 2003; Belostotsky et al., 2012). Work by collaborators determined that five patient-identified *TRNT1* mutations (*T154I*, *M158V*, *L166S*, *R190I*, and *I223T*) affect both the structure and enzymatic activity of tRNA-

NT *in vitro* (Leibovitch et al., 2018). All mutations had reduced thermostability, *M158V* and *I223T* had reduced affinity for CTP, and *R190I* had reduced ability to add nucleotides to the non-canonical tRNA^{Ser(AGY)} (Leibovitch et al., 2018). Mutations in *TRNT1/CCAI* may impact mt-tRNA more than cytoplasmic tRNA due to non-canonical structures or the more limited tRNA pool in the mitochondria.

With the vast majority of the proteins functioning in the mitochondria encoded in the nucleus (1000 to 1500 nuclear-encoded proteins compared to 8 (yeast) to 13 (mammals) mitochondrial-encoded proteins (Kumar et al., 2002; Lopez et al., 2000)), the maintenance of translation homeostasis is required to prevent the accumulation of pre-proteins and misfolded proteins while at the same time ensuring adequate protein supply for the mitochondria, required because the, (Battersby & Richter, 2013; Eisenberg-Bord & Schuldiner, 2017; Raimundo, 2014; Suhm & Ott, 2016; Topf et al., 2016; Yun & Finkel, 2014). Cytoplasmic translation can be regulated by eIF2 α phosphorylation (eIF2 α -P) which inhibits the formation of the ternary complex (eIF2-GTP-tRNA^{iMet}) for translation initiation, or by inhibition of eIF4E to prevent formation of the 5' cap binding complex (Dever et al., 2016; Holcik & Sonenberg, 2005; Proud, 2005; Sonenberg & Hinnebusch, 2009). Additionally, cytoplasmic translation can be slowed by reduced synthesis of ribosomal proteins (Schleit et al., 2013; Steffen et al., 2008; Wang et al., 2008a), drug inhibition of the cytoplasmic ribosome (Lamb et al., 1968), or reduced TOR signaling (via both S6K and 4E-BPs) (Cherkasova & Hinnebusch, 2003; Laplante & Sabatini, 2012). Mitochondrial translation is regulated by cytoplasmic translation to establish homeostasis in OXPHOS protein production, such that inhibition of cytosolic translation directly results in changes in mitochondrial translation, with some mRNA gaining translational capacity but others losing, independent of a nutrient shift (Couvillion et al., 2016). In contrast, cytoplasmic translation

responded similarly to a nutrient shift whether or not mitochondrial translation was inhibited or mtDNA was absent, indicating that nuclear and mitochondrial translation synchronization is controlled by cytoplasmic signals (Couvillion et al., 2016).

If mt-tRNA are impacted more than cytoplasmic tRNA by defects in TRNT1 function, a decrease in mitochondrial translation could result in a loss of translation homeostasis of OXPHOS proteins and trigger stress responses due to changes in ATP, mitochondrial membrane potential, defects in import, or unfolded protein accumulation (Battersby & Richter, 2013; Eisenberg-Bord & Schuldiner, 2017; Raimundo, 2014; Suhm & Ott, 2016; Yun & Finkel, 2014). Pathways that sense mitochondrial stress include: the mitochondrial unfolded protein response (mtUPR) (Jovaisaite & Auwerx, 2015; Pellegrino et al., 2013), the mitochondrial precursor over-accumulation stress (mPOS) (Wang et al., 2008a), the accumulation unfolded protein response (amUPR) (Wang & Chen, 2015), and the retrograde response (RTG) (Trendelewa & Zvyagilskaya, 2018).

The mtUPR is a protective transcriptional response to increase nuclear production of mitochondrial protein-folding chaperones and proteases while slowing general cytoplasmic protein translation (by eIF2 α -P) to maintain homeostasis for optimal mitochondrial function, found to date in *C. elegans* and mammals but not yeast (Jovaisaite & Auwerx, 2015; Pellegrino et al., 2013; Shpilka & Haynes, 2017; Topf et al., 2016). It is induced by the accumulation of unfolded proteins, oxidative stress, perturbed ETC function, or mtDNA depletion (Baker et al., 2012; Michel, Canonne et al., 2015; Rainbolt et al., 2013; Sekito et al., 2000). Reduction of cytoplasmic translation was proposed to reduce the available proteins for mis-folding in the mitochondria, thus protecting from stress (Baker et al., 2012).

In yeast, similar to the mtUPR, mitochondrial dysfunction caused by the deletion of mitochondrial protein chaperones was suppressed by decreasing cytoplasmic translation by

ribosomal protein deletions (*RPL20B*) or caloric restriction (Schleit et al., 2013). It was suggested that mitochondrial defects were suppressed by reducing cytoplasmic translation to balance protein homeostasis (Schleit et al., 2013). Another example of reducing cytoplasmic translation to cope with mitochondrial stress is found in the yeast model for human autosomal dominant Progressive External Ophthalmoplegia (adPEO) (Wang et al., 2008a). Expression of a dominant allele of Aac2 (Adenine nucleotide translocase) in yeast models the mitochondrial degeneration observed in adPEO by reducing mitochondrial gene expression. Mitochondrial dysfunction was suppressed by reducing cytoplasmic translation using cyclohexamide, ribosomal protein deletions (*RPL6B*), or deletion of TOR components (*TOR1* and *SCH9*) (Wang et al., 2008a). This supports the model that in times of mitochondrial dysfunction, slowing cytoplasmic translation to maintain balance between nuclear and mitochondrial OXPHOS protein production is advantageous.

In addition to the mtUPR, other pathways such as mPOS, amUPR, and the RTG pathway all respond to mitochondrial stress (D'Amico et al., 2017; Topf et al., 2016). The mPOS pathway is signaled by over accumulation of precursors and misfolding in the cytoplasm and results in the modulation of cytoplasmic ribosomal biogenesis, sequestering the 60S ribosome subunit, stabilization of unfolded proteins in the cytoplasm, and increase in cap-independent translation (Kaukonen et al., 2000; Wang & Chen, 2015; Wang et al., 2008b). The amUPR, like mPOS, is signaled by the accumulation of mis-targeted protein accumulation in the cytosol and results in reduction of cytoplasmic protein synthesis but also the upregulation of proteosomal activity and production of mitochondrial protein chaperones (Monaghan & Whitmarsh, 2015; Topf et al., 2016; Wrobel et al., 2015). The RTG pathway is signaled by decreased ATP concentrations (Zhang et al., 2013) or decrease in mitochondrial membrane potential (Miceli et al., 2012) and is responsible for upregulating

the first three reactions of the Krebs cycle during mitochondrial dysfunction maintain glutamate production required for amino acid biosynthesis (Liu & Butow, 2006; Quirós et al., 2016; Trendelewa & Zvyagilskaya, 2018; Zaman et al., 2008).

Defects in *TRNT1* function may result in the observed mitochondrial phenotypes because: (1) defective cytoplasmic tRNA 3'CCA addition impairing cytoplasmic translation and reducing levels of key nuclear-encoded mitochondrial proteins; (2) defective mt-tRNA 3'CCA addition reduces mitochondrial translation and possibly impairs OXPHOS leading to reduced ATP production; or (3) the loss of homeostasis between the cytoplasmic and mitochondrial translation systems results in a cellular stress response that contributes to the observed pathobiology. It is likely that all three causes of phenotypes are occurring, but by determining the degree to which they impact cellular function we will further our understanding of SIFD and mitochondrial diseases.

TRNT1/CCAI mutations impact cytoplasmic tRNA (hypothesis 1) and mt-tRNA (hypothesis 2) 3'CCA addition, but the presence of OXPHOS defects in all of these studies suggests a greater impairment of mt-tRNA function leading to dysfunctional mitochondrial translation (Giannelou et al., 2018; Sasarman et al., 2015; Wedatilake et al., 2016). In addition to OXPHOS defects previously reported, I show here that mutations in *TRNT1/CCAI* result in stress response signaling which alleviate or contribute to pathobiologies depending on the need to balance cytoplasmic and mitochondrial translation homeostasis (Liwak-Muir et al., 2016; Sasarman et al., 2012; Wedatilake et al., 2016). When *TRNT1/Cca1* function is impaired, this stress response signaling is proposed to slow cytoplasmic translation, resulting in *TRNT1/CCAI* mutants phenotype suppression under respiring conditions (where mitochondrial translation is impacted by *TRNT1/CCAI*

mutations) but synthetic under non-respiring (non-mitochondrial translation requiring) conditions.

3.3. Results

3.3.1. *TRNT1/CCAI* mutant phenotype is consistent with mitochondrial dysfunction

I previously described the identification and analysis of patient mutations in *TRNT1* and their analysis in yeast on plasmids (Chakraborty et al., 2014). From these patient-identified mutations, analogous mutations were made at the endogenous *CCAI* locus (Figure 3.1). *TRNT1/CCAI* wild type and mutant strains were spotted on a variety of plates at different temperatures, which showed that all mutant *TRNT1* genes complement a *cca1Δ* showing that they all still have CCA-adding activity (Figure 3.2A) (Chakraborty et al., 2014). The *TRNT1-R99W* and *K107R* mutations in the N-terminal region result in increased viability compared to wild type *TRNT1* at both increased temperature and on a non-fermentable carbon source (glycerol) (Figure 3.2A). This was possibly due to the role of this region in the organization of the catalytic domain (Goring et al., 2013). Mutations at R64 in yeast suppressed the *cca1-1* (*cca1-E189K*) mutation without affecting protein structure, suggesting a role of the N-terminal domain in regulating the catalytic function (Goring et al., 2013).

Analogous mutations in yeast, *cca1-L179V* and *cca1-V188S*, are temperature sensitive when grown in media containing a non-fermentable carbon source (respiration required, glycerol) demonstrating that in yeast, as in humans, mutations in *CCAI* result in impaired mitochondrial function (Figure 3.2B); this phenotype was consistent in two commonly used lab strains (S288C and W303) (Figure 3.2C).

Figure 3.1.

<i>Saccharomyces</i>	MLRSTISLLMNSAAQKTMNTSNFVLNAPKITLTKEQNICNLLNDYTDLYNQKYHNKP--	58
<i>Homo</i>	-----MKLQSPFEQSLFTEGLKSLTELFV-----K	25
<i>Arabidopsis</i>	-----ELTDKERKIFDRLLSTLRYCN-----	21
<i>Thermotoga</i>	-----RVDPKILNLFRLLGKFGDEVN-----	21
A		
<i>Saccharomyces</i>	EPLTIRITGGWVRDKLLGQGSHELDIAINVMSEGEQFATGLNEYLQQHY-----A	107
<i>Homo</i>	ENHEIRIAGGAVRDLLNGVKPQDIDFATTATP-----TQMKEMFQSAG-----	68
<i>Arabidopsis</i>	LDTQIRVAGGVRDKLLGKESDIDIAIDNMSGSEFLDKFKEYLSSRD-----	69
<i>Thermotoga</i>	--MPVYVVGGFVRDLLLGKINLIDIVVEGNA-----LEFAEYAKRFLP-----	63
. ** * * * * * : * : :		
R99W K107R		
<i>Saccharomyces</i>	KYGAKPHNIHKIDKNPEKSKHLETATTKLFGVEVDFVNLRSKEYTELSRIPKVCFGTPEE	167
<i>Homo</i>	-----IRMINNRGEKHGTITARLHEENFEITTLRIDVTTDG--RHAEEVFTTDWQ	116
<i>Arabidopsis</i>	---EEVQGDTVIERNPQSKHLETAKLRIYDQWIDFVNLRSSEYNTENSRIPTMKFGTAKD	126
<i>Thermotoga</i>	-----GKLVKHKDFMTASFLKGGRLRIDIATARLEYYESPAKLPDVMSTIKK	111
B T154I M158V L166S C R190I D		
<i>Saccharomyces</i>	-DALRRDATLNALFYNIHK--GEVEFTKRGLQDLKDGVLSTPLPAKQTLDPLVLR	223
<i>Homo</i>	KDAERRDLTINSMFLGFD--GTLFTYFN--CYEDLKNKKVVFVGHAKQRQEDYLILR	171
<i>Arabidopsis</i>	-DAFRRLTINSLFYNIINS--GAVELTERGIDDLKSGKIITPLPAKATLDPLVLR	182
<i>Thermotoga</i>	-DLYRRDFTINAMAIKLNPDKFGLLIDFFG--GYRDLKEGVIVLHT--LSVDDPTILR	167
* * * * * : * : * : * * * : * : * : * :		
I223T E		
<i>Saccharomyces</i>	LIRFASRFNFT---IDPEVMAEMGDPQINAVFNSKISRERVGVEMEKILVGPTPLLALQL	283
<i>Homo</i>	YFRFYGRIVDKPGDHPETLEATAE---NAKGLACISGERIWWELKILVGNHVNHLIHL	231
<i>Arabidopsis</i>	AVRFGARFGFT---LDEELKEAASSEEVVRVALGEKISRERIGNEIDLMISGNPVSATY	242
<i>Thermotoga</i>	AIRFQRFDRIETTERLLKQAVEEGYLERTTGF---EL-QELEKILEKNPLKSIRR	225
. ** * . : * : * : * : * :		
<i>Saccharomyces</i>	IQRHLENVIFFWHNDSSVVKFNEENCQDMDKINHVNNDNILNSHLKSFIELYPMFLEKL	343
<i>Homo</i>	IYDLDVAPYIGLPANAS-----LEEFDKVSKNV	259
<i>Arabidopsis</i>	LSDLKLFSVVFALPSSAEPSP-----PENCGSLSQSYLEAMWSLLKTPRPGKFSGEQRR	297
<i>Thermotoga</i>	MAGFDVIKHLFPKTYTTP-----SMDEKMENLFRNI	256
: :		
I326T		
<i>Saccharomyces</i>	PILREKIGRSPGFQONFILSAILSPMANLQIIGNPKKKINNLSVSTESIVKEGLK---	399
<i>Homo</i>	DGFSPPK-----VTLLASLFKVQDDVTCLDLRLKIAKEEKNLGLF---	300
<i>Arabidopsis</i>	ALYAAMFLPFRKTVYKDTKGKSIIPVNVHIFKFSMKRRTSDAETVMNIHQTTTERFRSLIPS	357
<i>Thermotoga</i>	PWVEENFG-----EVDRFYAVLVHVFLEFYDDESWEKVRDRYSR-----	295
:		
<i>Saccharomyces</i>	SKNDAAVIAKTVD---SICSYEELAK---FADRSQKKSEIGIFLRNFNG-----EWE	447
<i>Homo</i>	VKNRKDLIKATDSS-DPLKPYQDFIID----SREPDAITRVCELLKYQGE-----	342
<i>Arabidopsis</i>	LEVKKDVELDELTTWAADILEHWSITLNDPVIATSKIRVLTGFLLRDIKDFWRVSLTS	417
<i>Thermotoga</i>	RNLINERHVEKSAPALLEMLSERVPASFVYPLVKGVSNETICHFLAYLSG-----	343
: . :		
<i>Saccharomyces</i>	TAHFASLSDAFLKIPKLETKK-IELLFQNYNEFYSYIFDNNLNNCHELKP-IVDGKQMAK	505
<i>Homo</i>	-----HCLLKEMQQWSIPPFPVSGHDIRKVG--ISSGKEIGA	380
<i>Arabidopsis</i>	LLLSATVDGSDNDHQDIGQLDFQLERMRETYLTVEATIHGLGLDKIWDAPK-LVNGREIMQ	476
<i>Thermotoga</i>	-----EKEGLFKSYLLKIKNTKLEKINGEYLRKIGITSKIGI	385
. * :		
K416E		
<i>Saccharomyces</i>	LLQMKGP-WLGKINNEAIRWQFDNPTGTDQELITHLKAILPKYL---	546
<i>Homo</i>	LLQQLREQ-WKSGYQMEKDELLSYIKKT-----	405
<i>Arabidopsis</i>	IAELKGSRLIREWQKLLTWQLAYPNGTAEECKEWMRDIAKARQRIE	521
<i>Thermotoga</i>	VLEKILMKKLDGDRDEEEILEEVLASLETEGKL-----	416

Figure 3.1. Multiple sequence alignment of tRNA-NT. Alignments shown from *Saccharomyces cerevisiae*, human, *Arabidopsis thaliana*, and *Thermotoga maritima*. Additional sequences aligned were tRNA-NT were *Kluyveromyces lactis*, *Neurospora crassa*, *Aspergillus niger*, and *Xenopus danio* but removed for space. Conserved sites required for CCA addition are shown in green, patient-identified mutations in this analysis are shown in yellow. *TRNT1-R99W* (c.295C>T) and *TRNT1-K107R* (c.320A>G) mutant alleles were a gift from Matthew Leibovitch and Paul Joyce. The *pADH1-TRNT1*-mutant-CEN-*URA3* plasmids were generated using site-directed mutagenesis on *pADH1-TRNT1*-CEN-*URA3* (pAR1208) with primers containing each of the six *TRNT1* missense mutations: c.461C>T (p.*T154I*) (pAR1209), c.472A>G(p.*M158V*) (pAR1210), c.497T>C(p.*L166S*) (pAR1211), c.569G>T(p.*R190I*) (pAR1212), c.668T>C(p.*I223T*) (pAR1213), c.977T>C(p.*I326T*) (pAR1214), c.1246A>G(p.*K416E*) (pAR1215). Analogous mutations for *T154I*, *M158V*, *L166S*, and *R190I* were designed in yeast as follows: c.461C>T (p.*T154I*) as c.492ACA>ATT (p.*T175I*; c.472T>C(p.*M158V*) as c.504CTT>GTG (p.*L179V*); c.497T>C(p.*L166S*) as c.574GTG>TCA (p.*V188S*; c.569G>T(p.*R190I*) as c.643ACA>ATA (p.*R220I*).

Figure 3.2.

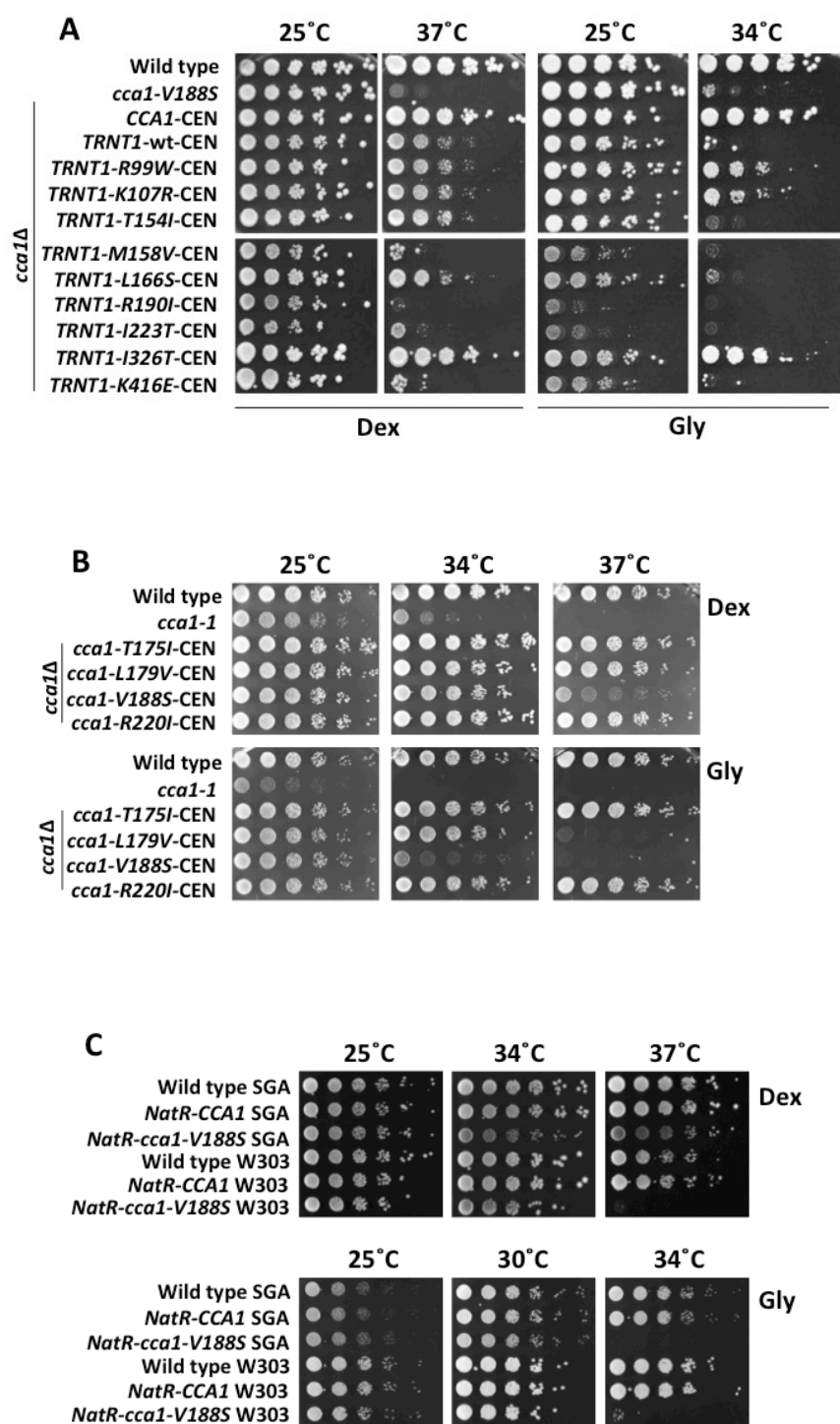


Figure 3.2. *TRNT1/CCA1* mutant phenotype was consistent with mitochondrial dysfunction.

(A) Complemented *cca1*Δ strains containing *TRNT1* wild type and patient identified mutations on CEN-plasmids. Ten-fold serial dilutions of wild type (ADR22), *cca1-V188S* (EKK130), *CCA1*-CEN (ADR6840), *cca1*Δ *TRNT1*-wt-CEN (EKK100), *cca1*Δ *TRNT1-R99W*-CEN (EKK374), *cca1*Δ *TRNT1-K107R*-CEN (EKK376), *cca1*Δ *TRNT1-T154I*-CEN (EKK332), *cca1*Δ *TRNT1-M158V*-CEN (EKK368), *cca1*Δ *TRNT1-L166S*-CEN (EKK334), *cca1*Δ *TRNT1-R190I*-CEN (EKK370), *cca1*Δ *TRNT1-I223T*-CEN (EKK372), *cca1*Δ *TRNT1-I326T*-CEN (EKK336), and *cca1*Δ *TRNT-K416E*-CEN (EKK338) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates and grown at the indicated temperatures for 2 to 4 days.

(B) Complemented *cca1*Δ strains containing patient-identified analogous *CCA1* mutations on CEN-plasmids. Ten-fold serial dilutions of wild type (ADR22), *cca1-1* (EKK002), *cca1*Δ *cca1-T175I*-CEN (ADR8363), *cca1*Δ *cca1-L179V*-CEN (ADR8363), *cca1*Δ *cca1-V188S*-CEN (ADR8367), and *cca1*Δ *cca1-R220I*-CEN (ADR8369) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates and grown at the indicated temperatures for 2 to 4 days.

(C) Analogous *CCA1* mutations in the endogenous *CCA1* gene in two commonly used yeast strains. Ten-fold serial dilutions of wild type SGA (ADR9360), *NatR-CCA1* SGA (EKK138), *NatR-cca1-V188S* (ADR9214), wild type W303 (ADR22), *NatR-CCA1* W303 (EKK132), and *NatR-cca1-V188S* W303 (EKK130) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates and grown at the indicated temperatures for 2 to 4 days.

TRNT1/CCAI mutant strains also are sensitive to increased reactive oxygen species, shown by sensitivity to growth on hydrogen peroxide (Figure 3.3A) and synthetic interactions with the loss of cytoplasmic (SOD1) or mitochondrial (SOD2) oxygen radical scavengers (Figure 3.3B). Furthermore, *TRNT1/CCAI* mutants are sensitive to DTT (dithiothreitol) that creates a reducing cellular environment and promotes protein unfolding (Figure 3.3C).

3.3.2. Acute loss of Cca1

Since the analogous *CCAI* mutants were only mildly temperature sensitive, I decided to study the acute loss of Cca1 with an auxin inducible degron (AID). The degron is recognized by the yeast SCF E3 ubiquitin ligase and activated by a co-expressed plant F-box protein, *TIR1*, to trigger proteasomal degradation of the tagged protein following the addition of auxin (Nishimura et al., 2009). Growth of *CCAI-AID* with auxin was lethal, consistent with the essential function of Cca1 (Figure 3.4A).

To assess cytoplasmic translation following acute degradation of Cca1, we compared the amount of eIF2 α -P to total eIF2 α , a standard measure of cytoplasmic translation inhibition (Holcik & Sonenberg, 2005). After one hour of Cca1 degradation, eIF2 α -P was induced prior to polysome loss, indicating that although the bulk cytoplasmic protein translation was unchanged, a stress response has been induced as shown by the presence of phosphorylated eIF2 α (Figure 3.4C, D). Induction of eIF2 α -P at two-hour post Cca1 degradation corresponded with reduced ribosome formation, as indicated by a reduction of the 40S, 60S, and 80S ribosomes; further degradation of Cca1 reduces polysome formation as expected (Figure 3.4E, F). The control strain containing *CCAI* wild type and the *TIR1* protein showed no difference in polysome formation, although readings were out of range

Figure 3.3.

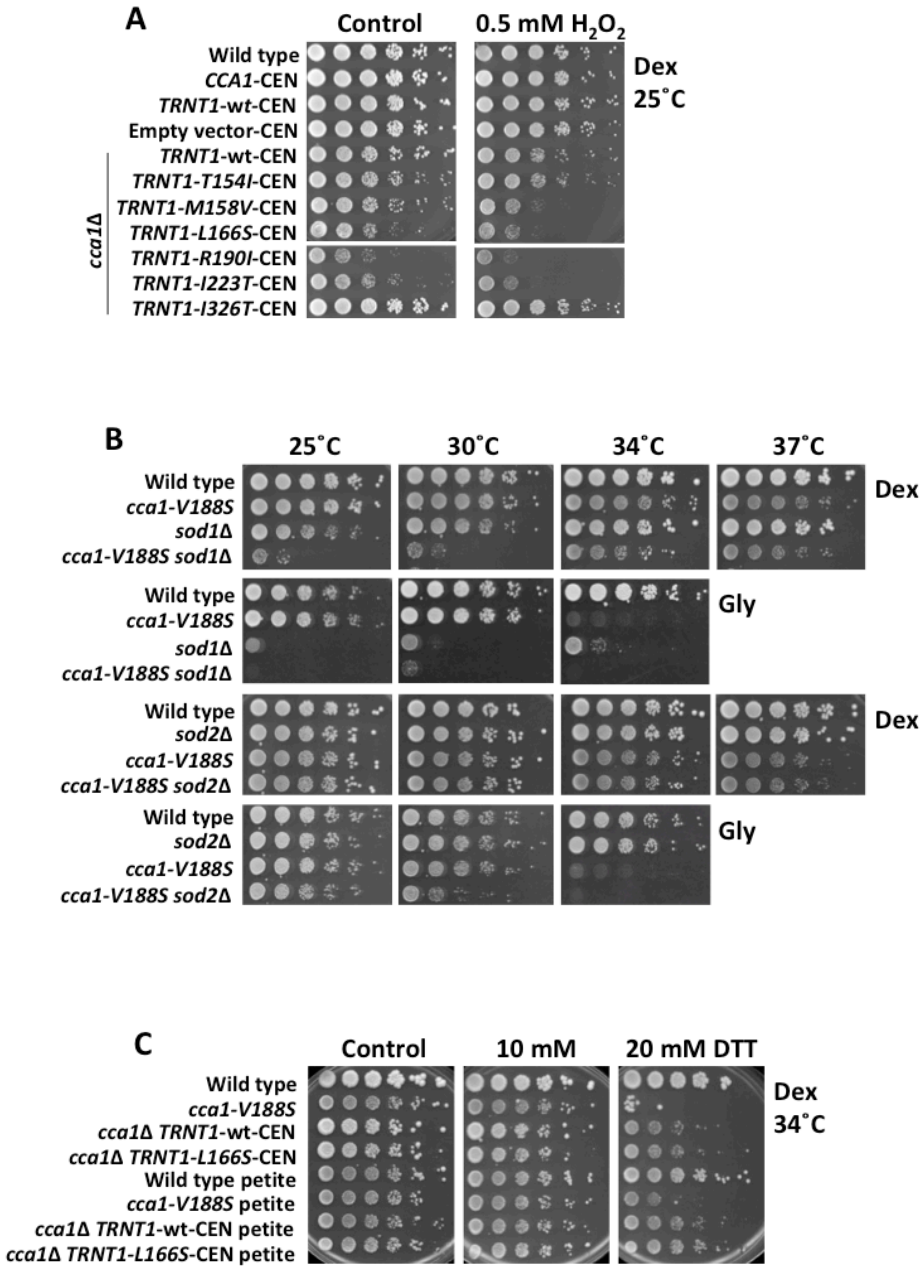


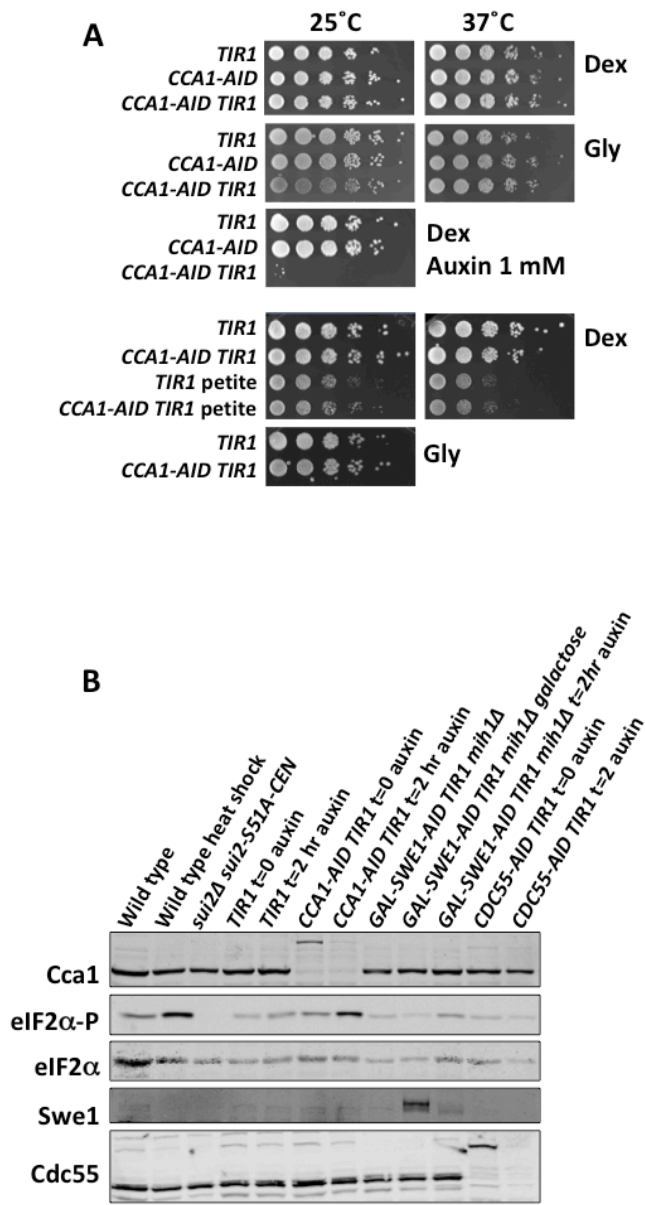
Figure 3.3. *TRNT1/CCA1* mutants are sensitive to reactive oxygen species and reduced environments.

(A) *TRNT1* wild type and mutants are sensitive to growth in the presence of reactive oxygen species. Ten-fold serial dilutions of wild type (ADR22), *CCA1*-CEN (ADR6836), *TRNT1*-wt-CEN (ADR6834), Empty vector (ADR6838), *cca1* Δ *TRNT1*-wt-CEN (ADR6791), *cca1* Δ *TRNT1-T154I*-CEN (ADR6793), *cca1* Δ *TRNT1-M158V*-CEN (ADR6933), *cca1* Δ *TRNT1-L166S*-CEN (ADR6935), *cca1* Δ *TRNT1-R190I*-CEN (ADR6937), *cca1* Δ *TRNT1-I223T*-CEN (ADR6939), and *cca1* Δ *TRNT1-I326T*-CEN (ADR6941) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) with 0.5mM H₂O₂ and grown at 25°C for 3 to 4 days.

(B) Deletion superoxide dismutases for reactive oxygen scavenging *SOD1* (cytosolic) or *SOD2* (mitochondrial) are both synthetic with *cca1-V188S*; this was in the deletion collection and synthetic genetic array background. Ten-fold serial dilutions of wild type (ADR9360), *cca1-V188S* (ADR9214), and *SOD* deletion strains (from the deletion collection but not kept) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates and grown at the indicated temperatures for 2 to 4 days.

(C) *TRNT1* wild type, *TRNT1-L166S*, and analogous *cca1-V188S* are sensitive to reduced environments, exasperated by the loss of mitochondrial DNA. Ten-fold serial dilutions of wild type (ADR4006), *cca1-V188S*, *cca1* Δ *TRNT1*-wt-CEN (EKK100), *cca1* Δ -*TRNT1-L166S*-CEN (EKK334), wild type petite (EKK275), *cca1-V188S* petite (EKK273), *cca1* Δ *TRNT1*-wt-CEN petite (EKK277), and *cca1* Δ -*TRNT1-L166S*-CEN petite (EKK346) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) with 10 or 20mM DTT and grown at 34°C for 3 to 4 days. No phenotype was observed at lower temperatures (data not shown).

Figure 3.4.



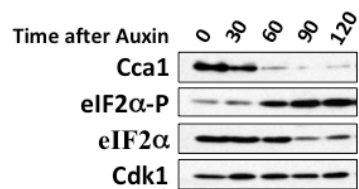
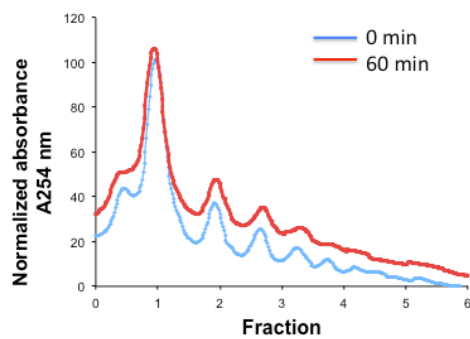
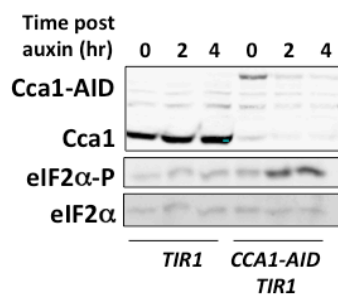
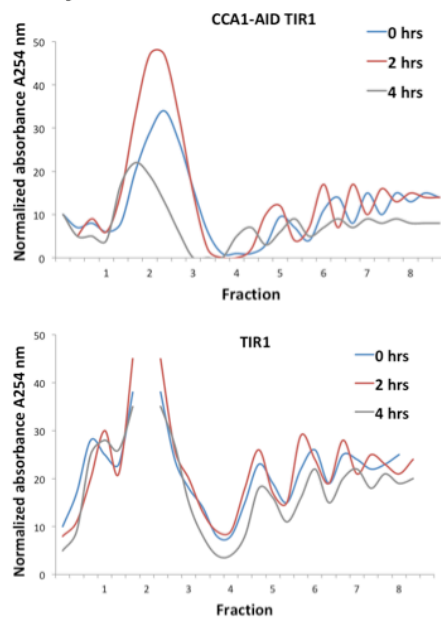
C**D****E****F**

Figure 3.4. Acute degradation of Cca1 using an auxin inducible degron (the AID tag) results in increased eIF2 α -P.

(A) Growth of *CCA1-AID* with auxin was lethal, consistent with the essential function of Cca1. Ten-fold serial dilutions of *TIR1* (ADR6431), *CCA1-AID*, *CCA1-AID TIR1* (ADR8264), *TIR1* petite (EKK005), and *CCA1-AID TIR1* petite (EKK003) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates, or YEP dextrose containing 1mM auxin and grown at the indicated temperatures for 2 to 4 days.

(B) eIF2 α -P was specific to degradation of Cca1-AID. Wild type (ADR4006), *sui2 Δ sui2-S51A* (EKK036), *TIR1* (ADR6431), *CCA1-AID TIR1* (ADR8264), and *CDC55-AID TIR1* (ADR6385) strains were grown over night at 25°C in YEP dextrose at 25°C to 2 to 4x10⁷ cells/mL, diluted, grown to 0.5x10⁷ cells/mL when auxin was added for 0 or 2 hrs. Strain *GAL-SWE1-AID TIR1 mih1 Δ* (ADR6918) was grown over night in YEP raffinose at 25°C to 2 to 4x10⁷ cells/mL, diluted, grown to 0.5x10⁷ cells/mL, induced with galactose for 30 min, then auxin was added for 2 hrs. 3x10⁷ cells were pelleted and frozen in liquid nitrogen; samples were mechanically lysed in 1X sample buffer and analyzed by western blot analysis.

(C) Increased eIF2 α phosphorylation occurs prior to polysome loss. Strain *CCA1-AID TIR1* (ADR8264) was grown overnight at 25°C in YEP dextrose at 25°C to 2 to 4x10⁷ cells/mL, diluted, grown to 0.5x10⁷ cells/mL when auxin was added for 0, 30, 60, 90, and 120 minutes. 3x10⁷ cells were pelleted and frozen in liquid nitrogen; samples were mechanically lysed in 1X sample buffer and analyzed by western blot analysis. **(D)** The sample at 60 min was also processed for polysomes: cells were pelleted, washed with 0.1mg/mL cycloheximide and frozen in liquid nitrogen. Cells were mechanically lysed, centrifuged at 5000 rcf at 4°C then 250,000 rcf and processed using the ISCO gradient fractionation system as per manufactures instructions. Polysomes analysis following 60 min of acute Cca1 degradation, preformed by Thet Naing in the Holcik Laboratory.

(E) Extended degradation of *CCA1* results in polysome loss. Strain *CCA1-AID TIR1* (ADR8264) was grown overnight at 25°C in YEP dextrose at 25°C to 2 to 4x10⁷ cells/mL, diluted, grown to 0.5x10⁷ cells/mL when auxin was added for 0, 2, or 4 hrs. 3x10⁷ cells were pelleted and frozen in liquid nitrogen, samples mechanically lysed in 1X sample buffer and analyzed by western blot analysis. **(F)** The samples were also processed for polysomes: cells were pelleted, washed with 0.1 mg/mL cycloheximide and frozen in liquid nitrogen. Cells were mechanically lysed, centrifuged at 5000 rcf at 4°C then 250,000 rcf and processed using the ISCO gradient fractionation system as per manufactures instructions. Polysomes analysis was preformed by Erin Kennedy and Laura Frost in the Stephen Lewis Laboratory at Dalhousie, Moncton Campus.

for the top of the 80S peak.

I analyzed other AID-tagged protein degradation to test if the degradation of AID-tagged proteins resulted in non-specific eIF2 α -P. Only degradation of Cca1-AID results in increased eIF2 α -P, supporting that this stress response was specific to loss of Cca1 (Figure 3.4B).

3.3.3. Mitochondrial translation in *TRNT1/CCA1* mutants

Given the mitochondrial and cytoplasmic translation phenotypes observed, the rate of mitochondrial translation *in vivo* was directly assessed. *TRNT1* wild type and mutant, Cca1 degon, and *cca1-V188S* have altered mitochondrial translation as observed with decreased levels of some mitochondrial-encoded proteins (Figure 3.5A). In the degon and *TRNT1* mutants the rate translation of Var1, Cox1, and Cox2 are reduced, and Atp6 was not translated properly (determined by size shift) in the degon or *cca1-V188S* at all temperatures, indicating altered mitochondrial translation (Figure 3.5A). Identification of mitochondrial-encoded proteins was done based on the molecular weight of the proteins and by previously identified western blot banding patterns (Augustin et al., 2005, Kuzmenko et al., 2015; Wang et al., 2010).

While all of the wild type and mutant strains had no significant difference in uptake of the [³⁵S] methionine/cysteine label (p-values > 0.05), the degon exposed to auxin for two hours showed reduced uptake of the [³⁵S] methionine/cysteine label (p-value = 0.0019) compared to no exposure to auxin (Figure 3.5B); analyzed using unpaired Student's t-tests of the percent of total label incorporated into the cell over the 15 min incubation. This reduced uptake did not completely account for differences in protein translation products, as observed by little to no differences in Cob and Cox3 translation between the strains analyzed

Figure 3.5.

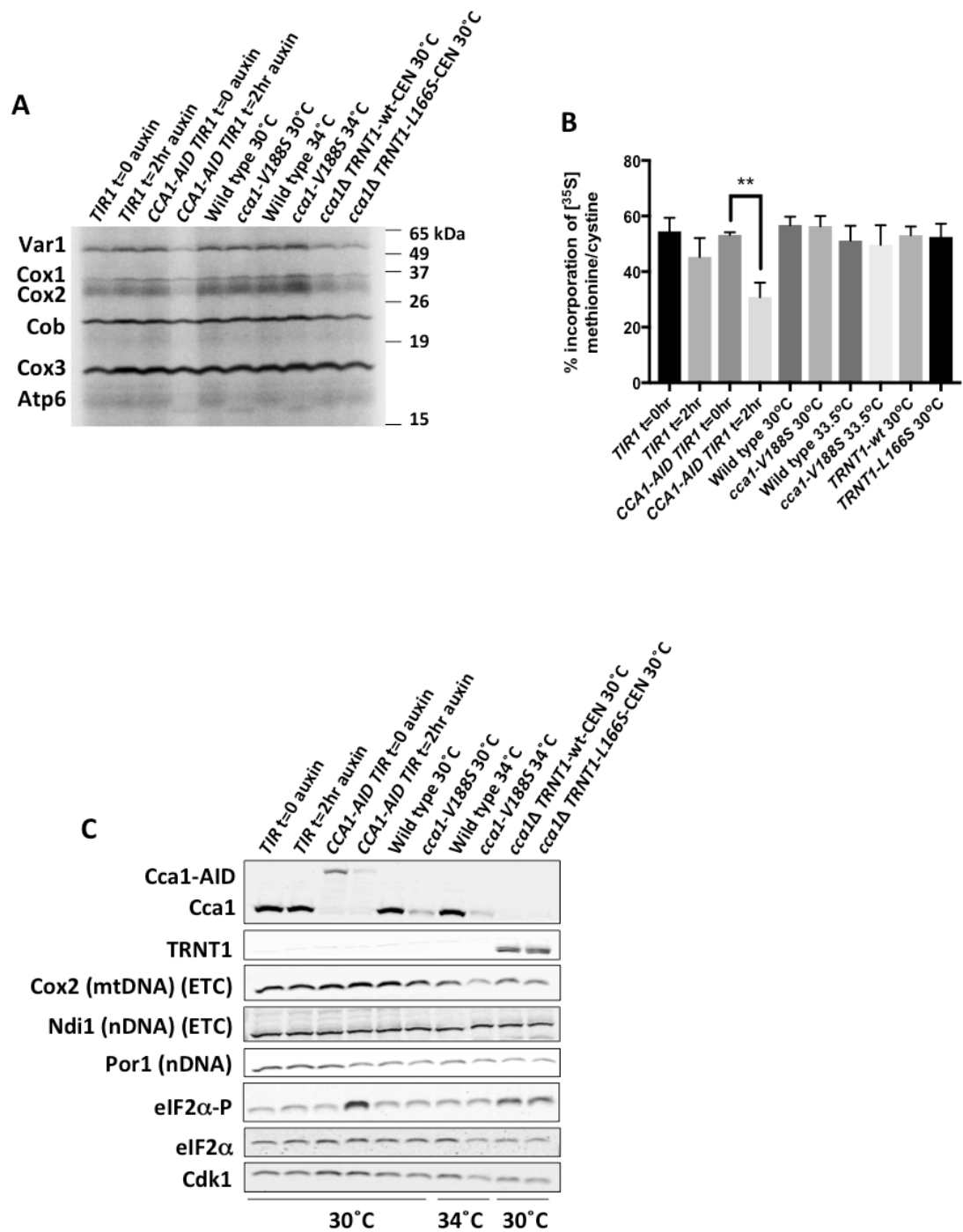


Figure 3.5. Mitochondrial translation was altered in *TRNT1* wild type and mutants, *cca1-V188S*, and *Cca1* degron.

(A) *In vivo* labeling of mitochondrial translation. Strains *TIR1* (ADR6431), *CCA1-AID TIR1* (ADR8264), wild type (ADR4006), *cca1-V188S* (EKK130), *cca1Δ TRNT1*-wt-CEN (EKK100), and *cca1Δ TRNT1-L166S*-CEN (EKK334) were grown overnight in YEP raffinose/galactose media, washed and resuspended in SC-met at 3×10^7 cells/mL for one hour. 0.5mL of 1.5×10^7 cells/mL had cytoplasmic translation stopped by cycloheximide for 10 min, labeled with 1mCi [35 S] methionine/cysteine for 15 minutes at 30°C or 34°C with frequent mixing. Cells were pelleted, frozen in liquid nitrogen and mechanically lysed with 1X sample buffer before running on a 12.5% polyacrylamide gel. The gel was fixed, dried, and exposed to a phosph-imager screen for 48 hrs and scanned at 50 microns.

(B) [35 S] methionine/cysteine uptake in *TRNT1* and *CCA1* mutant strains. Strains *TIR1* (ADR6431), *CCA1-AID TIR1* (ADR8264), wild type (ADR4006), *cca1-V188S* (EKK130), *cca1Δ TRNT1*-wt-CEN (EKK100), and *cca1Δ TRNT1-L166S*-CEN (EKK334) were grown overnight in YEP raffinose/galactose media at 30°C, washed and resuspended in SC-Met at 3×10^7 cells/mL for one hour. 0.5mL of 1.5×10^7 cells/mL had cytoplasmic translation stopped by cycloheximide for 10 min, labeled with 1 mCi [35 S] methionine/cysteine for 15 minutes at 30°C or 33.5°C with frequent mixing. 10mL of the 0.5ml mixture was taken at t=0 and of the supernatant of t=15, representing [35 S] methionine /cysteine not taken into the cells. The 10mL was measured by scintillation counting and each strain uptake determined relative to its control. Bars represent three replicates with the error bars representing SEM from n=3 experiments. Student's t-test between *CCA1-AID TIR1* t=0hr and *CCA1-AID TIR1* t=2hr had a p-value of 0.019; no other pair-wise comparisons had statistically different means (all p-values > 0.05)

(C) *TRNT1/CCA1* mutants do not change the steady state levels of mitochondrial proteins. Strains *TIR1* (ADR6431), *CCA1-AID TIR1* (ADR8264), wild type (ADR4006), *cca1-V188S* (EKK130), *cca1Δ TRNT1*-wt-CEN (EKK100), and *cca1Δ TRNT1-L166S*-CEN (EKK334) were grown overnight in YEP glycerol at 25°C to 2 to 4×10^7 cells/mL, diluted and grown to 0.5×10^7 cells/mL at 25°C, transferred to the indicated temperatures and grown for three hours to 1 to 2×10^7 cells/mL; the AID tagged strains were grown to 1×10^7 cells/mL when auxin was added for two hours at 30°C. 3×10^7 cells were pelleted and frozen in liquid nitrogen. Samples were mechanically lysed in 1X sample buffer and analyzed by western blot analysis.

(Figure 3.5A). Upregulation of the PDR pathway may impair [³⁵S] methionine/cysteine uptake.

Matched western samples show even loading of cells, relative amounts of nuclear encoded mitochondrial proteins Ndi1 (complex IV ETC) and porin (mitochondrial outer membrane pore), and the decreased levels of Cca1 due to degon degradation or *cca1-V188S* mutation (Figure 3.5C). Steady state levels of Cox2 are not significantly different and induction of the eIF2 stress response as shown by eIF2 α -P was observed in the degon and *TRNT1* wild type and mutants.

3.3.4. Functional mitochondria are required for eIF2 α -P signaling

To further investigate the eIF2 α -P stress response observed in the Cca1 degon, eIF2 α -P/total eIF2 α was assessed in *TRNT1* and *CCA1* mutants. *TRNT1* wild type and mutants and *cca1-V188S* result in increased eIF2 α -P, although in *cca1-V188S* this change was only observed at high temperature (Figure 3.6). Additionally, *cca1-V188S* protein was expressed at lower levels compared to wild type at high temperature or when grown under respiratory conditions (Figure 3.6B). eIF2 α -P is a competitive inhibitor of eIF2B which prevents the recycling of the eIF2 complex (brings initiator tRNA to the ribosome) and subsequently slows cytoplasmic translation (Proud, 2005; Williams et al., 2000). The induction of eIF2 α -P suggests that impaired mitochondrial translation in the *TRNT1/CCA1* mutants results in a stress response to slow cytoplasmic translation.

TRNT1-wild type and *TRNT1* mutants have significantly greater eIF2 α -P compared to wild type cells (Figure 3.7A, B, C); eIF2 α -P/eIF2 α from five separate experiments was statistically analyzed using the Student's t-test, p-values presented in Figure 3.7C. Yeast can survive as rho⁰ petites with no mtDNA, and therefore no mitochondrial translation, making

yeast are a robust model frequently used to study mitochondrial defects (Altmann & Linder, 2010). In this study, exposure and growth in low doses of ethidium bromide induces yeast to lose their mitochondrial DNA, making them rho⁰ petites (Goldring et al., 1971); there is no mitochondrial translation without mitochondrial DNA. When these strains were made petite by ethidium bromide, the *TRNT1* wild type and mutants no longer had greater levels of eIF2 α -P than wild type petite (Figure 3.7A) and differences in growth phenotype between wild type and mutants were abolished (Figure 3.7D). Although *TRNT1-I326T* had increased eIF2 α -P, this was not as great as other mutants and when made petite a significant decrease did not occur, likely due to the lower initial value and the occurrence of eIF2 α -P in wild type petite samples. This demonstrates that functioning mitochondrial translation and/or OXPHOS are needed induce eIF2 α -P signaling. Loss of mitochondrial translation results in loss of OXPHOS due to the essential OXPHOS components encoded on mtDNA.

Further loss of Cca1 function in *cca1-V188S* cells grown at 37°C still causes induction of eIF2 α -P in petite strains confirming that 3'CCA addition to tRNAs was required for translation in all compartments, such that increased loss of 3' CCA will impact cytoplasmic translation (Figure 3.8A, B). The eIF2 α -P response also occurs in the petite degdon following two hours degradation, consistent with the observed loss of cytoplasmic translation at this level of degradation (Figure 3.8C). Further work should look at less degradation time to see the eIF2 α -P level in petites prior to polysome loss.

Petite strains made by exposure to ethidium bromide have no mitochondrial DNA, resulting in no mitochondrial translation and no OXPHOS. Alternatively, the nuclear encoded *COQ5* gene can be deleted to result in non-functional electron transport chain and inhibition of OXPHOS while allowing mitochondrial translation (Bonawitz et al., 2007). In contrast to the *TRNT1* petites that grow similarly to the wild type petites (Figure 3.7D), the

Figure 3.6.

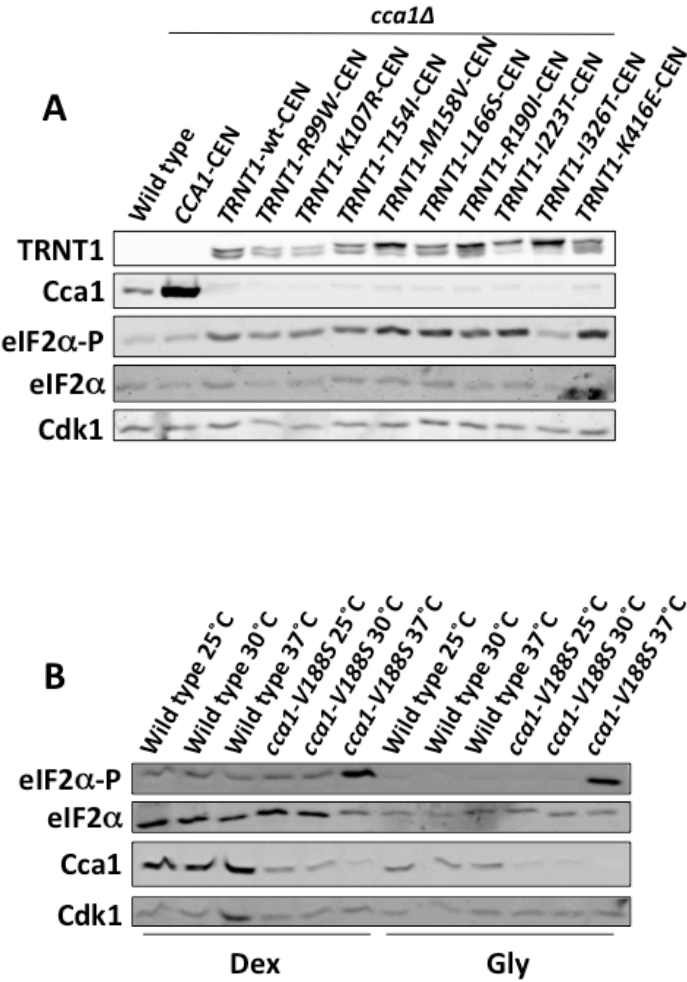
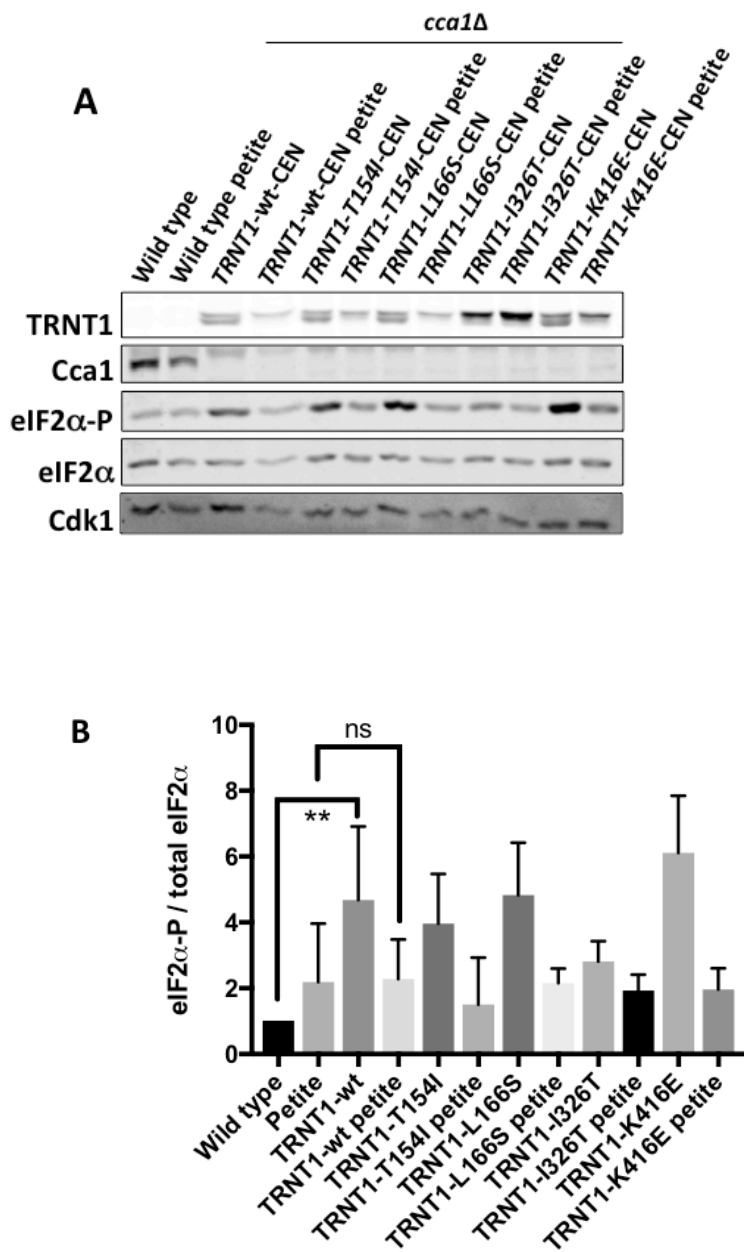


Figure 3.6. *TRNT1* wild type and mutants, and *cca1-V188S* have increased eIF2 α -P.

(A) Wild type (ADR22), *CCA1*-CEN (ADR6840), *cca1* Δ *TRNT1*-wt-CEN (EKK100), *cca1* Δ *TRNT1*-*R99W*-CEN (EKK374), *cca1* Δ *TRNT1*-*K107R*-CEN (EKK376), *cca1* Δ *TRNT1*-*T154I*-CEN (EKK332), *cca1* Δ *TRNT1*-*M158V*-CEN (EKK368), *cca1* Δ *TRNT1*-*L166S*-CEN (EKK334), *cca1* Δ *TRNT1*-*R190I*-CEN (EKK370), *cca1* Δ *TRNT1*-*I223T*-CEN (EKK372), *cca1* Δ *TRNT1*-*I326T*-CEN (EKK336), and *cca1* Δ *TRNT*-*K416E*-CEN (EKK338) were grown overnight in YEP dextrose at 25°C to 2 to 4 x10⁷ cells/mL, diluted and grown to 1 to 2x10⁷ cells/mL, 3 x10⁷ cells were pelleted and frozen in liquid nitrogen; samples were mechanically lysed in 1X sample buffer and analyzed by western blot analysis.

(B) Wild type (ADR22) and *cca1-V188S* (EKK130) were grown overnight in YEP dextrose at 25°C to 2 to 4 x10⁷ cells/mL, diluted and grown to 0.5x10⁷ cells/mL at 25°C, transferred to the indicated temperatures and grown for three hours in YEP dextrose (Dex) or glycerol (Gly) to 1 to 2 x10⁷ cells/mL, 3x10⁷ cells were pelleted and frozen in liquid nitrogen; samples were mechanically lysed in 1X sample buffer and analyzed by western blot analysis.

Figure 3.7.



C

	Wild type petite	TRNT1-wt-CEN	TRNT1-wt-CEN petite	TRNT1-T154I-CEN	TRNT1-T154I-CEN petite	TRNT1-L166S-CEN	TRNT1-L166S-CEN petite	TRNT1-I326T-CEN	TRNT1-I326T-CEN petite	TRNT1-K416E-CEN	TRNT1-K416E-CEN petite
Wild type	ns	**	*	**	ns	**	**	**	**	**	**
Wild type petite		ns	*	ns	**	*	**	ns	**	**	**
TRNT1-wt-CEN			ns	ns	*	ns	*	ns	*	ns	ns
TRNT1-T154I-CEN				*							
TRNT1-L166S-CEN						**					
TRNT1-I326T-CEN								ns			
TRNT1-K416E-CEN										**	

ns = p-value > 0.05
 * = p-value < 0.05
 ** = p-value < 0.01

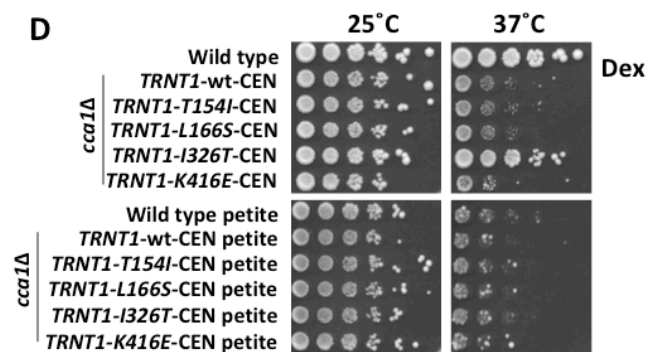


Figure 3.7. Functioning mitochondria are needed to slow cytoplasmic translation by induction of eIF2 α -P.

(A) Petite strains of *TRNT1* mutants do not induce high levels of eIF2 α -P. Wild type (ADR22), wild type petite (EKK275), *cca1* Δ *TRNT1*-wt-CEN (EKK100), *cca1* Δ *TRNT1*-wt-CEN petite (EKK277), *cca1* Δ *TRNT1-T154I*-CEN (EKK332), *cca1* Δ *TRNT1-T154I*-CEN petite (EKK344), *cca1* Δ *TRNT1-L166S*-CEN (EKK334), *cca1* Δ *TRNT1-L166S*-CEN petite (EKK346), *cca1* Δ *TRNT1-I326T*-CEN (EKK336), *cca1* Δ *TRNT1-I326T*-CEN petite (EKK348), *cca1* Δ *TRNT1-K416E*-CEN (EKK338), and *cca1* Δ *TRNT1-K416E*-CEN petite (EKK350) strains were grown overnight in YEP dextrose at 25°C to 2 to 4x10⁷ cells/mL, diluted and grown 1 to 2x10⁷ cells/mL. 3x10⁷ cells were pelleted and frozen in liquid nitrogen; samples were mechanically lysed in 1X sample buffer and analyzed by western blot analysis.

(B) Statistical analysis of eIF2 α -P in petite wild type and *TRNT1* mutants

Western band quantification of total eIF2 α and eIF2 α -P was analyzed using the Odyssey infrared imaging system (LI-COR) as directed by the manufacturer with the associated software. Total signal was normalized to the wild type for each antibody (eIF2 α /Sui2 or eIF2 α -P) and the proportion of eIF2 α -P/ eIF2 α was determined for each sample. A minimum of three blots were analyzed for each mutant, samples are shown with error bars representing the standard error of the mean (SEM). Two unpaired t-tests are shown between wild type and *TRNT1*, and wild type petite and *TRNT1* petite. When not petite, *TRNT1*-wild type has significantly greater (** indicating p-value < 0.01) eIF2 α -P than wild type, but in the petite condition there was no difference between wild type and *TRNT1*-wild type in eIF2 α -P levels.

(C) Statistical significance of eIF2 α -P differences between wild type and *TRNT1* mutants when petite.

Pairs of samples were analyzed using Unpaired t-tests to determine if they are statically significant using Prism; ns = not significant, * = p-value < 0.05, ** = p-value < 0.01.

(D) Growth phenotype difference between *TRNT1* wild type and mutants abolished when petite.

Ten-fold serial dilutions of wild type (ADR22), wild type petite (EKK275), *cca1* Δ *TRNT1*-wt-CEN (EKK100), *cca1* Δ *TRNT1*-wt-CEN petite (EKK277), *cca1* Δ *TRNT1-T154I*-CEN (EKK332), *cca1* Δ *TRNT1-T154I*-CEN petite (EKK344), *cca1* Δ *TRNT1-L166S*-CEN (EKK334), *cca1* Δ *TRNT1-L166S*-CEN petite (EKK346), *cca1* Δ *TRNT1-I326T*-CEN (EKK336), *cca1* Δ *TRNT1-I326T*-CEN petite (EKK348), *cca1* Δ *TRNT1-K416E*-CEN (EKK338), and *cca1* Δ *TRNT1-K416E*-CEN petite (EKK350) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates and grown at the indicated temperatures for 2 to 4 days.

Figure 3.8.

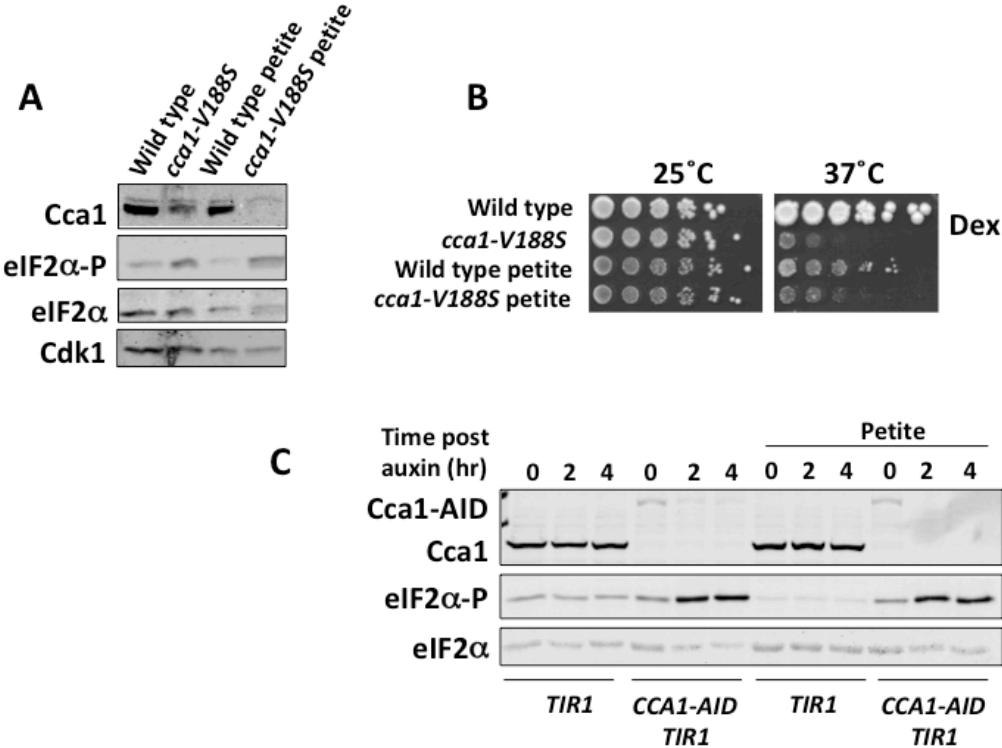


Figure 3.8. Increased loss of 3' CCA addition will impact cytoplasmic translation.

(A) Further loss of Cca1 function in *cca1-V188S* cells grown at 37°C still causes induction of eIF2 α -P in petite strains. Wild type (ADR22), *cca1-V188S* (EKK130), wild type petite (EKK275), and *cca1-V188S* petite (EKK273) strains were grown overnight in YEP dextrose at 25°C to 2 to 4x10⁷ cells/mL, diluted and grown for three hours at 37°C to 1 to 2x10⁷ cells/mL. 3x10⁷ cells were pelleted and frozen in liquid nitrogen; samples were mechanically lysed in 1X sample buffer and analyzed by western blot analysis.

(B) Growth phenotype difference decreased when *cca1-V188S* is petite. Ten-fold serial dilutions of wild type (ADR4006), *cca1-V188S* (EKK130), wild type petite (EKK275), and *cca1-V188S* petite (EKK273) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates and grown at the indicated temperatures for 2 to 4 days.

(C) The eIF2 α -P response also occurs in the petite degon following two hours degradation, consistent with the observed loss of cytoplasmic translation at this level of degradation. Strains *TIR1* (ADR6431), *CCA1-AID TIR1* (ADR8264), *TIR1* petite (EKK005), and *CCA1-AID TIR1* petite (EKK003) were grown overnight at 25°C in YEP dextrose at 25°C to 2 to 4x10⁷ cells/mL, diluted, grown to 0.5x10⁷ cells/mL when auxin was added for 0, 2, or 4 hrs. 3x10⁷ cells were pelleted and frozen in liquid nitrogen; samples were mechanically lysed in 1X sample buffer and analyzed by western blot analysis.

coq5 cca1-V188S does not grow similarly to *coq5Δ* alone (Figure 3.9A). Mitochondrial translation is normal in *coq5* mutants, suggesting that in *cca1-V188S* defective mitochondrial translation is generating the stress signal, not the loss of OXPHOS proteins. Further work needs to be done in the *coq5Δ* mutant, such as combining with *TRNT1* mutants and assessment of eIF2 α -P levels.

To further sensitize mitochondrial translation, I combined the *cca1-V188S* mutant with the deletion of *PUS1*. Pus1 is an enzyme required for pseudouridylation, the most common posttranscriptional modification of structural RNA, for both mitochondrial and cytoplasmic tRNA, with a proposed role in tRNA stabilization (Chen & Patton, 1999; Foster et al., 2000; Motorin et al., 1998; Spenkuch et al., 2014). Similar to defects in Cca1, this tRNA modification enzyme functions in both cytoplasmic and mitochondrial compartments and clinical phenotypes also present as a mitochondrial disease. Although no phenotype was observed for *pus1Δ* on either dextrose or glycerol, it was synthetic with *cca1-V188S* under both growth conditions (Figure 3.9B), suggesting that decreasing tRNA modification further exacerbates the *cca1-V188S* phenotype.

The Retrograde pathway is activated due to mitochondrial damage to ensure glutamate production for amino acid biosynthesis. To assess if this pathway was activated in the *cca1-V188S* mutant, the key regulatory protein of the RTG pathway, Rtg2, was deleted in combination with *cca1-V188S*. Deletion of *RTG2* prevents RTG signaling but did not change the growth phenotype in *cca1-V188S* (Figure 3.10). This suggests that the mitochondrial to cytoplasmic signaling I see in *TRNT1/CCA1* mutants was distinct from the RTG pathway.

Figure 3.9.

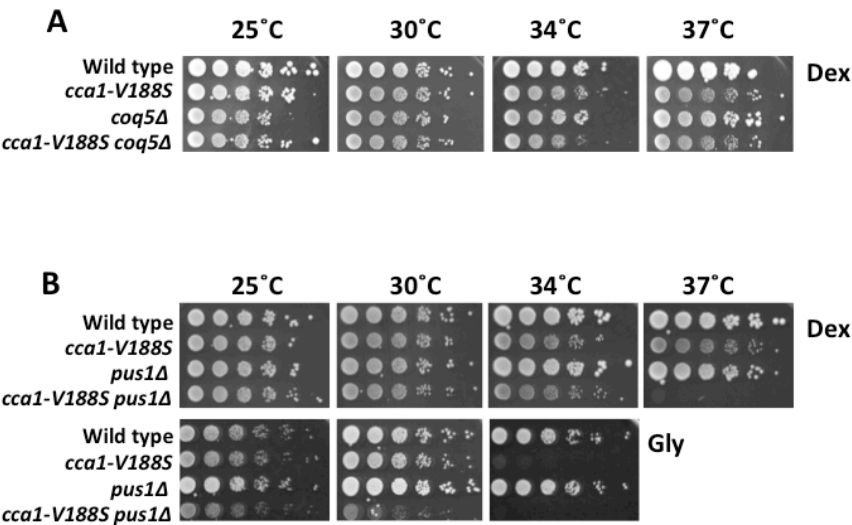


Figure 3.9. Impaired mitochondrial translation not defective OXPHOS results in increased growth phenotype of *cca1-V188S*

A. Disrupting OXPHOS did not alter the *cca1-V188S* growth phenotype.

Ten-fold serial dilutions of wild type (ADR9360), *cca1-V188S* (ADR9214), *coq5* Δ , and *coq5* Δ *cca1-V188S* were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) grown at the indicated temperatures for 2 days.

B. Decreasing tRNA modification further exacerbates the *cca1-V188S* phenotype. Ten-fold serial dilutions of wild type (ADR9360), *cca1-V188S* (ADR9214), *pus1* Δ , and *pus1* Δ *cca1-V188S* were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates and grown at the indicated temperatures for 2 to 4 days.

Figure 3.10.

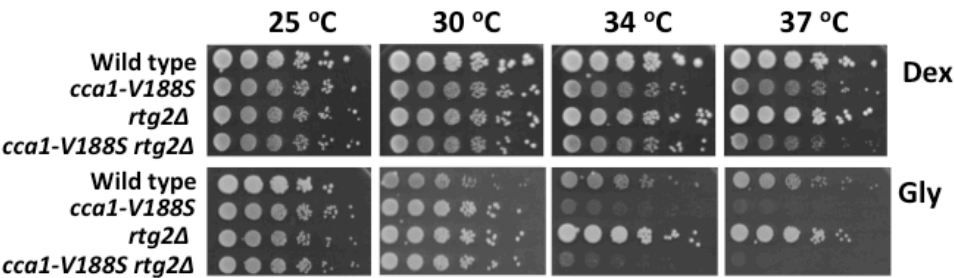


Figure 3.10. Mitochondrial to cytoplasmic signaling in *CCA1* mutants was distinct from the RTG pathway. Ten-fold serial dilutions of wild type (ADR9360), *cca1-V188S* (ADR9214), *rtg2Δ*, and *rtg2Δ cca1-V188S* were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates and grown at the indicated temperatures for 2 to 4 days.

3.3.5. Genetic alteration of eIF2 α -P

To further investigate the eIF2 α -P signaling, genetic alterations to eIF2 α (encoded by *SUI2* in yeast), *GCN2* encoding the eIF2 α -P kinase, and *GLC7* encoding the eIF2 α -P phosphatase, were combined with *TRNT1* and *CCA1* mutants. When *cca1-V188S* was combined with a mutant eIF2 α that cannot be phosphorylated (eIF2 α is encoded in yeast by *SUI2*, the phospho mutant is *sui2-S51A*) there was a suppressive effect under non-respiring conditions, indicating that eIF2 α -P was not suppressive under non-respiratory conditions when mitochondrial translation was not required in yeast (Figure 3.11A, Dex 37°C). In contrast, blocking eIF2 α -P by the *sui2-S51A* mutant was slightly synthetic under respiratory conditions (Figure 3.11A, Gly 33°C). Therefore, slowing cytoplasmic translation to balance protein homeostasis and reduce stress response signaling was suppressive in *TRNT1/CCA1* mutants when mitochondrial translation was required. In contrast, when *TRNT1* wild type and mutations are combined with *sui2-S51A*, a synthetic interaction was observed under respiration conditions with no mitochondrial translation (Gly 34°C) (Figure 3.11B), indicating aberrant stress response signaling when homeostasis between the translation systems was not required.

Glc7, the yeast PP1 homolog, removes the eIF2 α -P phosphorylation in yeast (Cherkasova et al., 2010; Wek et al., 1992). Lack of Glc7 would result in increased eIF2 α -P further decreasing cytoplasmic translation, opposite to *sui2-S51A*. A temperature sensitive allele of the eIF2 α -phosphatase *GLC7* (*glc7-10-ts*) showed a subtle synthetic interaction with *cca1-V188S* as measured by growth phenotype under non-respiring conditions (Dex 37°C), consistent with the suppressive effect observed under non-respiration requiring conditions with *sui2-S51A* (Figure 3.11A).

Figure 3.11.

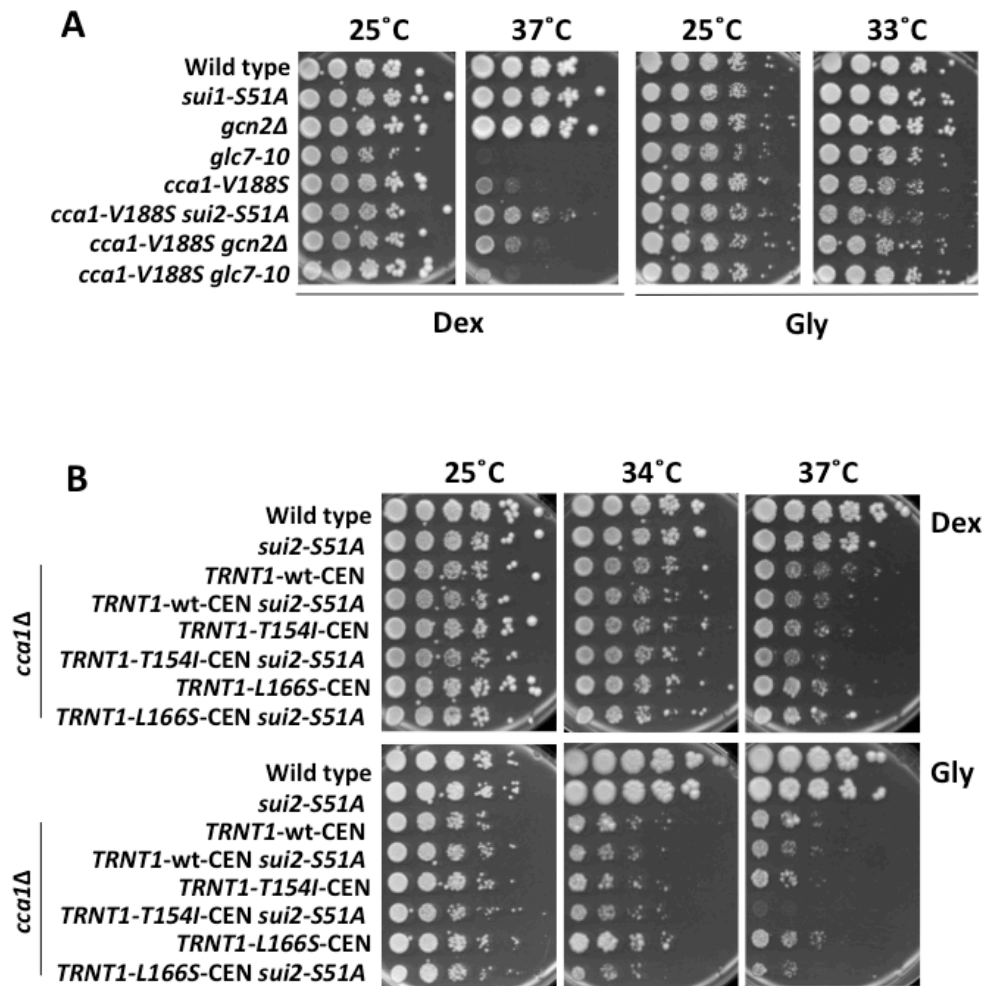


Figure 3.11. eIF2 α -P was synthetic under non-respiratory conditions when mitochondrial translation was not required.

(A) Genetic alteration of eIF2 α -P in *cca1-V188S*. Ten-fold serial dilutions of wild type (ADR22), *sui2-S51A* (EKK144), *gcn2 Δ* (EKK140), *glc7-10* (ADR9296), *cca1-V188S sui2-S51A* (EKK247), *cca1-V188S gcn2 Δ* (EKK367), and *cca1-V188S glc7-10* (EKK270) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates and grown at the indicated temperatures for 2 to 4 days.

(B) Genetic alteration of eIF2 α -P in *TRNT1* wild type and mutants. Ten-fold serial dilutions of wild type (ADR22), *sui2-S51A* (EKK144), *cca1 Δ TRNT1-wt-CEN* (EKK100), *cca1 Δ TRNT1-wt-CEN sui2-S51A* (EKK326), *cca1 Δ TRNT1-T154I-CEN* (EKK332), *cca1 Δ TRNT1-T154I-CEN sui2-S51A* (EKK328), *cca1 Δ TRNT1-L166S-CEN* (EKK334), and *cca1 Δ TRNT1-L166S-CEN sui2-S51A* (EKK330) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates and grown at the indicated temperatures for 2 to 4 days.

3.3.6. Decrease in cytoplasmic translation

Cytoplasmic translation can be slowed by deletion of ribosomal proteins (Schleit et al., 2013; Steffen et al., 2008; Wang et al., 2008a), drug inhibition of the cytoplasmic ribosome (Lamb et al., 1968), or reduced TOR signaling (Cherkasova & Hinnebusch, 2003; Laplante & Sabatini, 2012). To further assess the impact of reducing cytoplasmic translation in the *TRNT1/CCAI* mutants, I studied the mutants in combination with the methods to slow cytoplasmic translation listed above.

Deletion of the large ribosomal subunit *RPL20B* has been well documented to reduce cytoplasmic translation rates (Steffen et al., 2008). Deletion of *RPL20B* in glycerol slightly impaired growth of the *cca1-VI88S* mutant (Figure 3.12A), suggesting decreasing ribosome formation was possibly synthetic to mitochondrial function. Interestingly, a slight reduction of eIF2 α -P was observed when *RPL20B* was deleted in the *cca1-VI88S* (Figure 3.12B); this was not observed for *TRNT1* (Figure 13). The deletion of *RPL6B*, another ribosomal protein shown to suppress mitochondrial degeneration (Wang et al., 2008a), also did not suppress the *cca1-VI88S* growth phenotype (Figure 3.12C).

Deletion of *TOR1* nor *SCH9* (yeast S6 Kinase) did not change the growth phenotype of *cca1-VI88S* (Figure 3.14), and deletion of *SCH9* did not reduce eIF2 α -P in *cca1-VI88S* (Figure 3.12B). This suggests that the eIF2 α -P slowing of cytoplasmic translation impacts mitochondrial translation differently than caloric restriction or alteration of TOR signaling (Steffen et al., 2008), mitochondrial stress from chaperone deletion (Schleit et al., 2013), or reduced mitochondrial membrane potential (Wang et al., 2008a).

I used low doses of cycloheximide, an inhibitor of cytoplasmic translation elongation, to slow cytoplasmic translation in a titratable manner. In the presence of low doses of cycloheximide, *cca1-VI88S* growth was inhibited under non-respiring conditions

Figure 3.12.

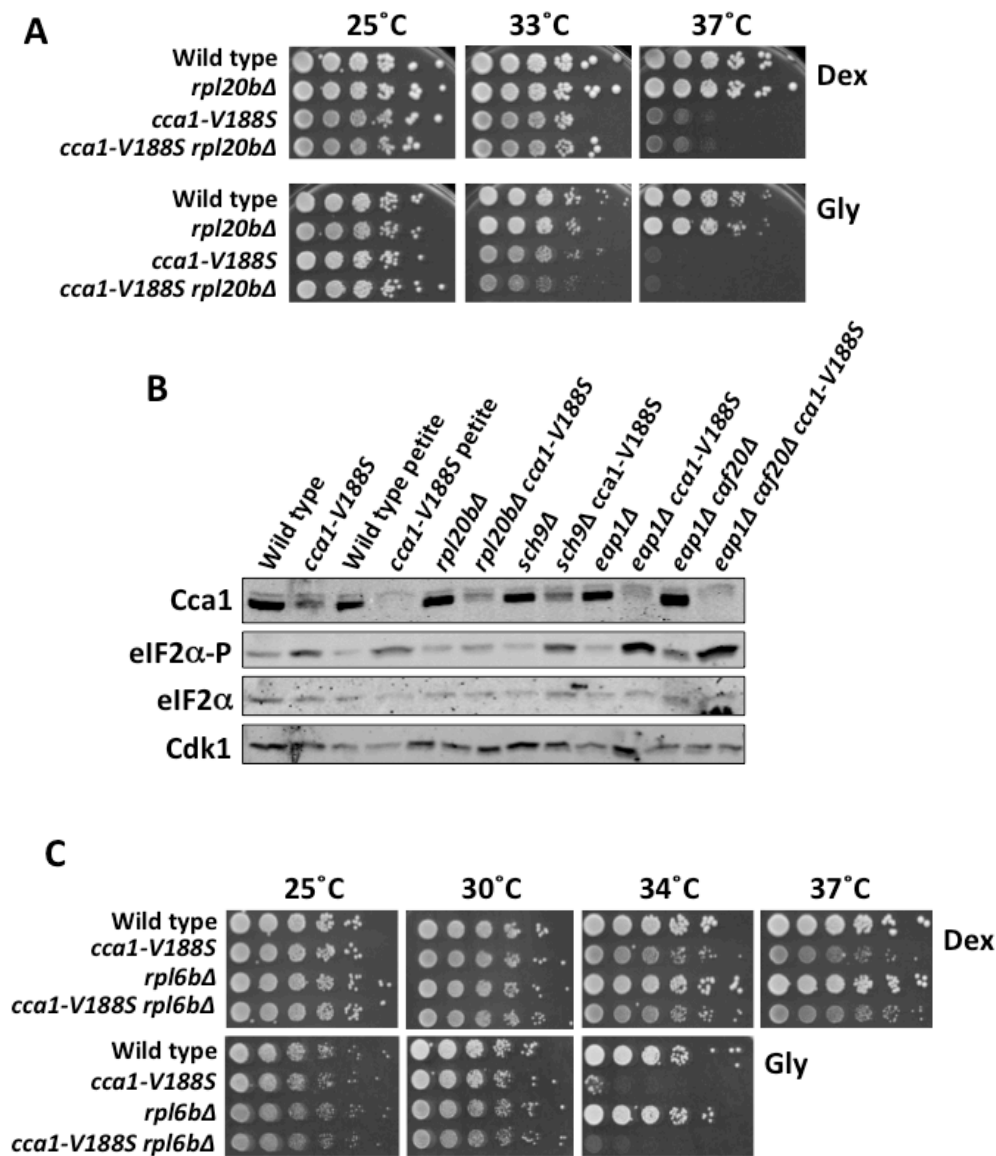


Figure 3.12. Reduction of cytoplasmic translation by alteration of the ribosome did not alter the *cca1-V188S* phenotype.

(A) Deletion of *RPL20b* did not impact the growth phenotype of *cca1-V188S*. Ten-fold serial dilutions of wild type (ADR22), *rpl20b* Δ (EKK134), *cca1-V188S* (EKK130), and *cca1-V188S rpl20b* Δ (EKK156) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates and grown at the indicated temperatures for 2 to 4 days.

(B) No change in eIF2 α -P in *cca1-V188S* in *rpl20b* Δ , but increased eIF2 α -P with *eap1* Δ . Wild type (ADR22), *cca1-V188S* (EKK130), wild type petite (EKK275), *cca1-V188S* petite (EKK273), *rpl20b* Δ (EKK134), *rpl20b* Δ *cca1-V188S* (EKK156), *sch9* Δ (EKK234), *sch9* Δ *cca1-V188S* (EKK236), *eap1* Δ (EKK238), and *eap1* Δ *cca1-V188S* (EKK240) strains were grown overnight in YEP dextrose at 25 °C to 2 to 4x10⁷ cells/mL, diluted and grown at 37°C for three hours to 1 to 2x10⁷ cells/mL. 3x10⁷ cells were pelleted and frozen in liquid nitrogen; samples were mechanically lysed in 1X sample buffer and analyzed by western blot analysis.

(C) Deletion of *RPL6B* did not impact the growth phenotype of *cca1-V188S*. Ten-fold serial dilutions of wild type (ADR9360), *cca1-V188S* (ADR9214) (*rpl6b* Δ from the deletion collection, tetra isolates not kept) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates and grown at the indicated temperatures for 2 to 4 days.

Figure 3.13.

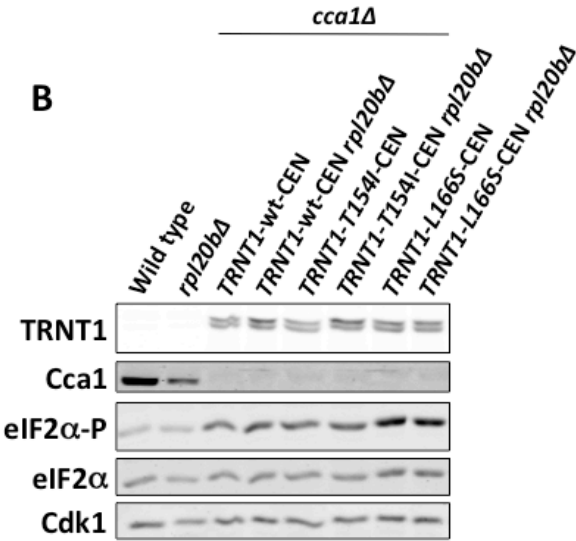
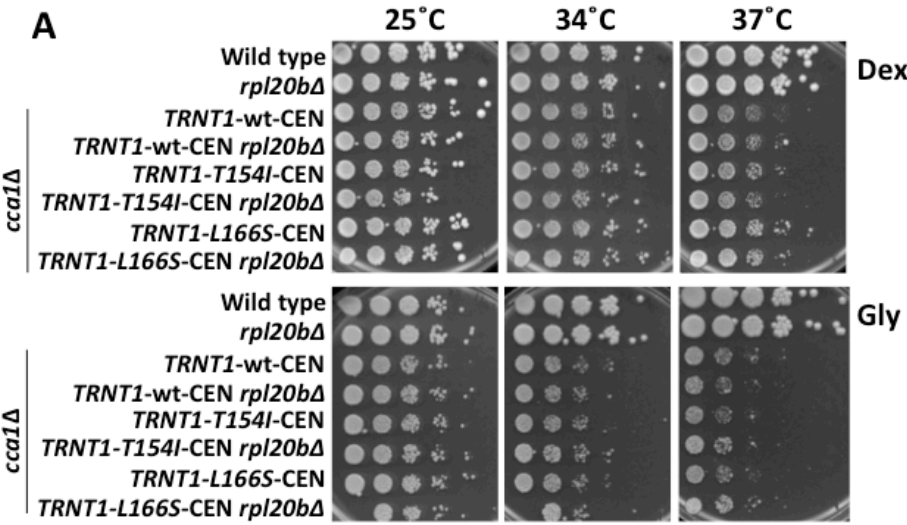


Figure 3.13. Reduction of cytoplasmic translation by deletion of *RPL20b* did not change the *TRNT1* wild type or mutant growth phenotypes or eIF2 α -P.

(A) Deletion of *RPL20B* did not impact the growth phenotype of *TRNT1* wild type or mutants. Ten-fold serial dilutions of wild type (ADR22), *rpl20b* Δ (EKK134), *cca1* Δ *TRNT1*-wt-CEN (EKK100), *cca1* Δ *TRNT1*-wt-CEN *rpl20b* Δ (EKK220), *cca1* Δ *TRNT1*-*T154I*-CEN (EKK332), *cca1* Δ *TRNT1*-*T154I*-CEN *rpl20b* Δ (EKK222), *cca1* Δ *TRNT1*-*L166S*-CEN (EKK334), and *cca1* Δ *TRNT1*-*L166S*-CEN *rpl20b* Δ (EKK224) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates and grown at the indicated temperatures for 2 to 4 days.

(B) No change in eIF2 α -P in *TRNT1* wild type or mutants in *rpl20b* Δ . Wild type (ADR22), *rpl20b* Δ (EKK134), *cca1* Δ *TRNT1*-wt-CEN (EKK100), *cca1* Δ *TRNT1*-wt-CEN *rpl20b* Δ (EKK220), *cca1* Δ *TRNT1*-*T154I*-CEN (EKK332), *cca1* Δ *TRNT1*-*T154I*-CEN *rpl20b* Δ (EKK222), *cca1* Δ *TRNT1*-*L166S*-CEN (EKK334), *cca1* Δ *TRNT1*-*L166S*-CEN *rpl20b* Δ strains were grown overnight in YEP dextrose at 25°C to 2 to 4x10⁷ cells/mL, diluted and grown 1 to 2x10⁷ cells/mL. 3x10⁷ cells were pelleted and frozen in liquid nitrogen; samples were mechanically lysed in 1X sample buffer and analyzed by western blot analysis.

Figure 3.14.

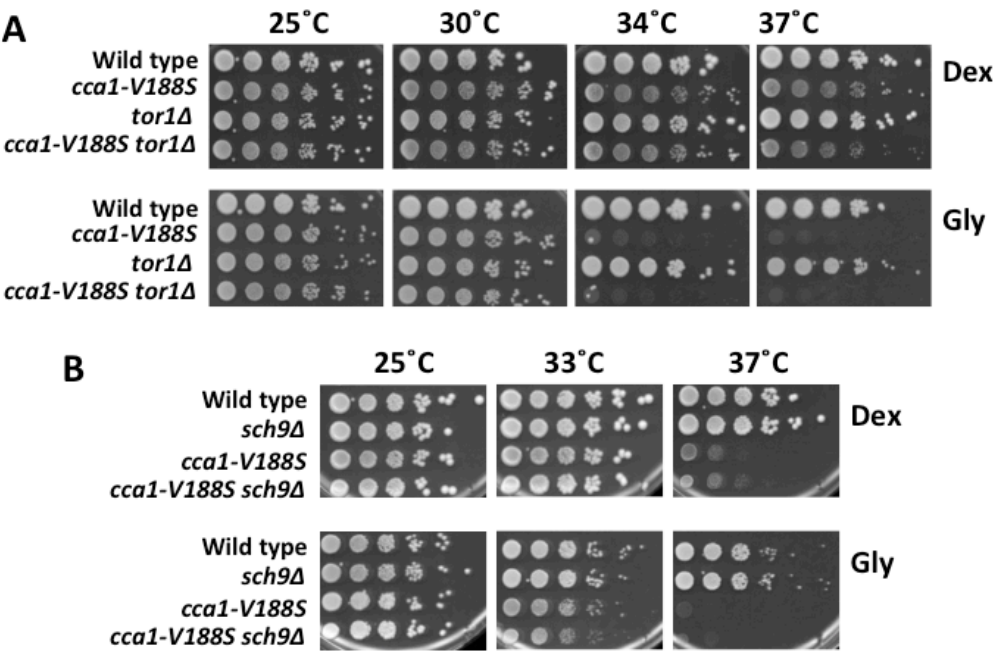


Figure 3.14. Deletion of *TOR1* nor *SCH9* did not change the growth phenotype of *cca1-V188S*

(A) Deletion of *TOR1*. Ten-fold serial dilutions of wild type (ADR9360), *cca1-V188S* (ADR9214), (*tor1* Δ from the deletion collection, tetra isolates not kept) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates and grown at the indicated temperatures for 2 to 4 days.

(B) Deletion of *SCH9*. Ten-fold serial dilutions of wild type (ADR22), *sch9* Δ (EKK234), *cca1-V188S* (EKK130), *cca1-V188S sch9* Δ (EKK236) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates and grown at the indicated temperatures for 2 to 4 days.

(Dex 37°C), but not in respiring conditions (Gly 33.5°C) (Figure 3.15A). This suggests that slowing cytoplasmic translation was synthetic when mitochondrial translation, and subsequently translation homeostasis, was not required. In the *TRNT1* wild type and *TRNT1-L166S* mutant, low doses of cycloheximide were synthetic under non-respiring conditions (Dex 37°C) but suppressive under respiratory conditions (Gly 34°C) (Figure 3.15B). This is consistent with slowing cytoplasmic translation being suppressive when high rates of mitochondrial translation, and subsequent translation homeostasis, are required. Using multiple doses of cycloheximide enabled me to find concentrations that inhibited the cytoplasmic ribosomes to the benefit of *TRNT1-wt* and *TRNT1-L166S* under respiring conditions. This variability of dose was possible with ribosome (*rpl20bΔ*, *rpl6bΔ*) or metabolic mutants (*tor1Δ*, *sch9Δ*) and may be why suppressive and synthetic effects were observed with cycloheximide and not other methods to slow cytoplasmic translation.

3.3.7. Increase in cytoplasmic translation

Decreasing cytoplasmic translation by eIF2 α -P or drug inhibition was suppressive to the *TRNT1/CCA1* mutants when respiration was required but synthetic under non-respiring conditions. Translation can also be regulated by sequestering of eIF4E (encoded by CDC33 in yeast) by eIF4E binding proteins (4E-BPs, *EAP1* and *CAF20* in yeast), preventing eIF4E from binding eIF4G which is required for 48S subunit formation (Dever et al., 2016; Sonenberg & Hinnebusch, 2009). A deletion of *EAP1* would increase availability of eIF4E increasing cap-dependent translation. 4E-BPs inactivated by TOR regulated phosphorylation to increase translation in times of nutrient availability (Gingras et al., 1998; Sonenberg & Hinnebusch, 2009).

Figure 3.15

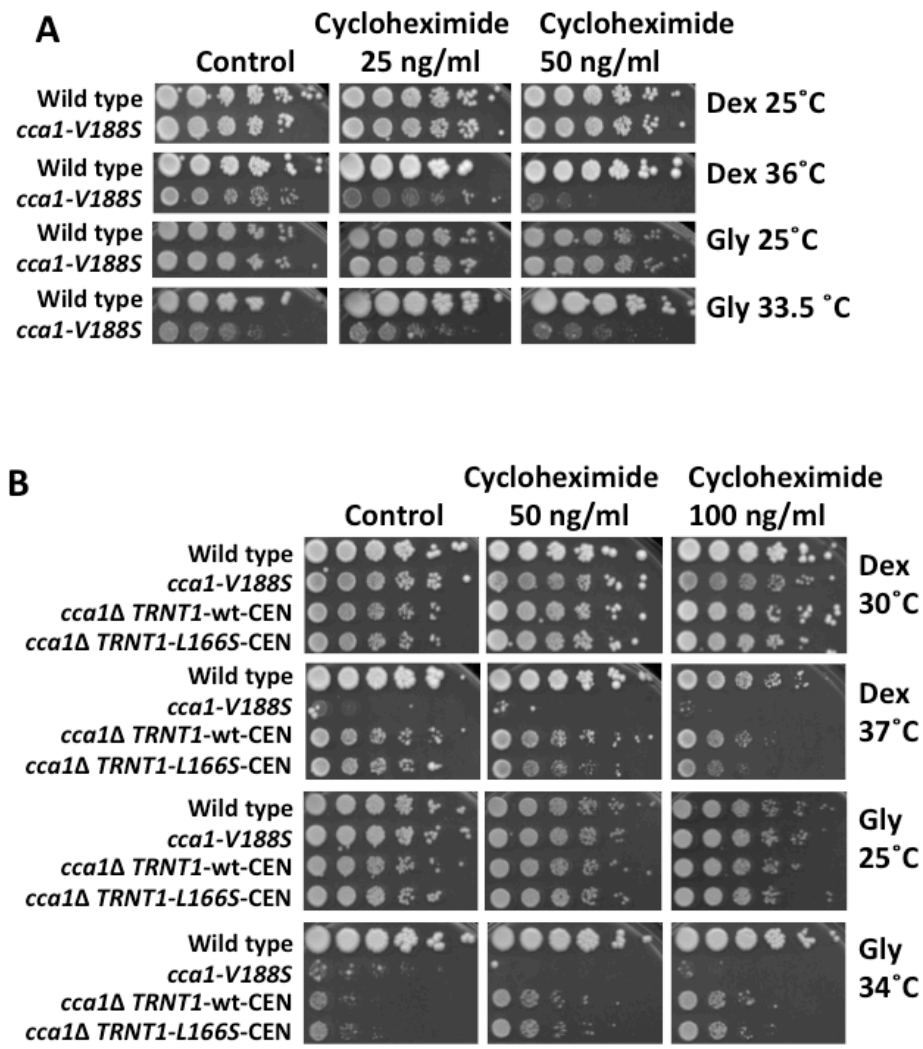


Figure 3.15. Slowing cytoplasmic translation was only suppressive when high rates of mitochondrial translation are required

(A) Slowing cytoplasmic translation in *cca1-V188S* was synthetic under non-respiratory conditions. Ten-fold serial dilutions of wild type (ADR4006) and *cca1-V188S* (EKK130) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates containing 0, 25, or 50 ng/ml cycloheximide and grown at the indicated temperatures for 2 to 6 days.

Cycloheximide plates were made fresh one day prior to use and stored in the dark.

(B) Slowing cytoplasmic translation in *TRNT1* wild type and mutants was synthetic under non-respiratory conditions but suppressive under respiratory conditions. Ten-fold serial dilutions of wild type (ADR4006), *cca1-V188S* (EKK130), *cca1Δ TRNT1*-wt-CEN (EKK100), *cca1Δ TRNT1-L166S*-CEN (EKK334) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates containing 0, 25, or 50 ng/ml cycloheximide and grown at the indicated temperatures for 2 to 6 days. Cycloheximide plates were made fresh one day prior to use and stored in the dark.

cca1-V188S has a synthetic interaction with *eap1Δ*, observed regardless of the presence of Caf20 (Figure 3.16A). As observed previously, addition of *sui2-S51A* to *cca1-V188S eap1Δ* was suppressive under non-respiring conditions (Dex 36°C) and synthetic under respiring conditions (Gly 32°C) (Figure 3.16A). Deletion of *EAP1* in the *cca1-V188S* background also results in increased eIF2α-P (Figure 3.12B). This suggests that the increased lethality in *cca1-V188S eap1Δ* was caused by the further increase in eIF2α-P on non-respiration requiring conditions, and inhibiting this phosphorylation in the *sui2-S51A cca1-V188S eap1Δ* suppressed some of this lethality (Figure 3.16A Dex 36°C), providing support to the synthetic affect of eIF2α-P when mitochondrial translation is not required. *TRNT1* wild type and mutants also have synthetic interactions with *eap1Δ*, consistent with results observed for *cca1-V188S* (Figure 3.16B).

3.3.8. Suppression of *TRNT1/CCA1* mutations

While examining various stress conditions, it was found that growth phenotype of *TRNT1/CCA1* mutants was suppressed when exposed to high osmolarity (Figure 3.17A). Petite cells have been shown to have altered cell wall composition leading to increased cell lysis and decreased resistance to calcofluor white, both of which are suppressed by high osmolarity (Wauters et al., 2001; Zaamoun et al., 1995). Mutations in nucleic acid metabolism and mitochondrial function have been associated with changes in cell wall biosynthesis and architecture in yeast (Evans et al., 1980; Lussier et al., 1997; Zhong et al., 2005). There are two main pathways involved in osmotic stress detection: the Cell Wall Integrity (CWI) pathway to cope with cell wall instability and the High Osmolarity Glycerol (HOG) pathway to survive hyperosmotic stress via glycerol production and transport coordination (Rodríguez-Peña et al., 2010). High osmolarity (1M sorbitol, 0.4M KCl)

Figure 3.16.

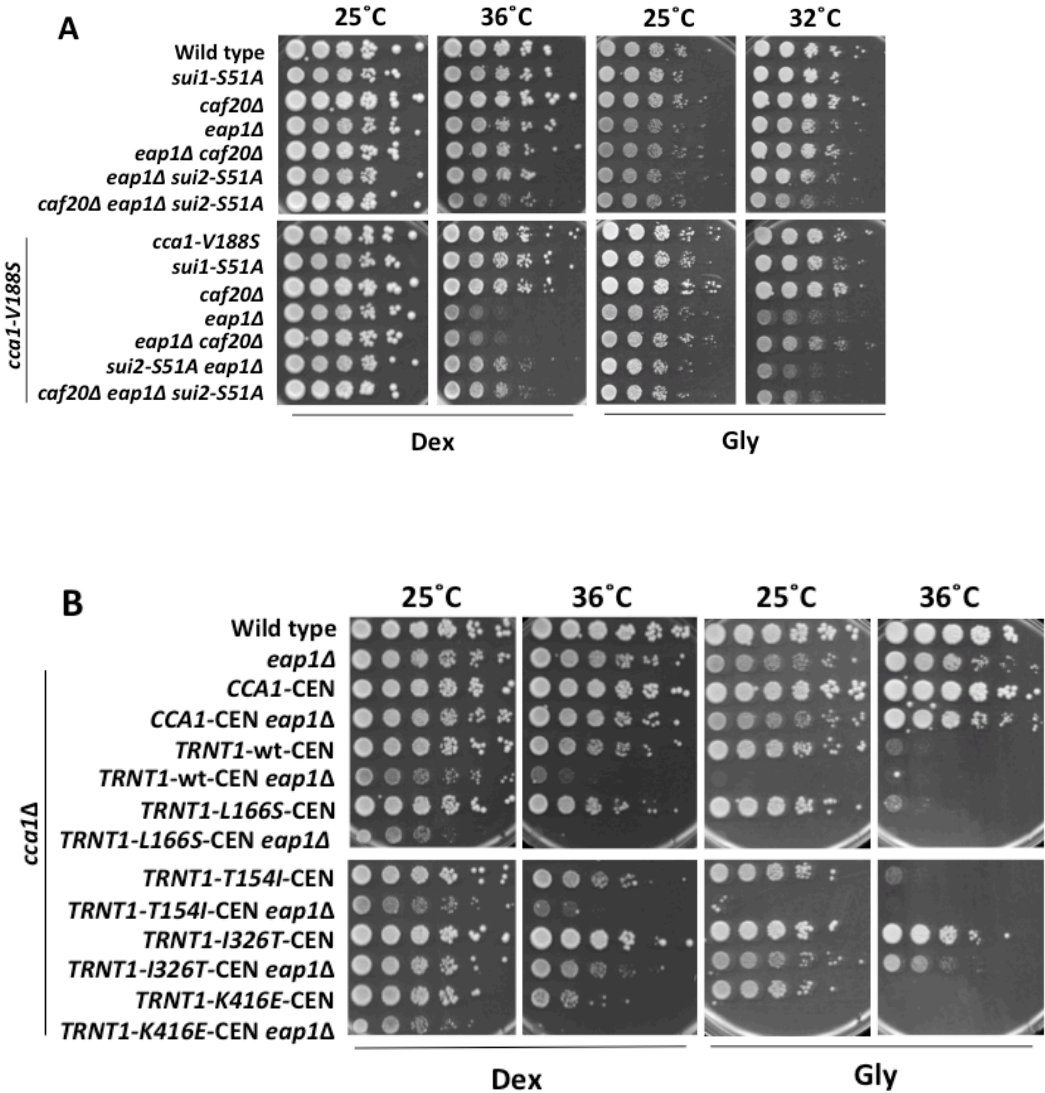


Figure 3.16. *cca1-V188S* and *TRNT1* wild type and mutants have a synthetic interaction with *eap1Δ*.

(A) *cca1-V188S* was synthetic with *eap1Δ* and addition of *sui2-S51A* to *cca1-V188S eap1Δ* was suppressive under non-respiring and synthetic under respiring conditions.

Ten-fold serial dilutions of wild type (ADR22), *sui2-S51A* (EKK144), *caf20Δ* (EKK265), *eap1Δ* (EKK238), *eap1Δ caf20Δ* (EKK316), *eap1Δ sui2-S51A* (EKK384), *caf20Δ eap1Δ sui2-S51A* (EKK400), *cca1-V188S* (EKK130), *cca1-V188S sui2-S51A* (EKK247), *cca1-V188S caf20Δ* (EKK267), *cca1-V188S eap1Δ* (EKK240), *cca1-V188S eap1Δ caf20Δ* (EKK318), *cca1-V188S eap1Δ sui2-S5A* (EKK382), and *cca1-V188S caf20Δ eap1Δ sui2-S51A* (EKK398) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates and grown at the indicated temperatures for 2 to 4 days.

(B) *TRNT1* wild type and mutants are have a synthetic interaction with *eap1Δ*. Ten-fold serial dilutions of wild type (ADR4006), *eap1Δ* (EKK238), *cca1Δ TRNT1*-wt-CEN (EKK100), *cca1Δ TRNT1*-wt-CEN *eap1Δ* (EKK463), *cca1Δ TRNT1-T154I*-CEN (EKK332), *cca1Δ TRNT1-T154I*-CEN *eap1Δ* (EKK465), *cca1Δ TRNT1-L166S*-CEN (EKK334), *cca1Δ TRNT1-L166S*-CEN *eap1Δ* (EKK467), *cca1Δ TRNT1-I326T*-CEN (EKK332), *cca1Δ TRNT1-I326T*-CEN *eap1Δ* (EKK469), *cca1Δ TRNT1-K416E*-CEN (EKK334), and *cca1Δ TRNT1-K416E*-CEN *eap1Δ* (EKK471) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates and grown at the indicated temperatures for 2 to 4 days. Unsheltered strains not kept due to decreased fitness, strain names here are sheltered with *CCA1*-CEN-*URA3*.

Figure 3.17.

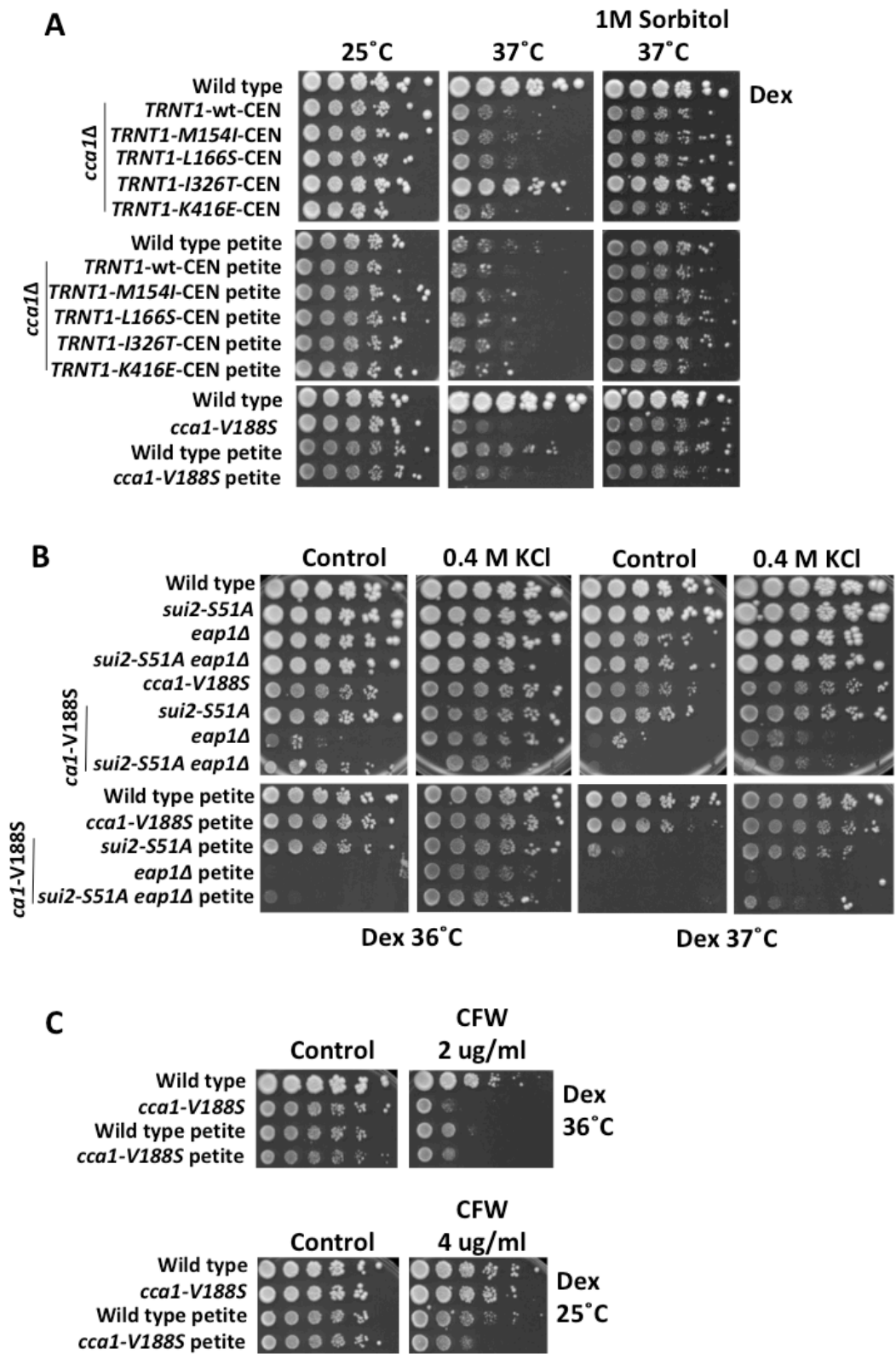


Figure 3.17. High osmolarity suppresses *TRNT1/CCA1* mutant growth phenotypes.

(A) High osmolarity suppresses growth phenotypes in mutants and petites. Ten-fold serial dilutions of wild type (ADR22), wild type petite (EKK275), *cca1Δ TRNT1*-wt-CEN (EKK100), *cca1Δ TRNT1*-wt-CEN petite (EKK277), *cca1Δ TRNT1-T154I*-CEN (EKK332), *cca1Δ TRNT1-T154I*-CEN petite (EKK344), *cca1Δ TRNT1-L166S*-CEN (EKK334), *cca1Δ TRNT1-L166S*-CEN petite (EKK346), *cca1Δ TRNT1-I326T*-CEN (EKK336), *cca1Δ TRNT1-I326T*-CEN petite (EKK348), *cca1Δ TRNT1-K416E*-CEN (EKK338), and *cca1Δ TRNT-K416E*-CEN petite (EKK350) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates and grown at the indicated temperatures for 2 to 4 days.

(B) High osmolarity suppresses growth phenotypes in mutants and petites of *cca1-V188S* combined with additional mutations. Ten-fold serial dilutions of wild type (ADR22), *sui2-S51A* (EKK144), *eap1Δ* (EKK238), *sui2-S51A eap1Δ* (EKK384), *cca1-V188S* (EKK130), *cca1-V188S sui2-S51A* (EKK247), *cca1-V188S eap1Δ* (EKK240), *cca1-V188S sui2-S51A eap1Δ* (EKK382), wild type petite (EKK275), *cca1-V188S* petite (EKK273), *cca1-V188S sui2-S51A* petite (EKK352), *cca1-V188S eap1Δ* petite (EKK358), and *cca1-V188S sui2-S51A eap1Δ* petite (EKK427) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) and grown at the indicated temperatures for 2 to 4 days.

(C) *cca1-V188S* and petites have cell wall defects increased by calcofluor white. Ten-fold serial dilutions of wild type (ADR22), *cca1-V188S* (EKK130), wild type petite (EKK275), *cca1-V188S* petite (EKK273) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) with 0, 2, or 4 ug/mL of calcofluor white, then grown at the indicated temperatures for 2 to 4 days. Plates with calcofluor white were made the day prior to use and stored in the dark.

suppresses defects in the CWI pathway (Levin, 2005) and induces the HOG pathway (Brewster et al., 1993; Hohmann, 2002). Previous studies identified that mutations in the HOG pathway suppressed mitochondrial-induced cell wall defects by reducing interior turgor pressure (Zhong et al., 2005; Zhou et al., 2009).

Double mutants of *cca1-VI88S* were all rescued with high osmolarity, including *eap1Δ*, *caf20Δ*, and *sui2-S51A* (Figure 3.17B). At 37°C, the *cca1-VI88S eap1Δ* petite strain was not rescued by high osmolarity, although the addition of *sui2-S51A* to this background was suppressive, supporting the synthetic role of eIF2α-P in non-respiratory conditions (Figure 3.17B). Cell wall defects are shown in *cca1-VI88S* by sensitivity to calcofluor white at increased temperature (Figure 3.17C). *cca1-VI88S* did not show increased cell lysis compared to wild type at 25°C, conditions used in this analysis and under which eIF2α-P was not induced (Figure 3.18). These results suggest defects in cell wall integrity result from *TRNT1/CCA1* mutations as observed in other mitochondrial mutants (Evans et al., 1980; Lussier et al., 1997; Wauters et al., 2001; Zaamoun et al., 1995; Zhong et al., 2005).

Negative regulation by protein phosphatases Ptp2 and Ptp3 turn off both the HOG pathway and CWI pathway, with Ptp2 having a greater role than Ptp3 (Mattison et al., 1999; Young et al., 2002). Deletion of *PTP2* with *cca1-VI88S* slightly suppressed the growth phenotype under respiration requiring conditions but deletion of *PTP3* with *cca1-VI88S* had no change (Figure 3.19A). Deletion of both phosphatases with *cca1-VI88S* was synthetic in non-respiration requiring conditions at high temperature and partially suppressive in respiration requiring conditions (Figure 3.19A). Deletion of HOG pathway components (*PBS2*, *SKO1*, or *SHO1*) did not alter the growth defect of *cca1-VI88S* cells (Figure 3.19B, C). This data suggests that while cell wall defects occur in *cca1-VI88S*, they are not rescued by modifications to CWI and HOG pathways.

Figure 3.18.

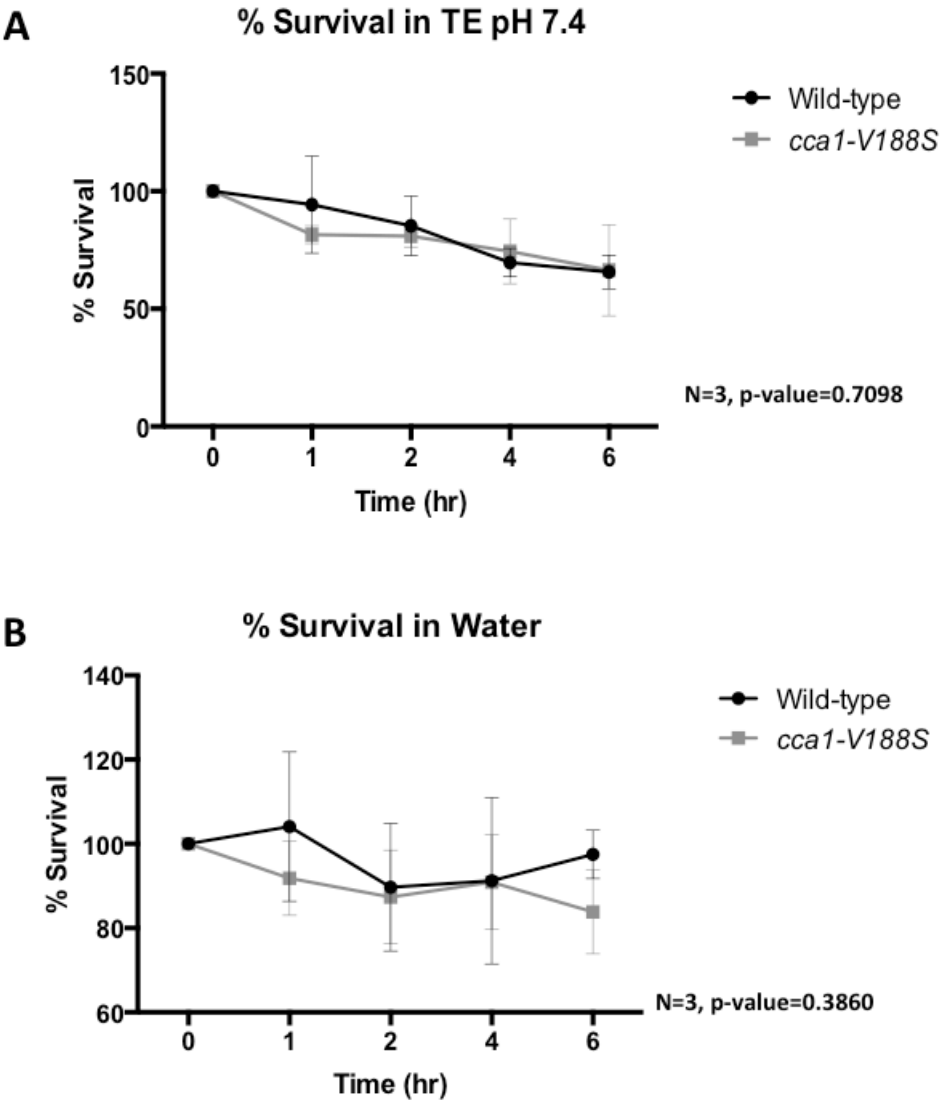


Figure 3.18. *ccaI-V188S* do not show increased lysis at 25°C in water or Tris-EDTA (TE). Wild type (ADR4006), *ccaI-V188S* (EKK130) were grown overnight in YEP dextrose at 25°C to 2 to 4×10^7 cells/mL, diluted and grown to 1 to 2×10^7 cells/mL. From this dilute culture cells were washed twice with TE (**A**) or water (**B**) and resuspended in TE or water for 1, 2, 4, 6 hrs. Samples were measured by OD for concentration and plated at approximately 300 colonies per plate on YEP dextrose. Colony forming units (cfu) were counted by scanning the plates on a flat-bed scanner and counting single colonies using ImageJ software. Data is of three replicates with error bars representing SEM.

Figure 3.19.

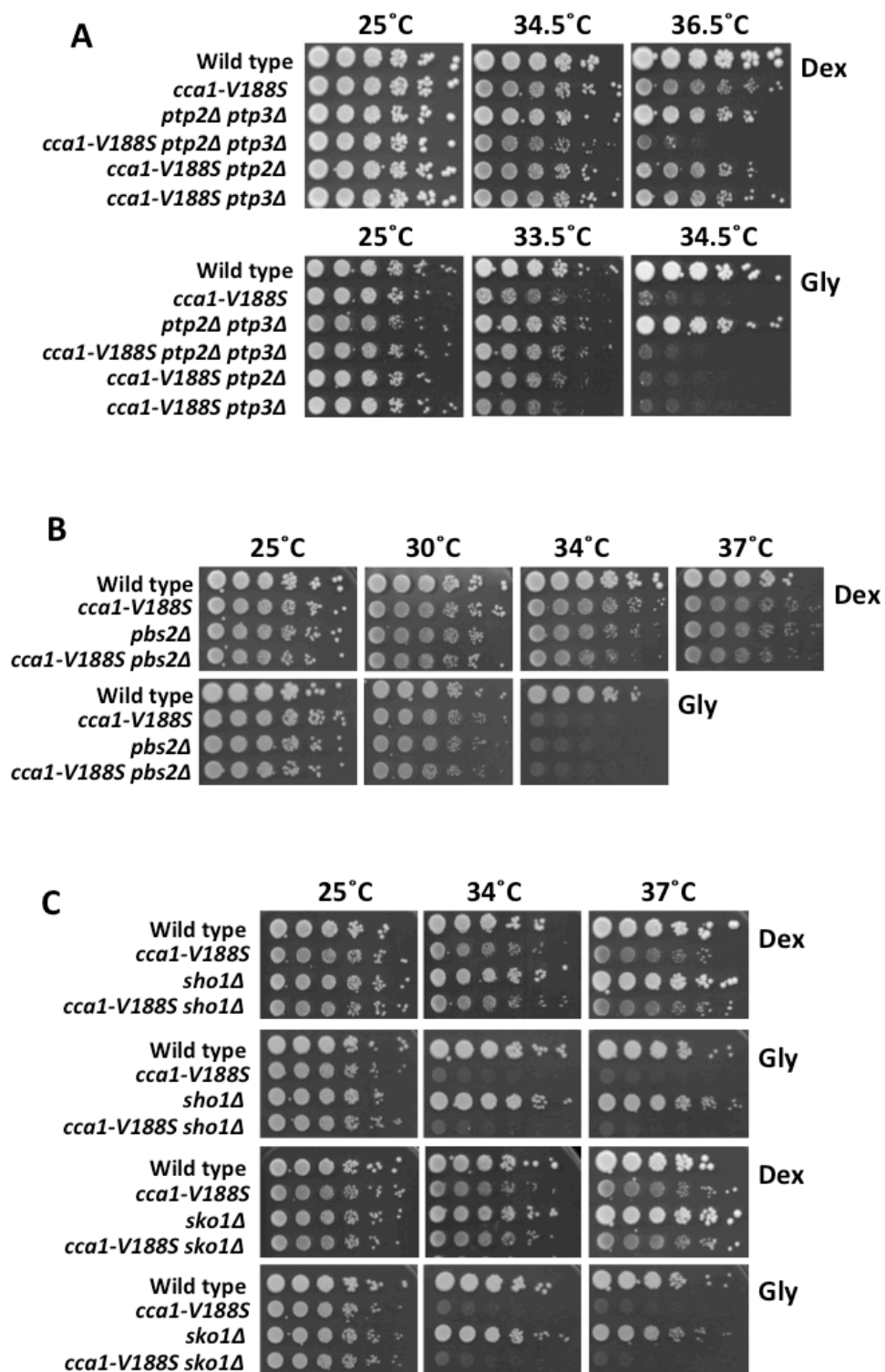


Figure 3.19. While cell wall defects occur in *cca1-V188S*, they are not rescued by changing CWI and HOG signaling

(A) Deletion of *PTP2* and *PTP3* was synthetic to *cca1-V188S* growth. Ten-fold serial dilutions of wild type (ADR4006), *cca1-V188S* (EKK130), *ptp2Δ ptp3Δ* (EKK501), *cca1-V188S ptp2Δ ptp3Δ* (EKK503), *cca1-V188S ptp2Δ* (EKK451), and *cca1-V188S ptp3Δ* (EKK455) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates and grown at the indicated temperatures for 2 to 4 days.

(B) Deletion of *PBS2*, *SHO1*, or *SKO1* of the HOG pathway did not impact *cca1-V188S* growth. Ten-fold serial dilutions of wild type (ADR9360) and *cca1-V188S* (ADR9214) (*pbs2Δ*, *sho1Δ*, and *sko1Δ* from the deletion collection, tetra isolates not kept) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates and grown at the indicated temperatures for 2 to 4 days.

3.4. Discussion

Defects in TRNT1 function may result in the observed mitochondrial phenotypes because: (1) defective cytoplasmic tRNA 3'CCA addition impairs cytoplasmic translation and reduces levels of key nuclear-encoded mitochondrial proteins; (2) defective mt-tRNA 3'CCA addition reduces mitochondrial translation and possibly impairs OXPHOS leading to reduced ATP production; or (3) the loss of homeostasis between the cytoplasmic and mitochondrial translation systems results in a cellular stress response and aberrant signaling contributes to the observed pathobiology.

The data presented in this study support hypothesis 2 and 3, where the phenotypes observed result from both impaired mitochondrial translation and stress response signaling. I show that *TRNT1/CCAI* mutations result in mitochondrial dysfunction, impaired mitochondrial translation, and induce a mitochondrial-dependent stress response (eIF2 α -P). In *TRNT1/CCAI* mutants when impaired mitochondrial translation was required (respiring conditions), the slowing of cytoplasmic translation by eIF2 α -P was suppressive, suggesting a homeostasis requirement between the two translation systems. When mitochondrial translation was not required (non-respiring conditions), this eIF2 α -P stress response was synthetic and slows growth unnecessarily; this may extend to patient cell types where OXPHOS requirements are low and aberrant signaling may contribute to the observed pathobiology. In cell types requiring greater amounts of mitochondrial translation, the impairment of TRNT1/Cca1 function may result in an imbalance of translation homeostasis and balancing mitochondrial and cytoplasmic translation could be a therapeutic target for SIFD.

3.4.1. Mutations in *TRNT1/CCA1* impair mitochondrial function

TRNT1/CCA1 mutants in yeast were sensitive to growth on non-respiratory carbon sources indicating impaired mitochondrial ETC required for respiration, as observed in patient cells (Figure 3.2) (Liwak-Muir et al., 2016; Sasarman et al., 2015; Wedatilake et al., 2016). Patient fibroblasts with *TRNT1* mutations were also found to have increased ROS production; damaged mitochondria result in increased intracellular ROS (Giannelou et al., 2018). Consistent with these observations, I show that *TRNT1/CCA1* mutants were sensitive to increased reactive oxygen species or loss of cytoplasmic or mitochondrial oxygen radical scavengers (Figure 3.3). Mitochondrial function is required for ROS stress resistance, supporting the previously concluded mitochondrial dysfunction occurring in *TRNT1/CCA1* mutants (Thorpe et al., 2004).

Suppression of *TRNT1/CCA1* mutant growth defect was observed under high osmolarity and mutants were sensitive to cell wall drugs, suggesting defects in cell wall stability, a phenotype associated with mitochondrial dysfunction (Figure 3.17) (Aviner et al., 2014; Segev & Gerst, 2018; Wauters et al., 2001; Zaamoun et al., 1995). Previous studies identified deletions in the HOG pathway that suppressed mitochondrial-induced cell wall defects by reducing interior turgor pressure (Zhong et al., 2005; Zhou et al., 2009). None of the deletions in the HOG pathway tested in this study changed the growth of *cca1-V188S*, indicating decreasing cytoplasmic pressure was not suppressive as observed in other mitochondrial mutants (Figure 3.19B) (Zhong et al., 2005; Zhou et al., 2009). Changes in CWI and HOG pathway regulation was also not suppressive, although these results may be difficult to interpret due to the dual functions of Ptp2 and Ptp3 in both opposing pathways (Figure 3.19A). Therefore, although high osmolarity was suppressive to *TRNT1/CCA1*

mutants, which is consistent with mitochondrial dysfunction as the cause of the observed phenotypes, the mechanism by which this functions has not been determined.

3.4.2. *TRNT1/CCA1* mutants induce eIF2 α -P and impair cytoplasmic translation

Since the analogous *CCA1* mutants were only mildly temperature sensitive, I decided to study the acute loss of Cca1 with an auxin inducible degron (AID). To assess cytoplasmic translation, I compared the amount of eIF2 α -P to total eIF2 α , a standard measure of cytoplasmic translation inhibition (Holcik & Sonenberg, 2005). Interestingly, the induction of eIF2 α -P occurred prior to polysome loss under acute degradation of Cca1 (Figure 3.4C, D), indicating that although the bulk cytoplasmic protein translation was unchanged, a stress response had been induced. Further degradation of Cca1 resulted in the expected loss of polysomes (Figure 3.4E, F), this as unsurprising given the central role of Cca1 in translation. Induction of eIF2 α -P occurred in both *cca1-V188S* and degron petite cells (Figure 3.8C). This requires further work because eIF2 α -P was not measured at 1 hr post auxin when previously shown to occur prior to polysome loss. At 2 hrs, this eIF2 α -P was likely from lack of 3' CCA on cytoplasmic tRNA, resulting in accumulation of uncharged tRNA and activation of Gcn2. More detailed analysis of the degron is needed to determine if the eIF2 α -P is affected in petite cells. This data is in support of hypothesis 1, which proposes defective cytoplasmic tRNA 3'CCA addition impacts cytoplasmic translation, although the role of this in mitochondrial dysfunction is unknown.

3.4.3. Mutations in *TRNT1/CCA1* impair mitochondrial translation

Given the affect on cytoplasmic translation by *TRNT1/CCA1* mutations, we assessed mitochondrial translation using *in vivo* radiolabeling of newly translated mitochondrial

encoded proteins. *TRNT1/CCAI* mutants had altered mitochondrial translation, with translation rates of Cob and Cox3 constant between strains, but Var1, Cox1, and Cox2, and Atp6 translation was reduced in the mutants (Figure 3.5). Multiple studies have shown defects in mitochondrial and cytoplasmic 3'CCA addition due to mutations in *TRNT1* (Sasarman et al., 2015; Wedatilake et al., 2016; DeLuca et al., 2016; Giannelou et al., 2018), but changes in mitochondrial tRNA availability observed in other *TRNT1* studies do not account for the differences observed here. The tRNAs and cognate codons that are affected in other *TRNT1* studies are present in both unaffected and affected proteins of this study (Sasarman et al., 2015; Wedatilake et al., 2016). Furthermore, when accounting for mitochondrial tRNA abundance and usage, no major difference separated Cox1, Cox2, and Var1 to account for less translation compared to Cox3 and Cob (Figure 3.5A) (Turk et al. 2013). Additionally, I observed that Atp6 was not translated properly in the *CCAI* mutants, indicating altered mitochondrial translation. The size shift observed in Atp6 (Figure 3.5A) could be from stalling at a tryptophan residue from the TGG codon, the only TGG codon found in the mtDNA encoded proteins assessed. This codon is 16 amino acids from the stop, potentially accounting for the size difference. Further work is needed to verify that this smaller band is incorrectly translated Atp6 and not another protein product. This data is in support of hypothesis 2, which proposes defective mt-tRNA 3'CCA addition reduces mitochondrial translation.

3.4.4. Functional mitochondria are required for eIF2 α -P signaling

Induction of eIF2 α -P in *TRNT1* wild type and mutants was observed at 25°C when grown on dextrose (Figure 3.6A, 3.7A), conditions under which growth was not observed to be different from wild type cells (Figure 3.7D, 25°C). In agreement, *TRNT1-T154I*, *M158V*,

and *L166S* were found to have similar 3' CCA addition activity as *TRNT1* wild type at 25°C (Leibovitch et al., 2018). This supports that eIF2 α -P signaling was occurring in *TRNT1* strains with functional TRNT1 protein. Under these same conditions, the signaling of eIF2 α -P in *TRNT1* wild type and mutants was absent in petite cells (Figure 3.7A). Petite cells in this study were made using ethidium bromide exposure, resulting in the loss of mitochondrial DNA, the absence of mitochondrial DNA also results in no mitochondrial translation. The loss of increased eIF2 α -P in petite cells suggests that mitochondrial translation was required for this signaling. Additionally, differences in growth phenotype between petite wild type and *TRNT1* wild type and mutants were abolished in petite strains, indicating that *TRNT1* wild type and mutants are able to fully rescue cytoplasmic function in petite cells (Figure 3.7D, 37°C). This data does not support hypothesis 1, as petite *TRNT1* mutants can fully rescue the petite wild type growth and have similar eIF2 α -P levels, indicating that the *TRNT1* mutants can support the cytoplasmic-translated requirements. Additionally, this suggests that the stress response signal of increased eIF2 α -P requires mitochondrial translation, which supports hypotheses 2 and 3.

To differentiate between impaired OXPHOS (ATP production) and impaired mitochondrial translation, I generated a *cca1-V188S* mutant with an essential component of the electron transport chain deleted (*COQ5*), preventing OXPHOS. In terms of ATP production and OXPHOS de-regulation, the *coq5* Δ is petite, but mitochondrial translation functions normally. Unlike in the ethidium bromide (no mitochondrial translation) *TRNT1* petites that had similar growth to the wild type petite (Figure 3.7D), *coq5* Δ (no OXPHOS, no ATP production) *cca1-V188S* petites had poorer growth than *coq5* Δ alone (Figure 3.9A). This suggest that the stress response signaling from the *cca1-V188S* mutant does not function through OXPHOS or lack of ATP production, but that defective mitochondrial

translation was likely the cause of the *ccal-V188S* phenotypes. This supports hypothesis 2, specifically that impaired mitochondrial translation and not impaired OXPHOS or ATP production results in the observed phenotype.

I also generated a *ccal-V188S* mutant with a strain lacking *PUS1*, an enzyme responsible for a tRNA stabilization modification whose depletion impacts mitochondrial tRNA greater than cytoplasmic tRNA (Chen & Patton, 1999; Foster et al., 2000; Motorin et al., 1998; Spenkuch et al., 2014). A synthetic interaction with *Pus1* suggests that additional impairment of tRNA metabolism or stability was synthetic, in both non-respiratory and respiratory conditions (Figure 3.9B). Mitochondrial tRNA with the lack of canonical structure are more dependent on base modifications for structural stabilization, as seen in *pus1Δ* reducing pseudouridines in both cytoplasmic and mitochondrial tRNAs but only impacting mitochondrial translation (Bykhovskaya et al., 2004; Helm, 2006; Patton et al., 2005). Defects in mitochondrial translation and further increase of growth defects by decreasing tRNA stability (*pus1Δ*), suggest that *TRNT1/CCAI* mutants have impaired mitochondrial translation due to a lack of mitochondrial tRNA 3'CCA addition (Figure 3.9B). This offers further support for hypothesis 2, which proposes defective tRNA function results in impaired mitochondrial translation.

I show, for the first time, that mutations in *TRNT1* result in mitochondrial translation defects and increased eIF2 α -P. I propose that eIF2 α -P reduces cytoplasmic translation in times of mitochondrial stress to reduce protein load and subsequently relieve stress on mitochondrial protein folding and complex assembly to prevent imbalance between cytoplasmic and mitochondrial proteins, separate from eIF2 α -P induction from uncharged tRNA as observed in *C. elegans* (Baker et al., 2012).

3.4.5. Decrease in cytoplasmic translation

When *cca1-V188S* was combined with a mutant eIF2 α that can not be regulated by phosphorylation (*sui2-S51A*), there was a suppressive effect under non-respiring conditions (Figure 3.11A Dex 37°C), indicating that the eIF2 α -P stress response signaling was synthetic to the cell when mitochondrial translation was not required; this was consistent in the *TRNT1* mutants. In contrast, the *sui2-S51A* mutation was synthetic under respiring conditions (Figure 3.11A Gly 33 °C), indicating that the eIF2 α -P stress response slowing cytoplasmic translation in times of mitochondrial dysfunction was suppressive to these cells. Additionally, *TRNT1/CCA1* mutant growth was improved by slowing cytoplasmic translation (cycloheximide) under respiring conditions when mitochondrial translation was required but worsened when mitochondrial translation was not required (Figure 3.15). These data are in support of hypothesis 3, where slowing of cytoplasmic translation was suppressive only when mitochondrial translation and homeostasis was required in *TRNT1/CCA1* mutants.

A lack of suppression by other methods to decrease translation (*rpl20b* Δ , *rpl6b* Δ , *tor1* Δ , *sch9* Δ) may indicate a fine tuning of translation repression not achieved by these mutations (Figure 3.12, 13, 14) (Cherkasova & Hinnebusch, 2003; Laplante & Sabatini, 2012; Schleit et al., 2013; Steffen et al., 2008; Wang et al., 2008a). This suggest that the slowing of cytoplasmic translation by low doses of cycloheximide was different from previously observed alteration of TOR signaling (Steffen et al., 2008), mitochondrial stress from chaperone deletion (yeast mtUPR) (Schleit et al., 2013), or reduced mitochondrial membrane potential (Wang et al., 2008a). When both translation systems are impacted, as found in the *TRNT1/CCA1* mutants, more sensitive adjustment may be required and only possible using different concentrations of cycloheximide. Furthermore, these methods of

reducing cytoplasmic translation were suppressive to dysfunctional mitochondrial that did not result from defects in mitochondrial translation. Because *TRNT1/CCAI* mutations impact mitochondrial translation, a global down regulation of OXPHOS proteins would be required, compared to up regulation of chaperones or members of specific ETC complexes as occurs in these mutants (Cherkasova & Hinnebusch, 2003; Laplante & Sabatini, 2012; Schleit et al., 2013; Steffen et al., 2008; Wang et al., 2008a). All of these factors may account for why phenotype differences were observed using cycloheximide but not other cytoplasmic translation modifications.

Petite cells made using ethidium bromide were not analyzed with cycloheximide due to upregulation of the pleiotropic drug resistance (PDR) pathway that occurs with the loss of mtDNA (Hallstrom & Moye-Rowley, 2000). The pleiotropic drug resistance (PDR) pathway is upregulated in response to drugs, heat shock, and mitochondrial dysfunction to enable tolerance to a wide range of toxic agents and anti-fungal drugs in yeast (Balzi & Goffeau, 1995; Hallstrom & Moye-Rowley, 2000; Prasad & Goffeau, 2012; Shahi et al., 2010). Not all petite mutations will up-regulate the PDR pathway, as deletion of *ATP2* (a nuclear-encoded component of the mitochondrial F1 ATPase) is OXPHOS incompetent but only slightly increases PDR induction and does not result in increased resistance to cycloheximide (Hallstrom & Moye-Rowley, 2000).

3.4.6. Increase of cytoplasmic translation

Loss of *EAP1* increases translation by removing the repression on eIF4E of the 48S ribosome formation for cap-dependent translation (Haghighat et al., 1995; Sonenberg & Hinnebusch, 2009). When *EAP1* was deleted in *TRNT1/CCAI* mutants, I observed a synthetic interaction under both respiratory and non-respiratory conditions (Figure 3.16).

This suggests that increasing in cytoplasmic translation was synthetic when combined with impaired TRNT1/Cca1 function, consistent with the suppressive effect of slowing cytoplasmic translation observed with cycloheximide and eIF2 α -P. As observed previously, inhibition of eIF2 α -P signaling by in *sui2-S51A* was suppressive to *cca1-V188S eap1 Δ* under non-respiratory conditions (Figure 3.16A Dex 36°C), showing the key role eIF2 α -P plays in *cca1-V188S*. This data is in support of hypothesis 3, where slowing is cytoplasmic translation is suppressive than increasing cytoplasmic translation would be synthetic when mitochondrial translation and homeostasis was required in *TRNT1/CCA1* mutants.

In response to various signals, TORC1 promotes translation through substrates such as ribosomal protein S6 kinase (Sch9 in yeast) and phospho-inhibition of 4E-BPs to promote translation (de al Cruz et al., 1997; Hara et al., 1998; Jastrzebski et al., 2007). In this analysis, deletion of *CAF20*, *TOR1*, or *SCH9* did not change the *cca1-V188S* growth phenotype (Figure 3.16A, 14), indicating the specificity of the *CCA1/TRNT1* and *EAP1* interaction.

3.4.7. Future work

The stress response pathway involved in sensing mutant *TRNT1/CCA1* mitochondrial dysfunction and resulting in eIF2 α -P has yet to be determined, although the TOR and RTG pathways are not involved. Further work needs to be done in the *coq5 Δ* mutant to differentiate between mitochondrial translation defects and stress resulting from impaired OXPHOS. This includes combining with TRNT1 mutants and assessment of eIF2 α -P levels. Analysis of components of the mPOS, amUPR, and yeast mtUPR-like pathways may indicate the signaling pathway and lead to future targets for SIFD therapies. The cause of suppression of the *TRNT1/CCA1* mutant growth defects by high osmolarity is still unknown

and future work in this area may also indicate potential therapeutic targets. Assessment of eIF2 α -P under high osmolarity conditions could be important to determine if mitochondrial function and cell wall impairment are linked or separate phenotypes.

Table 3.1. Yeast strains used in this analysis

Strains used in this study are derivatives of W303-1 (*ura3-1 leu2-3,112 trp1-1 his3-11 ase2-1 can1-100*), Y7092 (*MAT α can1 Δ ::STE2 pr -Sp $_$ his5 lyp1 Δ his3 Δ 1 leu2 Δ 0 ura3 Δ 0 met15 Δ 0*), or Y7096 from S288C (*MAT α his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0*) as indicated.

Strain	MAT	Background	Genotype
ADR22	α	W303-1	wild type
ADR941	a/ α	W303-1	wild type
ADR4006	α	W303-1	wild type <i>bar1Δ</i>
ADR6431	α	W303-1	<i>leu2::pGDP1-OsTIR1-LEU2</i>
ADR6511	α	W303-1	<i>cca1Δ::hphMX pADH1-CCA1-CEN-URA3</i>
ADR6791	α	W303-1	<i>cca1Δ::hphMX pADH1-TRNT1-CEN-TRP1</i>
ADR6793	α	W303-1	<i>cca1Δ::hphMX pADH1-TRNT1-T154I-CEN-TRP1</i>
ADR6834	α	W303-1	<i>pADH1-TRNT1-CEN-URA3</i>
ADR6836	α	W303-1	<i>pADH1-CCA1-CEN-URA3</i>
ADR6838	α	W303-1	<i>pADH1-empty-CEN-URA3</i>
ADR6840	α	W303-1	<i>cca1Δ::hphMX pADH1-CCA1-CEN-TRP1</i>
ADR6933	α	W303-1	<i>cca1Δ::hphMX pADH1-TRNT1-M158V-CEN-TRP1</i>
ADR6935	α	W303-1	<i>cca1Δ::hphMX pADH1-TRNT1-L166S-CEN-TRP1</i>
ADR6937	α	W303-1	<i>cca1Δ::hphMX pADH1-TRNT1-R190I-CEN-TRP1</i>

ADR6939	α	W303-1	<i>cca1Δ::hphMX pADHI-TRNT1-I223T-CEN-TRP1</i>
ADR6941	α	W303-1	<i>cca1Δ::hphMX pADHI-TRNT1-I326T-CEN-TRP1</i>
ADR7020	a/ α	W303-1	<i>cca1Δ::hphMX/CCA1</i>
ADR8264	a	W303-1	<i>CCA1-AID-kanMX pTIR-LEU2</i>
ADR8363	α	W303-1	<i>cca1Δ::hphMX pADHI-CCA1-T175I-CEN-TRP1</i>
ADR8365	α	W303-1	<i>cca1Δ::hphMX pADHI-CCA1-L179V-CEN-TRP1</i>
ADR8367	α	W303-1	<i>cca1Δ::hphMX pADHI-CCA1-V188S-CEN-TRP1</i>
ADR8369	α	W303-1	<i>cca1Δ::hphMX pADHI-CCA1-R220I-CEN-TRP1</i>
ADR8990	α	Y7096	<i>cca1Δi::hphMX pADHI-TRNT1-CEN-URA3</i>
ADR8992	α	W303-1	<i>cca1Δi::hphMX pADHI-TRNT1-CEN-URA3</i>
ADR9214	α	Y7096	<i>natMX-cca1-V188S</i>
ADR9215	α	Y7096	<i>natMX-BCK2 cca1-V188S</i>
ADR9296	α	W303-1	<i>glc7-10-TRP1, glc7Δ::LEU2, bar1Δ</i>
ADR9360	α	Y7096	wild type
EKK002	a	W303-1	<i>cca1-1 (cca1-ts-352)</i>
EKK003	a	W303-1	<i>CCA1-AID-kanMX pTIR-LEU2 petite</i>
EKK005	α	W303-1	<i>pTIR-TRP1</i>

EKK036	a	W303-1	<i>sui2Δi::hphMX Sui2-S51A-Cen-LEU2</i>
EKK100	α	W303-1	<i>cca1Δ::hphMX pADH1-TRNT1-CEN-TRP1</i>
EKK130	α	W303-1	<i>natMX-cca1-V188S</i>
EKK132	α	W303-1	<i>natMX-CCA1</i>
EKK134	a	W303-1	<i>rpl20bΔ::kanMX</i>
EKK138	α	Y7096	<i>natMX-CCA1</i>
EKK140	a	W303-1	<i>gcn2Δ::natMX</i>
EKK144	a	W303-1	<i>natMX-sui2-S51A</i>
EKK148	a	W303-1	<i>natMX-cca1-V188S</i>
EKK156	a	W303-1	<i>cca1Δ::hphMX rpl20bΔ::kanMX CCA1-CEN-URA3</i>
EKK220	α	W303-1	<i>cca1Δ::hphMX rpl20bΔ::kanMX pADH1-TRNT1-CEN-TRP1</i>
EKK222	α	W303-1	<i>cca1Δ::hphMX rpl20bΔ::kanMX pADH1-TRNT1-T154I-CEN-TRP1</i>
EKK224	α	W303-1	<i>cca1Δ::hphMX rpl20bΔ::kanMX pADH1-TRNT1-L166S-CEN-TRP1</i>
EKK234	α	W303-1	<i>sch9Δ::kanMX</i>
EKK236	α	W303-1	<i>natMX-cca1-V188S sch9Δ::kanMX</i>
EKK238	α	W303-1	<i>eap1Δ::kanMX</i>
EKK240	α	W303-1	<i>natMX-cca1-V188S eap1Δ::kanMX</i>
EKK247	α	W303-1	<i>hphMX-cca1-V188S natMX-sui2-S51A</i>
EKK265	α	W303-1	<i>caf20Δ::CaURA3</i>

EKK267	a	W303-1	<i>natMX-cca1-V188S caf20Δ::CaURA3</i>
EKK270	α	W303-1	<i>natMX-cca1-V188S glc7-10-TRP1, glc7Δ::Leu2</i>
EKK273	α	W303-1	<i>natMX-cca1-V188S petite</i>
EKK275	α	W303-1	wild type petite
EKK277	α	W303-1	<i>cca1Δ::HYG^R pADH1-TRNT1-CEN-TRP1</i> petite
EKK316	α	W303-1	<i>caf20Δ::URA3 eap1Δ::kanMX</i>
EKK318	α	W303-1	<i>natMX-cca1-V188S caf20Δ::URA3 eap1Δ::KAN^R</i>
EKK326	α	W303-1	<i>sui2-S51A cca1Δ::HYG^R pADH1-TRNT1-CEN-TRP1</i>
EKK330	α	W303-1	<i>sui2-S51A cca1Δ::HYG^R pADH1-TRNT1-L166S-CEN-TRP1</i>
EKK332	α	W303-1	<i>cca1Δ::hphMX pADH1-TRNT1-T154I-CEN-TRP1</i>
EKK334	α	W303-1	<i>cca1Δ::hphMX pADH1-TRNT1-L166S-CEN-TRP1</i>
EKK336	α	W303-1	<i>cca1Δ::hphMX pADH1-TRNT1-I326T-CEN-TRP1</i>
EKK338	α	W303-1	<i>cca1Δ::hphMX pADH1-TRNT1-K416E-CEN-TRP1</i>
EKK344	α	W303-1	<i>cca1Δ::hphMX pADH1-TRNT1-T154I-CEN-</i>

			<i>TRP1</i> petite
EKK346	α	W303-1	<i>cca1</i> Δ :: <i>hphMX pADH1-TRNT1-L166S</i> -CEN- <i>TRP1</i> petite
EKK348	α	W303-1	<i>cca1</i> Δ :: <i>hphMX pADH1-TRNT1-I326T</i> -CEN- <i>TRP1</i> petite
EKK350	α	W303-1	<i>cca1</i> Δ :: <i>hphMX pADH1-TRNT1-K416E</i> -CEN- <i>TRP1</i> petite
EKK358	α	W303-1	<i>natMX-cca1-V188S eap1</i> Δ :: <i>kanMX</i> petite
EKK367	α	W303-1	<i>natMX-cca1-V188S gcn2</i> Δ :: <i>hphMX</i>
EKK368	α	W303-1	<i>cca1</i> Δ :: <i>hphMX pADH1-TRNT1-M158V</i> -CEN- <i>TRP1</i>
EKK370	α	W303-1	<i>cca1</i> Δ :: <i>hphMX pADH1-TRNT1-R190I</i> -CEN- <i>TRP1</i>
EKK372	α	W303-1	<i>cca1</i> Δ :: <i>hphMX pADH1-TRNT1-I223T</i> -CEN- <i>TRP1</i>
EKK374	α	W303-1	<i>cca1</i> Δ :: <i>hphMX pADH1-TRNT1-R99W</i> -CEN- <i>TRP1</i>
EKK376	α	W303-1	<i>cca1</i> Δ :: <i>hphMX pADH1-TRNT1-K107R</i> -CEN- <i>TRP1</i>
EKK382	α	W303-1	<i>hphMX-cca1-V188S natMX-sui2-S51A</i> <i>eap1</i> Δ :: <i>kanMX</i>
EKK427	α	W303-1	<i>hphMX-cca1-V188S natMX-sui2-S51A</i> <i>eap1</i> Δ :: <i>kanMX</i> petite

EKK451	α	W303-1	<i>natMX-cca1-V188S ptp2Δ::hphMX</i>
EKK455	α	W303-1	<i>natMX-cca1-V188S ptp3Δ::kanMX</i>
EKK463	α	W303-1	<i>cca1Δ::hphMX eap1Δ::kanMX pADHI- TRNT1-CEN-URA3 pADHI-CCA1-CEN- URA3</i>
EKK465	α	W303-1	<i>cca1Δ::hphMX eap1Δ::kanMX pADHI- TRNT1-T154I-CEN-URA3 pADHI-CCA1- CEN-URA3</i>
EKK467	α	W303-1	<i>cca1Δ::hphMX eap1Δ::kanMX pADHI- TRNT1-L166S-CEN-URA3 pADHI-CCA1- CEN-URA3</i>
EKK469	α	W303-1	<i>cca1Δ::hphMX eap1Δ::kanMX pADHI- TRNT1-I326T-CEN-URA3 pADHI-CCA1- CEN-URA3</i>
EKK471	α	W303-1	<i>cca1Δ::hphMX eap1Δ::kanMX pADHI- TRNT1-K416E-CEN-URA3 pADHI-CCA1- CEN-URA3</i>
EKK501	α	W303-1	<i>ptp3Δ::kanMX ptp2Δ::hphMX</i>
EKK503	α	W303-1	<i>natMX-cca1-V188S ptp3Δ::kanMX ptp2Δ::hphMX</i>

3.5. Materials and Methods

3.5.1. Plasmid construction

The empty vector (EV, pRS415-*pADHI*) and *CCAI* (SCAW1-2) yeast expression plasmids containing the *URA3* marker and driven by the *ADHI* promoter (Sikorski and Heiter, 1989; Mumberg et al., 1995; Goring et al., 2013) were provided by P. Hanic-Joyce (Concordia University). The pADHI-*TRNT1*-CEN-*URA3* (pAR1200) plasmid was created by amplifying HeLa cells *TRNT1* cDNA and cloning into the plasmid *A163-3*, a vector constructed by replacing the *SacI*-*KpnI* MCS of pRS316 with the *pADHI* fragment from pRS415-*ADHI* (Sikorski and Heiter, 1989; Mumberg et al., 1995). The *pADHI*-*CCAI*-CEN-*URA3* (pAR1199) plasmid was constructed by digesting the *pADHI*-*CCAI* gene from vector SCAW1-2 and cloning it into the pRS316 vector; a *pADHI*-*CCA*-CEN-*TRP1* (pAR1216) plasmid was also generated by cloning the *ADHI*-*CCAI* fragment into pRS314. The *pADHI*-*TRNT1*-CEN-*TRP1* (pAR1208) plasmid was created by digesting the *pADHI*-*TRNT1* fragment from the *pADHI*-*TRNT1*-CEN-*URA3* (pAR1200) and cloning into the pRS314 vector. This work was done by post-doctorates and technicians in the Chakraborty and Holcik laboratory (Chakraborty et al., 2014).

The *pADHI*-*TRNT1*-mutant-CEN-*URA3* plasmids were generated using site-directed mutagenesis on *pADHI*-*TRNT1*-CEN-*URA3* (pAR1208) with primers containing each of the six *TRNT1* missense mutations: c.461C>T (p.*T154I*) (pAR1209), c.472A>G(p.*M158V*) (pAR1210), c.497T>C(p.*L166S*) (pAR1211), c.569G>T(p.*R190I*) (pAR1212), c.668T>C(p.*I223T*) (pAR1213), c.977T>C(p.*I326T*) (pAR1214), c.1246A>G(p.*K416E*) (pAR1215). The *pADHI*-*TRNT1*-mutant-CEN-*TRP1* plasmids were generated by cloning the *SacI*/*KpnI* fragment from the *pADHI*-*TRNT1*-mutant-*URA3* plasmids into pRS314 (*TRP1*) plasmid. Plasmids were confirmed by sequencing (StemCore Laboratories). All

plasmids were confirmed by sequencing (Applied Biosystems 3730 DNA Analyzer and MJ Research Tetrad Thermocyclers, StemCore Laboratories, Ottawa Hospital Research Institute). This work was done by post-doctorates and technicians in the Chakraborty and Holcik laboratory (sequences available in Chakraborty et al., 2014). I continued with these plasmids and used them to transform the *cca1* Δ strain for the plasmid shuffle experiments of the diploid *CCA1/cca1* Δ for meiosis analysis.

The *TRNT1-R99W* (c.295C>T) and *TRNT1-K107R* (c.320A>G) mutant alleles were a gift from Matthew Leibovitch and Paul Joyce. An EcoRI/PacI mutation containing fragment was digested from their pET-15b plasmid containing the mutant alleles and ligated into a similarly digested *pADHI-TRNT1-CEN-TRP1* (pAR1208) to generate *pADHI-TRNT1-R99W-CEN-TRP1* (pEK003) and *pADHI-TRNT1-K107R-CEN-TRP* (pEK004); mutations were confirmed by restriction digests and silent restriction sites BstYI and NlaIII respectively.

NAT^R-cca1-V188S was designed based on a multiple sequence alignment between *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, *Neurospora crassa*, *Aspergillus niger*, human, *Xenopus danio*, *Arabidopsis thaliana*, and *Thermotoga maritima* to assess conservation of the patient identified mutations described above; for space only *S. cerevisiae*, human, *A. thaliana*, and *T. maritima* are shown (Figure 3.3.1). Homologous mutations for *T154I*, *M158V*, *L166S*, and *R190I* were designed in yeast as follows: c.461C>T (p.*T154I*) in humans was designed as c.492ACA>ATT (p.*T175I*) from with a silent restriction site change involving an additional codon changed from GGC to GGA to introduce a KpnI site; c.472T>C(p.*M158V*) in humans was designed as c.504CTT>GTG (p.L179V) with a silent restriction site change involving an additional codon changed from CAA to CAG to introduce a BceAI site; c.497T>C(p.*L166S*) in humans was designed as

c.574GTG>TCA (p.*V188S*) with a silent restriction site change involving an additional codon changed from CAA to CAG to introduce a PstI site; c.569G>T (p.*R190I*) in humans was designed as c.643ACA>ATA (p.*R220I*) with a silent restriction site change involving an additional codon changed from ACT to ACG to introduce a BsiWI site. Although original amino acids were not always matching, changes were made to have consistent mutation amino acids resulting. Altered codons and inserted silent restriction sites were assessed to have similar codon bias (within 0.4X to 2.0X) as the wild type codon using the online Genscript.com tool. Restriction sites were engineered into the codons for the mutated residues for later identification. Mutant copies of the alleles were created using mutation containing primers with overlapping homology along with primers at the start and stop of the *ADHI* promoter and terminator sequences in *pADHI-CCAI-CEN-TRP1* (pAR1216). The resulting two PCR products were then used as template to create one PCR product from *ADHI* promoter start and end of the terminator. The *pADHI-CCAI-CEN-TRP1* (pAR1216) plasmid was then digested to remove the *CCAI* coding sequence and gap-repaired in wild type yeast using the PCR products: *pADHI-cca1-T153I-CEN-TRP1* (pAR1253); *pADHI-cca1-V188S-CEN-TRP1* (pAR1255); and *pADHI-cca1-T191I-CEN-TRP1* (pAR1256). Mutations were confirmed by restriction digest of the silent site introduced within 20 bp of the mutation.

The *EAPI-2μ-LEU2* plasmid was generated using gap repair in ADR4006 into the yEP13 plasmid. The *EAPI* sequence was amplified from 351 bp upstream to 250 bp downstream of the *EAPI* coding sequence with primers. Plasmid was purified from 3×10^7 cells of fresh yeast lysed in 1:1 phenol:chloroform and LiCl lysis buffer (2.5M LiCl, 50mM Tris HCl pH 8.0, 4% Triton-X-100, 62.5mM Na₂-EDTA) using an equal volume of glass beads and bead beating at 4°C 1 min. Following centrifugation the upper phase was

precipitated with 2.5 volumes of 100% ethanol and pelleted by centrifugation at 16.1 ref at room temperature. The pellet was washed with 70% ethanol and resuspended in water. The DNA was further cleaned by incubation at room temperature for 1 hour on 1% agarose with 100mM glucose. DNA was then transformed into electrocompetent DH10B (Invitrogen) cells and plasmid screened by restriction digestion and ability to suppress *eap1Δ* temperature sensitive phenotype. Chemically competent *Escherichia coli* strain Top10 (Invitrogen) or DH10B (Invitrogen) electrochemical competent cells were used for plasmid recovery following gap repair or plasmid ligation. Top10 cells were heat shocked for 30 seconds at 42°C and DH10B (Invitrogen) cells were incubated with 0.25μg of plasmid DNA on ice and transformed in 0.1 cm gal electroporation cuvettes at 25 μF, 200 Ohms, 1.66k Volts.

3.5.2. Yeast strain construction

Table 3.1 in Chapter 3 and Table A1.1 in Appendix 1 contain the lists of the strains used in this study. Strains used in this study are derivatives of W303-1a (*ura3-1 leu2-3,112 trp1-1 his3-11 ase2-1 can1-100*), Y7092 (*MATα can1Δ::STE2pr-Sp_his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*), or Y7096 from S288C (*MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) as indicated. All deletions and replacements were confirmed by PCR and/or sequencing (Applied Biosystems 3730 DNA Analyzer and MJ Research Tetrad Thermocyclers, StemCore Laboratories, Ottawa Hospital Research Institute). The sequences of all primers used in this study are contained in Table 5.1.

CCA1/cca1Δ::hphMX diploid yeast knockout strain (ADR7020) was created using PCR-targeted recombination in ADR941 (wild type diploid). Using gene specific primers, the gene was replaced with a hygromycin resistance cassette (*hphMX*) amplified from *pAG32-hphMX4* (Goldstein and McCusker, 1999).

The *cca1Δ::HYG^R pADHI-CCAI-CEN-URA3* sheltering strain was created by transforming *pADHI-CCAI-CEN-URA3* (pAR199) into the *CCAI/cca1Δ::hphMX* diploid (ADR7020), inducing meiosis, dissecting tetrads, and selecting for haploids containing both the *HYG^R* marker for the knockout and the *URA3* marker for the sheltering plasmid. All hygromycin resistant *strains* were also prototrophic for uracil, confirming deletion of *CCAI* is lethal. Plasmid shuffling strains containing *cca1Δ::hphMX pADHI-CCAI-CEN-URA3* and *pADHI-TRNT1-CEN-TRP1* plasmids were created by transforming *pADHI-TRNT1-CEN-TRP1* (*TRNT1* pAR1208, *TRNT1-R99W* pEK003, *TRNT1-K107R* pEK004, *TRNT1-T154I* pAR1209, *TRNT1-M158V* pAR1210, *TRNT1-L166S* pAR1211, *TRNT1-R190I* pAR1212, *TRNT1-I223T* pAR1213, *TRNT1-I326T* pAR1214, *TRNT1-K416E* pAR1215) plasmids into the *cca1Δ::hphMX pADHI-CCAI-CEN-URA3* sheltering strain and selecting for the presence of hygromycin, uracil, and tryptophan requirements.

Viable *cca1Δ::hphMX pADHI-TRNT1-CEN-TRP1* cells were found following spotting serial dilutions of the plasmid shuffle strains containing *cca1Δ::hphMX pADHI-CCAI-CEN-URA3 pADHI-TRNT1-CEN-TRP1* onto media containing 5- fluoroorotic acid (5-FOA; 1 g/L; Toronto Research Chemicals, ON, Canada), these are referred to as rescued strains. The presence of 5-FOA selects against cells that are *URA3* as the activity of the Ura3 protein results in the conversion of 5-FOA to 5-fluorouracil, a toxic compound. The rescued strains only contain *pADHI-TRNT1-CEN-TRP1* plasmids and this was confirmed by these strains inability to grow on 5- fluoroanthranilic acid (FAA) medium that selects against cells that are *TRP1*. FAA, like 5-FOA, is converted into a toxic compound, 5-fluorotryptophan (Toyn et al., 2000). Strains that survived on SC-trp 5-FOA were selected to contain only the *TRNT1* plasmids and to lose the sheltering *pADHI-CCAI-CEN-URA3* plasmid; these strains are referred to as rescued strains (*TRNT1* EKK100, *TRNT1-R99W*

EKK374, *TRNT1-K107R* EKK376, *TRNT1-T154I* EKK332, *TRNT1-M158V* EKK368, *TRNT1-L166S* EKK334, *TRNT1-R190I* EKK370, *TRNT1-I223T* EKK372, *TRNT1-I326T* EKK336, *TRNT1-K416E* EKK338).

The *cca1Δ::hphMX pADHI-cca1-V188S-CEN-TRP1* strains were created by the CCA1 mutant plasmids transformed into the sheltered *CCA1-CEN-URA3 cca1Δ::hphMX* (ADR6511) strain and shuffled as described above using 5-FOA (ADR8363, ADR8365, ADR8367, and ADR8369). All mutant *CCA1* plasmids allowed progression through sporulation and crossing, but the only CCA1 mutant allele to have a growth phenotype was *cca1Δ::hphMX cca1-V188S-CEN-TRP1* (ADR8367). Expression of the mutant Cca1 proteins was confirmed by western blot analysis. The *cca1-V188S* mutant allele was chosen to integrate into the W303-1A and Y7092 backgrounds.

Production of the endogenous *natMX-cca1-V188S* mutants first required two new *cca1Δi::hphMX* (i for internal) strains to be created where *CCA1* was deleted by PCR-targeted recombination to the internal region of nucleotides 428 to 1356 using a cassette amplified from pAG25 (Goldstein and McCusker, 1999). One strain was in ADR4006 (W303-1) (ADR8992) and one in Y7092 (query strain of the *MATa* deletion collection in S288C background) (ADR8990) (Tong and Boon, 2005), both transformations also containing the *pADHI-TRNT1-wt-CEN-URA3* (pAR1200) plasmid to shelter the loss of the essential *CCA1* gene. Deletion was confirmed by PCR analysis and loss of viability when exposed to 5-FOA. *CCA1* mutations were introduced into these two strains using a CRISPR/Cas9 plasmid pAR1279, a gift from and generated by Bruce Futscher, SUNY-Stonybrook. The pAR1279 (pRS425-*Cas9-pSNR52-kanMX6*) targets the upstream region of the *kanMX* or *hphMX* resistance cassettes for double strand breaks. Linearized *pADHI-cca1-V188S-CEN-TRP1* plasmid was transformed with pRS425-*Cas9-pSNR52-kanMX6* into

the two sheltered *cca1Δi::hphMX pADH1-TRNT1-CEN-URA3* (ADR8990 and ADR8992) to generate *cca1-V188S pADH1-TRNT1-CEN-URA3*. Plasmid loss was achieved by exposure to 5-FOA and western blot analysis for Cca1 and TRNT1 expression and genotype was confirmed by loss of hygromycin resistance, PCR, and restriction digest. The endogenous *cca1-V188S* phenotype was consistent between backgrounds (W303-1a and S288C) with strains expressing *pADH1-cca1-V188S-CEN-TRP1* plasmid in *cca1Δi::hphMX* (ADR8367). Strains were then marked 250 bp upstream of the start of *CCA1* by homologous recombination of the ClonNat resistance cassette amplified from pAG32 (*natMX*) (Goldstein and McCusker, 1999); marker was confirmed by PCR. The growth phenotype did not change upon introduction of the marker and the final strains were *natMX-cca1-V188S*; named EKK130 in W303-1 and ADR9214 in Y7092. The Y7092 *cca1-V188S* was also marked approximately 2500 bp upstream prior to the start of the upstream gene *BCK2* (ADR9215), no differences in growth phenotype were observed in this marked strain compared to unmarked or marked immediately upstream of *cca1-V188S*. *hphMX-cca1-V188S* was created by marker switching by homologous recombination using a *hphMX* cassette amplified off pAG32 (Goldstein and McCusker, 1999) (EKK158).

The *CCA1-AID-kanMX pTIR-TRP1* (ADR8264) strain was generated as an inducible AID degron system to control expression and degradation of one or multiple proteins in yeast (Nishimura, *et al.*, 2009). The *CCA1-AID-kanMX* was constructed by amplifying *AID* and *kanMX* off *pAID1*, a gift from Leon Chan, Thomas Eng and Vinny Guacci (Nishimura, *et al.*, 2009) and integrating the resulting PCR product. *leu2::pGPD1-OsTIR1-LEU2* was constructed by cutting *pTIR4*, a gift from Leon Chan, Thomas Eng and Vinny Guacci (Nishimura, *et al.*, 2009) with PmeI to integrate it at *LEU2* (ADR6431). The AID tag was added to the C-terminal of the *CCA1* protein; presence of the tag was confirmed by PCR and

western analysis and wild type protein levels and function when not exposed to auxin were confirmed by growth assays at a range of temperatures and nutrient conditions.

The *sui2-S51A* (EKK144) was introduced into using a CRISPR/Cas9 plasmid pAR1274, a gift from and generated by Bruce Futscher, SUNY-Stonybrook. Guide RNA (AATTGTCCCGTAGACG TATT and AATACGTCTACGGGACAATT) targeted *SUI2* and PCR generated fragments included the *sui2-S51A* mutation at c.154TCC>GCT (p.S51A) with a silent restriction site for SacI at c.146.GAATTG>GAGCTC for screening colonies were used to rescue the resulting double stranded break. Transformants were selected for the presence of LEU2, screened by PCR and SacI digestion from genomic DNA. Positive strains were then screened for loss of the LEU2 plasmid and had the natMX marker inserted 197 bp of stop using a PCR fragment amplified from pAG32 (Goldstein and McCusker 1999); insertion of the marker was check by PCR. Additional testing was done by in cross behavior and western blot analysis for eIF2 α and eIF2 α -P. This work was done by Dr. Adam Rudner.

ptp2 Δ ::hphMX, *cca1 Δ ::hphMX*, were constructed by amplifying *HYG^R* (*hphMX*) off pAG32 (Goldstein and McCusker 1999) and deleting *PTP2*, and *CCA1* respectively. *gcn2 Δ ::natMX* was constructed by amplifying *natMX* off pAG25 (Goldstein & McCusker, 1999) and deleting *GCN2*. *ptp3 Δ ::kanMX*, *eap1 Δ ::kanMX*, *sch9 Δ ::kanMX*, *rpl20b Δ ::kanMX* were constructed by amplifying *kanMX* off pFA6a-kanMX6 (Longtine et al., 1998) and deleting *PTP3*, *EAP1*, *SCH9*, or *RPL20B*. *caf20 Δ ::CaURA3* was constructed by amplifying *CaURA3* off pAR747 (Larin et al. 2015) and deleting *CAF20*. Unmarked *bar1 Δ* was constructed using pJGsst1 (J. Thorner, University of California, Berkeley, CA). *glc7-10-TRP1*, *glc7 Δ ::LEU2* were a gift from Sue Biggins, Fred Hutchinson Cancer

Research center, and were crossed to the *natMX-cca1-VI88S* strain to make the appropriate strains for analysis.

Strains used from the *MATa* yeast deletion array (Thermo Fisher Scientific), including deletion of *TOR1*, *RTG2*, *PBS2*, *SKO1*, *SHO1*, *SOD1*, *SOD2*, *RPL6B*, *COQ5*, *SWI6*, *PUS1* were verified by PCR and crossed to an SGA query strain containing *natMX-cca1-VI88S* (ADR9214) made by targeted PCR recombination. Suppression of the *natMX-cca1-VI88S* viability phenotype was achieved by sheltering the mutation with *CCA1-CEN-UR3A* (pAR1199).

3.5.3. Petite production

Exponentially growing cells were grown to a concentration of 1 to 2×10^7 cells/mL in complete medium (YEP dextrose), washed twice with distilled water, and 1×10^7 cells were re-suspended in 1mL in minimal medium (0.67% YBN) containing 2% glucose (SD) plus 25 $\mu\text{g/mL}$ ethidium bromide (filter sterilized) and grown to saturation, approximately 2 days at 30°C. 100 μL of this saturated culture was resuspended in 900 μL of minimal medium (0.67% YBN) containing 2% glucose (SD) plus 25 $\mu\text{g/mL}$ ethidium bromide and again grown to saturation. The culture was streaked for single colonies on a complete media (YEP dextrose) agar plate and single colonies were assayed for growth phenotypes and inability to grow on a non-fermentable carbon source (YEP glycerol). Lack of mtDNA was later confirmed by the absence of Cox2 protein by western blot.

3.5.4. Growth Assays

Exponentially growing cells were grown to a concentration of 1 to 2×10^7 cells/mL in complete medium (YEP dextrose) or synthetic complete medium minus tryptophan (SC-trp).

Cells were concentrated to 1.85×10^8 cells/mL and ten-fold serial dilutions were spotted onto YEP (Sunrise Science Products, CA, USA), SC-leu (Sunrise Science products), or SC-ura-trp (Sunrise Science Products); carbon sources were either 2% dextrose or 5% glycerol. Cells were grown at 25, 30, 33.5, 34, 36, or 37°C as indicated. Plates were imaged by scanning at 300 dpi on an Epson Perfection V550 Photo scanner.

Cycloheximide (MilliporeSigma Canada, ON, Canada) was used in plates at concentrations ranging from 20 to 400 ng/mL. Stock solutions were made as 10 mg/mL in water. Hygromycin (MilliporeSigma Canada, ON, Canada) was used in plates at concentrations ranging from 1 to 25 ng/mL. Stock solutions were made as 50 mg/mL in PBS. G418 (Thermo Fisher Scientific, Invitrogen, ON, Canada) was used in plates at 200 µg/mL. Stock solutions were made as 50 mg/mL in water. Clonazepam (Werner Bioagent) was at 100 µg/mL. Stock solutions were made as 100 mg/mL in water. Calcofluor white (Sigma-Aldrich) was used in plates at concentrations ranging from 1 to 6 µg/mL.

3.5.5. Western Blots

Cell extracts were prepared by mechanical lysis of frozen cell pellets containing 3.0×10^7 cells in a Mini-Beadbeater (BioSpec Products) into 1X SDS sample buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 125mM Tris-HCl pH 6.8, 1mM PMSF) and an equal volume of acid washed glass beads (BioSpec Products) for 60 seconds. Equal quantities of total protein were loaded on a 10% polyacrylamide gel and separated by SDS-PAGE. Membranes were stained with Ponceau S to ensure equal protein loading.

Membranes were pre-blocked with 1:5 Odyssey Blocking Buffer (PBS, LI-COR Biosciences). The following antibodies were used at 4°C overnight incubations for western

blots in 1:5 Odyssey Blocking Buffer (PBS, LI-COR Biosciences) and 0.02% NaN₃; α -Cdk1 at 1:1000 5% glycerol (Rudner Lab); α -TRNT1 (NBP1-86589) at 1:2000 (Novous Biologicals, ON, Canada); α -Sui2 at 1:1000 (Rudner Lab); α -Phospho-eIF2 α (Ser51) (#9721) at 1:1000 (Cell Signaling Technology); α -Cox2 at 1:500 (Invitrogen); α -Ndi1 at 1:500 (gift from Dr. Takao Yagi); α -Por1 at 1:2000 (Abcam). Anti-rabbit or anti-mouse fluorescent Alexa⁸⁰⁰-conjugated secondary antibodies (LI-COR) were used at 1:20,000 and 1:10000, respectively, also at 4°C for overnight incubation. Membranes were scanned and analyzed with the associated software using the Odyssey infrared imaging system (LI-COR) as directed by the manufacturer. The membranes were stripped with New Blot Nitro Stripping Buffer (LI-COR) for 20 min at room temperature, washed with TBS-T and re-probed as required.

3.5.6. Antibody production

α -Cca1 was generated as follows: coding sequence for the protein Cca1₁₇₋₅₄₇ starting from the third in-frame ATG was amplified by PCR and cloned into a pGEX-2T as a BamHI/Sall fragment by Paul Joyce (Concordia University) and kept in our collection as pAR1244. The GST fusion protein expression was induced using 0.25mM IPTG, cells were lysed in 1X PBS with 1mM EDTA, 1mM EGTA, 1mM PMSF, 15mM DTT, 0.5% Triton X-100 and 200 μ g/mL lysozyme. Lysate was batch bound to glutathione agarose and eluted with 50mM Tris pH 8.1, 0.5M NaCl and 5mM reduced glutathione. 1mg of each GST fusion protein was injected into rabbits every 4 weeks for 8 to 16 weeks (uOttawa animal facility). Rabbit serum was harvested, clarified by centrifugation and used as primary antibody. This work was done by summer students Alick Wang and Camille Fortinez and Dr. Adam Rudner.

α -Sui2 was generated as follows: coding sequence for the truncated protein Sui2 was amplified by PCR and cloned into a pGEX6P-1 (Promega) as a BamHI/EcoRI fragment to create pAR1395. Antigen production and injections proceeded as described for α -Cca1. Rabbit serum was harvested, clarified by centrifugation and used as primary antibody. This work was done by summer student Zach Comeau and Dr, Adam Rudner.

3.5.7. *In vivo* labeling of mitochondrial proteins

Cells were grown at 25°C overnight in non-fermentable media (YEP 2.5% raffinose + 2.5% galactose) to an OD of 0.4 to 0.8. If temperature change was required, cells were then transferred to the restrictive temperature for two hours prior to the next step. Degradation of *CCA1-AID* using auxin was done at 25°C in YEP 2.5% raffinose + 2.5% galactose for 2 hrs. Cells were washed twice with SD-met 2.5% raffinose + 2.5% galactose media, resuspended in SD-met 2.5% raffinose + 2.5% galactose media at 3×10^7 cells/mL, and incubated with shaking for 60 min. Cycloheximide was added at 0.2 mg/mL (from a 20 mg/mL stock in 100% ethanol) and incubated shaking for 10 minutes. 0.5 mL with 1.5×10^7 cells were labeled with [35 S]methionine/cysteine (EXPRES35S Protein Labeling Mix, [35S]-, 1 mCi (74MBq), 50mM Tricine (pH 7.4), 10mM 2-mercaptoethanol, Perkin Elmer, ON, Canada) in 1mL for 15 minutes with frequent inversion at 30°C or restrictive temperature as required. Samples were pelleted and frozen in liquid nitrogen. Pellets were mechanically lysed in 1X sample buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 125mM Tris-HCl pH 6.8, 1mM PMSF), 10 μ L were separated on 12.5% polyacrylamide gel, cutting free label from the bottom prior to fixation. Gels were fixed for 2 x 30 min in fixative (25% methanol v/v, 7% glacial acetic acid 5% glycerol) and dried for 3 hrs at 65°C on a gel dryer prior to exposure to a phosphorimager screen. The

phosphoimager screen was scanned by a Typhoon 9410 Variable Mode Imager at 50 micrometer resolution after seven days exposure.

3.5.8. Polysome analysis

Cells were grown to an OD of 0.6 to 0.7 then collected in cold centrifuge bottles containing cycloheximide final concentration 0.1 mg/mL. Cells were pelleted at 2000 rcf at 4°C, washed twice with 0.1 mg/mL cycloheximide and stored at -80°C. From this point Thet Naing (Holcik laboratory) or Laura Gillis (Lewis laboratory, with my assistance) processed the samples. Pellets were lysed in cold lysis buffer (20 mM Tris-Cl pH 8.0, 140 mM KCl, 1.5mM MgCl₂, 0.5mM DTT, 0.1mg/mL cycloheximide) with glass beads; samples were centrifuged at 2600 rcf. 1% Triton X-100 (final concentration) was added to the supernatant and centrifuged at approximately 5000 rcf. Supernatant was then spun at 250,000 rcf at 4°C. Samples were then processed using the ISCO gradient fractionation system as per manufactures instructions.

3.5.9. Statistical analyses

All results are expressed as mean and standard error of the mean with a minimum of 3 biological replicates, unless otherwise noted. The Student's t-test was used to determine statistical significance (Booher et al., 1993) (Graph Pad Prism 5).

Table 3.2. Primers used in this analysis

Primer	Description	Sequence
oAR35	5' check for upstream sequence	GGACGAGGCAAGCTAAACAG
oAR36	3' check for downstream sequence	TGGTCGCTATACTGCTGTCG
oAR1311	<i>CCAI</i> -F	GCCTGAGCCATTGACTCTTC
oAR1312	<i>CCAI</i> -R	TAAATTAGCCATGGGGGACA
oAR1412	c.1246 AAA>GAA (K416E) Forward, site directed mutagenesis	AACAGTTGCGAGAACAGTGGG AAAAAAGTGGTTACCAAATG
oAR1413	c.1246 AAA>GAA (K416E) Reverse, site directed mutagenesis	CATTTGGTAACCACTTTTTTCC CACTGTTCTCGCAACTGTT
oAR1422	<i>PTPs2</i> -upstream	ATTAATAGTTTACAATAAAATAGGATC GACGTTGCTATTGCGTACGCTGCAGGT CGAC
oAR1423	<i>PTP2</i> -downstream	AAACGAAAAGTGTTTGTATAATAGGAG AAAAACAATTCTAATCGATGAATTCGA GCTCG
oAR1424	<i>PTP2</i> -check upstream	TTTCTTTGAACACCGCGCTG
oAR1427	<i>PTP3</i> -upstream	CTTCTCCATCAGCAACACAGATCTACT TATCATATAGAACCGTACGCTGCAGGT CGAC
oAR1428	<i>PTP3</i> -downstream	ATTAATAAAATAGAGATCAAATACATT CATATTAGCCTAAATCGATGAATTCGA GCTCG
oAR1429	<i>PTP3</i> -check upstream	CACCGGCGGATGTAACACTA

oAR1461	<i>CCA1-AID</i> upstream	TTAATAACTCATTTAAAAGCCATACTA CCAAAATACCTGGCGGCCGCTCTAGAA CTAGT
oAR1462	<i>CCA1-AID</i> downstream	CTTGTTCTACATGTCTTATGTATACTA ATAAGCTACTACCTCGAGGTGACGGT ATCG
oAR1463	<i>CCA1-AID</i> check downstream	CAGCACCAATTGCAAATAACGAC
oAR1466	<i>CCA1</i> c.492ACA>ATT (p.T175I) F	CCTAAAGTGTGCTTTGGAATTCCCGAG GAAGACGCTTTAAG
oAR1467	<i>CCA1</i> c.492ACA>ATT (p.T175I) R	CTTAAAGCGTCTTCCTCGGGAATTCCA AAGCACACTTTAGG
oAR1468	<i>CCA1</i> c.504CTT>GTG (p.L179V) F	GATGCTACATTGAACGCCGTGTTCTAT AACATTCATAAAGG
oAR1469	<i>CCA1</i> c.504CTT>GTG (p.L179V) R	CCTTTATGAATGTTATAGAACACGGCG TTCAATGTAGCATC
oAR1470	<i>CCA1</i> c.574GTG>TCA (p.V188S) F	AAAGGTGAATCAGAAGATTTACCAAG AGAGGTCTGCAGGATCTAA
oAR1471	<i>CCA1</i> c.574GTG>TCA (p.V188S) R	TTAGATCCTGCAGACCTCTCTTGGTGA AATCTTCTGATTACCTTT
oAR1472	<i>CCA1</i> c.643ACA>ATA (p.R220I) F	GTTCTCCGTACGCCGCTTCCTGCAAAA CAAATATTTTTGGATG
oAR1473	<i>CCA1</i> c.643ACA>ATA (p.R220I) R	CATCCAAAAATATTTGTTTTGCAGGAA GCGGCGTACGGAGAAC
oAR1551	<i>CCA1</i> downstream sequencing	GGTGACAGAAAGCATTGTG
oAR1552	<i>ADHI</i> promoter	TCTCACCATATCCGCAATGA

oAR1552	<i>ADHI</i> terminator	CGCAAATTAAAGCCTTCGAG
oAR1624	<i>CCAI</i> internal deletion upstream	CCCGAGAAATCCAAGCATCTGGAAACT GCCACTACTAAGCAGCTGAAGCTTCGT ACGC
oAR1625	<i>CCAI</i> internal deletion downstream	GGAATCTTTAAAAATGCATCTGATAGA GATGCAAAATGTGTACGACTCACTATA GGG
oAR1668	Insert marker 3' <i>CCAI</i> upstream	GCAACGCCTTCGATGCGAATATATGTT TTGCTGATAACAGCTGAAGCTTCGTAC GC
oAR1669	Insert marker 3' <i>CCAI</i> downstream	GCGATAATTTTAAGAGAATTGCAGACG CTGTACATAGATCTACGACTCACTATA GGG
oAR1697	<i>SCH9</i> deletion upstream	ACCCTTTTCGTGGTCCTTTT
oAR1698	<i>SCH9</i> deletion downstream	AAACTAGAAATTTGCAGGAACAA
oAR1735	<i>GCN2</i> deletion check	CAGCCTAGAATCTTTTAAAGCAA
oAR1736	<i>RPL20B</i> deletion check	GGAAGGCGGTGTACCATTTA
oAR1737	<i>SCH9</i> deletion check	ACCCTTTTCGTGGTCCTTTT
oEK013	<i>RPL20B</i> deletion upstream	TGAAACATTTCATTTACATGTAATTGTC TGCAATAAAGCCAGCTGAAGCTTCGTA CGC
oEK014	<i>RPL20B</i> deletion downstream	ATAATGTAAGAGAAGAAACAGGATATA TTTTAAAATTTCCCTACGACTCACTATA GGG
oEK015	<i>GCN2</i> deletion upstream	CAATAATTTTCCGTTCCCTTAACACA

		TACTATGTATAACAGCTGAAGCTTCGT ACGC
oEK016	<i>GCN2</i> deletion downstream	TTAACTGATGCGTTATAGCGCCGCACA GATCTTTAAAGGCTACGACTCACTATA GGG
oEK023	<i>RPL20B</i> deletion check	ATTAGCGCACCCACAGTTCT
oEK025	<i>EAPI</i> deletion upstream	CTACACAAAGAACTAGCATAGTAGGAC TTTAACCCATACAAGCTGAAGCTTCGT ACGC
oEK026	<i>EAPI</i> deletion downstream	GTTTTTGTCGTTTCCTGTCAAGTACTC GCTCGTTACGCATCTACGACTCACTAT AGGG
oEK027	<i>EAPI-HA</i>	CCAGTTACCACCAGGATTAAACTCTAA AAAGAATATAAAATCCATGGAAAAGAG AAG
oEK029	<i>EAPI</i> downstream check	CGACTAGTAAATATCAAAGGCGAAA
oEK030	<i>CAF20</i> deletion upstream	CCTGAATATAGGATTAAGAACTTTTAT TTAATTTACGACAGCTGAAGCTTCGT ACGC
oEK031	<i>CAF20</i> deletion downstream	GTACACGTACAGCTGCCTGAAGAAAGC ATGAGCGAATGACTACGACTCACTATA GGG
oEK033	<i>CAF20-MYC</i>	CATTTGCTGCTTTGGAAAGTGAAGACG AAGACGACGAAGCACGTACGCTGCAGG TCGAC
oEK034	<i>CAF20</i> downstream check	GGGGAAGCCTTTCTCTTTCA

oEK043	<i>CDC33-TAP</i> upstream	CAGTGCCAATGGTAGACACCCTCAACC ATCAATCACCTTGTCATGGAAAAGAG AAG
oEK044	<i>CDC33-TAP</i> downstream	CATGATGAGTTTATACGTGCATCCTAA CTAGTAGAATGTACGACTCACTATAGG G
oEK045	<i>CDC33</i> downstream check	TGTGGTGCGTCTTTCATACC
oEK047	<i>TOR1</i> deletion downstream check	GGGCGCTAAATTAGGGAAAC
oEK052	<i>RTG2</i> deletion downstream check	TTCTTGCTGAAGATGGTTCC
oEK053	<i>PBS2</i> deletion downstream check	TTCTAGCCTTGAGCCGTAA
oEK054	<i>SKO1</i> deletion downstream check	GACGAAAAGGAATGGCAAA
oEK055	<i>SHO1</i> deletion downstream check	CTGGTCCAGTCGGTAAGGAA
oEK056	<i>SOD2</i> deletion downstream check	TCTCTAAACAACCGCCCATT
oEK058	<i>RPL6B</i> deletion downstream check	ACACATTGGGAGGACGACTC
oEK060	<i>COQ5</i> deletion downstream check	CGTTAGTGCCCAATCTTGAAA
oEK061	<i>SOD1</i> deletion downstream check	CGAGATCGACAAGGAACACA
oEK066	<i>SWI6</i> deletion downstream check	TGTGTCGGTGTCATTTACCG
oEK068	<i>PUS1</i> deletion downstream check	GATACGTTGGGCCAGTAAA
oEK069	<i>EAP1</i> insert into yEP13 upstream	CTTGAGCCACTATCGACTACGCGATC ATGGCGACCACGGCGCAGAAAACCTGA AAAT
oEK071	<i>EAP1</i> insert into yEP13 downstream	GATGTCGGCGATATAGGCGCCAGCAAC CGCACCTGTGTTCCGAGCGTGACGTTA CTA

Chapter 4: Redundant regulation of Cdk1 tyrosine dephosphorylation in *S. cerevisiae*

This chapter includes works completed with Dr. Adam Rudner and honours and summer students in the Rudner laboratory, undertaken as the *TRNT1/CCAI* project was initiated and preliminary work was being done to assess if it was an adequate project for a doctorate degree. Consistent with Chapters 2 and 3, this study analyzes the highly complex pathways of phosphorylation regulation using yeast and has implications in human health and disease. Dr. Noel Lianga, Sophie Pilon, Carole Doré, and Jean-Sebastien Deneault generated preliminary data and strains. Dr. Elizabeth Williams quantified western blot analyses and Michael Drysart, an honours student I assisted in supervising, assisted with unpublished work associated in Appendix 2. Dr. Adam Rudner produced Figures 1C, 1D, and repeated experiments for images of Figure 2. I contacted the journal Genetics and requested copyright permission for use of the article in this thesis. I was informed by Genetics that using the published article in a thesis and/or dissertation is an acceptable form of author reuse and no copyright permission is required. Unpublished data associated with this project is presented in Appendix 2.

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Redundant regulation of Cdk1 tyrosine dephosphorylation in *Saccharomyces cerevisiae*

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Short title: Multiple phosphatases regulate Cdk1 dephosphorylation.

Article Summary

Nobel prize-winning work of Paul Nurse showed that Cdc25-dependent dephosphorylation of Cyclin dependent kinase (Cdk1) is a universally conserved mechanism regulating mitotic entry. In budding yeast, Cdk1 is dephosphorylated when the *cdc25* homologue, *MIH1*, is deleted, indicating an additional phosphatase functions redundantly with Mih1. In this manuscript we identify two phosphatases, Ptp1 and PP2A^{Rts1}, that function in conjunction with Mih1. This redundancy suggests novel mechanisms for how cells may activate pools of Cdk1 at different times and in different cellular locations.

Abstract

Cdk1 activity drives both mitotic entry and the metaphase-to-anaphase transition in all eukaryotes. The kinase Wee1, and the phosphatase Cdc25, regulate the mitotic activity of Cdk1 by the reversible phosphorylation of a conserved tyrosine residue. Mutation of *cdc25* in *Schizosaccharomyces pombe* blocks Cdk1 dephosphorylation and causes cell cycle arrest. In contrast, deletion of *MIH1*, the *cdc25* homologue in *Saccharomyces cerevisiae*, is viable. Although Cdk1-Y19 phosphorylation is elevated during mitosis in *mih1Δ* cells, Cdk1 is dephosphorylated as cells progress into G1 suggesting additional phosphatases regulate Cdk1 dephosphorylation. Here we show that the phosphatase Ptp1 also regulates Cdk1 dephosphorylation *in vivo* and can directly dephosphorylate Cdk1 *in vitro*. Using a novel *in vivo* phosphatase assay we also show that PP2A bound to Rts1, the budding yeast B56-regulatory subunit, regulates dephosphorylation of Cdk1 independently of a function regulating Swe1, Mih1, or Ptp1, suggesting that PP2A^{Rts1} either directly dephosphorylates Cdk1-Y19 or regulates an unidentified phosphatase.

Introduction

Mitotic onset is regulated in all eukaryotes by an increase in Cdk1 activity caused by the dephosphorylation of Cdk1 on a conserved inhibitory tyrosine (tyrosine 19 in budding yeast; (Nurse 1990)). The Wee1 kinase phosphorylates and inhibits Cdk1 (Gould and Nurse 1989; Parker *et al.* 1992), and the Cdc25 phosphatase acts as a mitotic inducer by dephosphorylating and activating Cdk1 (Dunphy and Kumagai 1991; Gautier *et al.* 1991). *wee1* mutants in fission yeast shorten G2 by prematurely activating Cdk1 (Nurse 1975; Russell and Nurse 1987), whereas *cdc25* mutants cannot accumulate sufficient Cdk1 activity to enter mitosis and arrest (Russell and Nurse 1986). Both Wee1 and Cdc25 are the targets of numerous cell cycle checkpoints, all of which delay mitotic entry by activating Wee1 or inhibiting Cdc25 (Kellogg 2003). In budding yeast, Swe1 (the Wee1 homologue) and Mih1 (the Cdc25 homologue) also function prior to mitosis (Russell *et al.* 1989; Booher *et al.* 1993; Harvey and Kellogg 2003; Pal *et al.* 2008), but our recent work revealed that overexpression of Swe1, or activation of a Swe1-dependent checkpoint, arrests cells in metaphase (Liang *et al.* 2013).

Deletion of *cdc25* in fission yeast is lethal and arrests cells in G2, indicating the essential role of Cdk1-Y15 dephosphorylation in fission yeast (Russell and Nurse 1986). In contrast, although deletion of *MIH1* exhibits high Cdk1-Y19 phosphorylation during mitosis, these cells have only mild delays in mitotic entry and anaphase onset, and initiate Cdk1-Y19 dephosphorylation at anaphase onset (Russell *et al.* 1989; Rudner *et al.* 2000; Pal *et al.* 2008; Liang *et al.* 2013). This behaviour argues that at least one additional phosphatase functions redundantly with Mih1.

Russell and colleagues (Millar *et al.* 1992) identified the fission yeast Pyp3 as a phosphatase that functions redundantly with Cdc25. Increased expression of *pyp3*, or the

budding yeast and mammalian homologues, *PTP1*, PTP1B and TC-PTP1, respectively, suppress the temperature sensitivity of *cdc25-22*, and the *pyp3Δ* mutant exacerbates the defects of *cdc25-22* (Gould *et al.* 1990; Millar *et al.* 1992; Hannig *et al.* 1993), suggesting that Ptp1 has a conserved function dephosphorylating Cdk1.

Protein phosphatase 2A (PP2A) may also act redundantly with Mih1. PP2A is a hetero-trimeric complex composed of a catalytic subunit, a scaffolding subunit and one of two B-regulatory subunits, Cc55 (homologous to B55/B) or Rts1 (homologous to B56/B') (Healy *et al.* 1991; Stark 1996; Zhao *et al.* 1997; Shu *et al.* 1997). Deletion of *CDC55* or *RTS1* elevates Cdk1-Y19 phosphorylation (Minshull *et al.* 1996; Zapata *et al.* 2014) and both mutants display synthetic interactions when combined with *mih1Δ* (Pal *et al.* 2008; Costanzo *et al.* 2010), suggesting that PP2A may function with Mih1. However, these synthetic interactions may instead be explained by negative regulation of Swe1 by PP2A^{Cdc55} and PP2A^{Rts1} (Harvey *et al.* 2011). In addition, although PP2A is capable of tyrosine dephosphorylation *in vitro*, it is believed to function solely as a serine/threonine phosphatase *in vivo* (Foulkes *et al.* 1983; Janssens and Goris 2001; Shi 2009; Tonks 2013).

Here we show that Ptp1, the budding yeast homologue of fission yeast Pyp3, also regulates Cdk1 dephosphorylation *in vivo*. However, despite delayed Cdk1-Y19 dephosphorylation in anaphase, *mih1Δ ptp1Δ* cells are as healthy as *mih1Δ* cells, suggesting the existence of an additional redundant phosphatase. Using an *in vivo* Cdk1-Y19 phosphatase assay we show that PP2A^{Rts1}, but not PP2A^{Cdc55}, regulates dephosphorylation of Cdk1-Y19 independently of its role regulating Swe1. Although we are not able to confirm whether PP2A^{Rts1} directly dephosphorylates Cdk1-Y19 or regulates an unidentified phosphatase, our results suggest that Mih1, Ptp1, and PP2A^{Rts1} function redundantly to

regulate the spatial and temporal activation of Cdk1, providing a mechanism for the observed step-wise activation of Cdk1 prior to anaphase onset.

Results

Ptp1 regulates Cdk1 tyrosine dephosphorylation

In fission yeast, the Pyp3 phosphatase acts redundantly with Cdc25 *in vivo* and *in vitro* (Millar *et al.* 1992), so we examined the role of the budding yeast homologue, Ptp1, in regulating tyrosine phosphorylation on Cdk1. Asynchronously growing *ptp1Δ* cells have elevated Cdk1-Y19 phosphorylation (Fig. 1A; See Tables S1 and S2 for a complete list of all strains used in this study), but this increase is not as dramatic as what is observed in *mih1Δ* cells.

mih1Δ ptp1Δ double mutants are viable and healthy, so we examined the effect of mutating both phosphatases on entry into and progression through mitosis. Deletion of *MIH1* causes an increase in Cdk1-Y19 phosphorylation during mitosis, delays mitotic entry and the length of mitosis increases (Fig. 1B; (Lianga *et al.* 2013)). *ptp1Δ* cells behave similarly to wild type, but in *mih1Δ ptp1Δ* cells Cdk1-Y19 levels remain high as they progress through mitosis and re-enter G1. Surprisingly, despite this persistent phosphorylation, we observe no change in the timing of degradation of the mitotic cyclins Clb2 and Clb5, the anaphase inhibitor Pds1/Securin, and the Cohesin subunit Mcd1/Sccl in *mih1Δ ptp1Δ* cells compared to *mih1Δ* cells. Although Cdk1-Y19 phosphorylation does not appear to drop as *mih1Δ ptp1Δ* cells exit mitosis, the lower levels present after G1 arrest caused by the mating pheromone, α -factor (α f; $t=0$), suggest that an additional phosphatase may function redundantly with Mih1 and Ptp1.

Although the level of Cdk1-Y19 phosphorylation in *mih1Δ* and *mih1Δ ptp1Δ* cells is high relative to wild type, it is lower than the extent of phosphorylation in wild type cells arrested by the actin-depolymerizing drug latrunculin A (latA), which activates a Swe1-dependent checkpoint, or in cells over expressing Swe1 (*GAL-SWE1*) (Fig. 1C). These two treatments arrest the cell cycle and depend on Y19 in Cdk1 (Booher *et al.* 1993; Lew and Reed 1995), suggesting that small differences in Cdk1-Y19 phosphorylation have large phenotypic effects.

We assessed the extent of inhibition of Cdk1 activity caused by Y19 phosphorylation *in vivo* by measuring the Clb2-associated histone H1 kinase activity in synchronous mitotic cells (Fig. 1D). By normalizing the activity to the total Cdk1 present in each kinase reaction, we are able to compare the relative activity and Y19 phosphorylation of each Cdk1/Clb2 complex present in a cell. Cdk1 precipitated from synchronous mitotic *mih1Δ* and *mih1Δ ptp1Δ* cells (t=75 minutes after release from G1 arrest) has approximately 65% of wild type activity, while arrest caused by latA or *GAL-SWE1* further reduces Cdk1 activity to 50% of wild type. The reduction in Cdk1 activity in these cells is mirrored by concomitant increases in Cdk1-Y19 phosphorylation. These measurements also allow us to infer the relative stoichiometry of Cdk1-Y19 phosphorylation and suggest that small decreases in Cdk1 activity (and increased Y19 phosphorylation) in latA or *GAL-SWE1* arrested cells halts cell cycle progression. This data is consistent with our prior observation that approximately 30% of the Cdk1 remains active during an arrest caused by *GAL-SWE1* (Liang *et al.* 2013).

Cdk1 precipitated from mitotic *swe1Δ* cells (t=60) has three-fold higher activity than Cdk1 from synchronous mitotic wild type cells (t=60) (Fig. 1D) suggesting that two-thirds of the Cdk1 in mitotic wild type cells is phosphorylated on Y19. We were surprised, however, that mitotic Cdk1 activity in wild type cells doesn't differ from the activity of Cdk1

precipitated from wild type or *swe1Δ* cells arrested in mitosis by nocodazole, despite the lack of Cdk1-Y19 phosphorylation. We hypothesize that an unidentified mechanism reduces Cdk1 activity in nocodazole-arrested cells, and may be used to control the increase in total Cdk1 activity as Clb2 protein rises during the arrest.

PP2A^{Rts1} acts redundantly with Mih1 and Ptp1

Mutation of the B-regulatory subunits of PP2A, *CDC55* and *RTS1*, also cause an increase in Cdk1-Y19 phosphorylation (Fig. 1A; (Minshull *et al.* 1996; Zapata *et al.* 2014)). *cdc55Δ* and *rts1Δ* are also synthetically lethal or sick, respectively, in combination with *mih1Δ*, and these interactions are completely suppressed by deletion of *SWE1* (Fig. 1E; (Pal *et al.* 2008; Costanzo *et al.* 2010)). In addition, the temperature sensitivity of *rts1Δ* is partially suppressed by *swe1Δ* (Fig. S1A). The increase in Cdk1-Y19 phosphorylation in these mutants and their interaction with *mih1Δ* can at least be partially attributed to up-regulation of Swe1 protein in both *cdc55Δ* and *rts1Δ* cells (Minshull *et al.* 1996; Harvey *et al.* 2011; Zapata *et al.* 2014), and down-regulation of Mih1 in *cdc55Δ* cells (Pal *et al.* 2008), but may also be caused by other functions of PP2A.

To test if PP2A regulates Cdk1-Y19 phosphorylation independently of its function regulating Swe1 and Mih1, we developed an *in vivo* phosphatase assay in which we monitor Cdk1-Y19 dephosphorylation in mitotically-arrested cells that first induce high levels of Swe1 and Cdk1-Y19 phosphorylation using galactose induction of a *GAL-SWE1-AID* gene, then rapidly repress *SWE1* transcription with glucose, and degrade the Swe1-AID protein with the addition of auxin (Fig. 2). This experimental design allows us to assess the role of PP2A independently of its function regulating Swe1, and in the absence of Swe1-dependent re-phosphorylation of Cdk1. *GAL-SWE1-AID* replaces the endogenous *SWE1*, so when

grown on glucose these strains behave effectively as *swe1Δ* cells, suppressing the lethality of several of the mutant combinations tested.

In *mih1Δ* cells, Cdk1 is dephosphorylated between two and four hours after glucose and auxin addition (Fig. 2A and B). If only glucose, but not auxin, is added, Swe1-AID is degraded slowly and Cdk1-Y19 phosphorylation persists throughout the experiment. Cdk1 is dephosphorylated slowly in *mih1Δ ptp1Δ* cells (Fig. 2A), consistent with our observation that these cells are defective in the dephosphorylation of Cdk1-Y19 in anaphase (Fig. 1B). To simplify our analysis of these experiments we add α f during the release from nocodazole, arresting cells in the subsequent G1. We noticed that although adding either 25ng/ml or 1μg/ml of α f will arrest cells in G1 (Fig. S2A and B), Cdk1-Y19 dephosphorylation in *mih1Δ ptp1Δ* cells is similar to *mih1Δ* cells when treated with a higher concentration of α f (Fig. 2B). However, the triple mutant combination, *mih1Δ ptp1Δ rts1Δ*, completely blocks dephosphorylation of Cdk1 at both concentrations of pheromone (Fig. 2A and B), suggesting that PP2A^{Rts1} may directly target Cdk1-Y19, or it may negatively regulate an unidentified fourth phosphatase that targets Cdk1. Consistent with redundancy among these three phosphatases, the deletion of *PTP1* in *mih1Δ rts1Δ* cells causes lethality that is fully suppressed by deletion of *SWE1* (Fig. 1E). Although *mih1Δ ptp1Δ rts1Δ* cells block Cdk1-Y19 dephosphorylation, they do not remain arrested in mitosis as judged by the degradation of Clb2, Clb5, Pds1 and Mcd1 (Fig. S2A and B).

In order to investigate whether PP2A^{Cdc55} also functions independently of Swe1 to regulate Cdk1-Y19 dephosphorylation we utilized strains containing *CDC55-AID* rather than a deletion of *CDC55* because *cdc55Δ* cells grow poorly in media containing raffinose or galactose, both of which are used in this experiment (Fig. S1B). In the presence of auxin, *CDC55-AID* is lethal in combination with *mih1Δ*, and grows poorly on raffinose and

galactose media (Fig. S1B and C) suggesting *CDC55-AID* behaves as a null mutant when grown on auxin. In the *in vivo* phosphatase assay, *mih1Δ CDC55-AID* and *mih1Δ ptp1Δ CDC55-AID* cells induce lower levels of Swe1-AID, but enough is produced to allow the accumulation of high levels of Cdk1-Y19 phosphorylation (Fig. 2A). As in *mih1Δ* cells, Cdk1 is dephosphorylated between two and four hours after glucose and auxin addition, suggesting that PP2A^{Cdc55} regulates Cdk1-Y19 phosphorylation indirectly through Swe1 and Mih1. We were surprised that *mih1Δ ptp1Δ CDC55-AID* cells dephosphorylate Cdk1-Y19 more rapidly than *mih1Δ ptp1Δ* cells, suggesting that PP2A^{Cdc55} may have an additional function negatively regulating Cdk1-Y19 dephosphorylation.

Ptp1 dephosphorylates Cdk1 *in vitro*

To validate our genetic results we tested if purified phosphatases can dephosphorylate Cdk1 *in vitro* (Fig. S3). Immunopurified Swe1-phosphorylated Cdk1/Clb2 complexes were incubated with Mih1, Ptp1, PP2A^{Cdc55} or PP2A^{Rts1} purified from yeast, or with lambda phosphatase. Both Mih1 and Ptp1 can dephosphorylate Cdk1 *in vitro*, but both forms of PP2A and lambda phosphatase cannot (Fig. 3). The dephosphorylation of a fortuitous background protein (indicated by an asterisk) confirms that purified PP2A^{Cdc55} and lambda phosphatase are active in this experiment, and that Mih1 and Ptp1, which do not dephosphorylate this protein, display Cdk1-Y19 specificity. PP2A^{Rts1}, which our prior experiments suggested may directly dephosphorylate Cdk1, had no activity in this experiment, but we have no evidence that the purified enzyme is active.

Discussion

We have identified two phosphatases, Ptp1 and PP2A^{Rts1}, that work redundantly with Mih1 to regulate Cdk1-Y19 dephosphorylation. Past work had suggested that Ptp1 may regulate Cdk1-Y19 dephosphorylation in budding yeast (Hannig *et al.* 1993), and we have shown that combining deletions in *PTP1* and *MIH1* severely impairs Cdk1-Y19 dephosphorylation during anaphase. *mih1Δ ptp1Δ* cells still dephosphorylate Cdk1-Y19 during a prolonged G1 arrest, suggesting that at least one additional phosphatase may regulate Cdk1-Y19 dephosphorylation.

Cdk1-Y19 dephosphorylation in *mih1Δ ptp1Δ* cells could be caused by turnover of Cdk1, as newly synthesized Cdk1 would not be re-phosphorylated by Swe1, which is absent in G1 (Fig. 1B). Although we did not directly measure the half-life of Cdk1 in budding yeast, we think this mechanism is unlikely, or plays only a minor function, because the half-life of Cdk1 in cultured mammalian cells in G1 is approximately 18 hours (Welch and Wang 1992; Gannon *et al.* 1998).

To test if PP2A functions redundantly with Mih1 and Ptp1, we developed a novel *in vivo* phosphatase assay that allowed us to examine whether PP2A regulates Cdk1-Y19 phosphorylation independently of its known role regulating Swe1 and Mih1. We discovered that PP2A^{Rts1} either directly dephosphorylates Cdk1-Y19, or inhibits a fourth phosphatase that does (Fig. 2). In contrast, we have shown that PP2A^{Cdc55} does not regulate Cdk1-Y19 dephosphorylation independently of its function inhibiting Swe1 and activating Mih1, and cannot dephosphorylate Cdk1-Y19 *in vitro*. Acute loss of Cdc55 using an auxin-inducible degron, in fact, appears to cause more rapid dephosphorylation of Cdk1-Y19, suggesting that PP2A^{Cdc55} may negatively regulate Cdk1-Y19 dephosphorylation (Fig. 2A). This function of

PP2A^{Cdc55} may function through PP2A^{Rts1}, as deletion of *RTS1* is epistatic to this behaviour of *CDC55-AID*.

Although we have demonstrated that purified Mih1 and Ptp1 dephosphorylate Cdk1-Y19 *in vitro* (Fig. 3), we were unable to rigorously test PP2A^{Rts1} function *in vitro*. PP2A^{Rts1} substrates are thought to be phosphorylated only on serine and threonine residues (Janssens and Goris 2001; Shi 2009), but past work has shown that PP2A can acquire activity against tyrosine residues *in vitro* in specific reaction conditions, or in the presence of PTPA (encoded by *Rrd1* and *Rrd2* in budding yeast), whose *in vivo* role is poorly understood (Cayla *et al.* 1990; Van Hoof *et al.* 1994; Van Hoof 2001).

We found it surprising that *mih1Δ ptp1Δ* cells initiate anaphase and enter G1 with little change in the level of Cdk1-Y19 phosphorylation. Our measurements of Cdk1-Y19 phosphorylation and Cdk1 activity in these cells suggest that an additional one-third of the active Cdk1/Clb2 complexes are inhibited in these cells compared to wild type, which increases to half when cells are arrested by overexpression of *Swe1* or activation of a *SWE1*-dependent checkpoint (Fig. 1D). This difference suggests that there is sufficient Cdk1 activity in *mih1Δ ptp1Δ* cells to drive entry into mitosis and the metaphase-to-anaphase transition, and that the difference between cell cycle arrest and progression may reflect a small change in the extent of inhibition of Cdk1, or the targeted inhibition of a pool of Cdk1. Our data leads to the hypothesis that PP2A^{Rts1} acts with Mih1 and Ptp1 to activate this pool of Cdk1 activity.

One aim of this study was to assess if preventing Cdk1-Y19 dephosphorylation would block anaphase onset in a similar way as we have described in cells overexpressing *Swe1*, or arrested by *latA* treatment (Liang *et al.* 2013). Although *mih1Δ ptp1Δ* cells block Cdk1-Y19 dephosphorylation during anaphase in an unperturbed cell cycle (Fig. 1B), and

mih1Δ ptp1Δ rts1Δ cells block dephosphorylation in the *in vivo* phosphatase assay (Fig. 2), both mutant combinations do not block anaphase onset as judged by activation of the APC, and loss of the Cohesin subunit Mcd1 (Fig. S2). These results may indicate that Cdk1-Y19 dephosphorylation is not needed to trigger anaphase, but we think it is more likely that Cdk1-Y19 phosphorylation does not reach the level needed for cell cycle arrest in these experiments. *rts1Δ* cells, like *CDC55-AID* cells, induce *GAL-SWE1-AID* poorly, and lower levels of Cdk1-Y19 phosphorylation accumulate in these cells prior to auxin and dextrose addition (Fig. 2).

Numerous studies have suggested that inhibitory phosphorylation regulates Cdk1 activation in a stepwise manner in both vertebrates and yeast (Stern and Nurse 1996; Pomerening *et al.* 2003; 2005; Deibler and Kirschner 2010; Harvey *et al.* 2011). Lower levels of Cdk1 activity are needed to induce the entry into mitosis than the metaphase-to-anaphase-transition, and Cdk1-Y19 phosphorylation functions at both these thresholds (Rahal and Amon 2008; Lianga *et al.* 2013). Our results suggest that at least three phosphatases regulate Cdk1 dephosphorylation and the regulation of their activity may provide a mechanism for cells to activate pools of Cdk1 at different times and in different cellular locations. PP2A^{Rts1} is localized to the pericentromere (Gentry and Hallberg 2002; Kitajima *et al.* 2006; Riedel *et al.* 2006), and its activity there may allow kinetochore activation of Cdk1 and assist in triggering anaphase onset.

Cdc25 inhibitors have been pursued as promising chemotherapeutics that could block cell cycle progression in cancerous cells, but these compounds have had only limited clinical success (Lavecchia *et al.* 2012). If similar redundancy exists in human cells as we have uncovered in budding yeast, then several different phosphatase inhibitors may be needed to effectively block Cdk1 activation and arrest the cell cycle.

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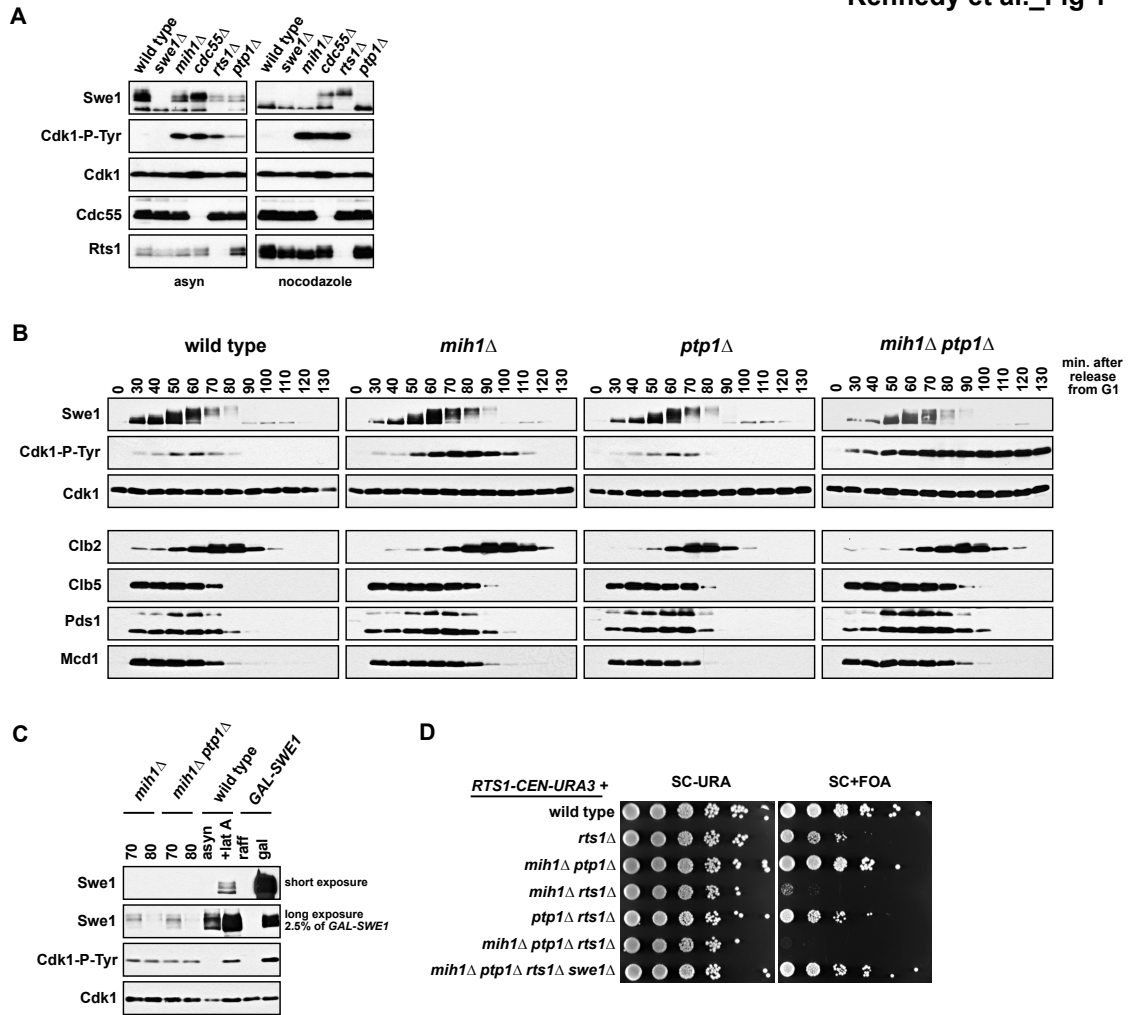


Figure 1. *PTP1* regulates Cdk1-Y19 phosphorylation *in vivo*.

(A) *mih1Δ*, *cdc55Δ*, *rts1Δ* and *ptp1Δ* cells have increased Cdk1-Y19 phosphorylation. Wild type, *swe1Δ*, *mih1Δ*, *cdc55Δ*, *rts1Δ*, and *ptp1Δ* cells were grown at 25°C asynchronously (asyn) or arrested in mitosis with nocodazole, harvested, lysed, and blotted with the indicated antibodies. (B) Wild type, *mih1Δ*, *ptp1Δ*, and *mih1Δ ptp1Δ* cells were grown at 25°C, arrested with mating pheromone, and released into the cell cycle at t=0. Cells were harvested, lysed and blotted with the indicated antibodies. (C) A comparison of Cdk1-Y19 phosphorylation in *mih1Δ* and *mih1Δ ptp1Δ* cells in samples from (B) at t=70 and t=80, wild type cells treated with latA (2.5μM) and cells overexpressing Swe1 from a *GAL-SWE1* gene that replaces the endogenous *SWE1*. Cells arrested by latA or Swe1 overexpression have significantly more Swe1 than *mih1Δ* and *mih1Δ ptp1Δ*, and have slightly higher levels of phosphorylated Cdk1-Y19. (D) Differences in Cdk1-Y19 phosphorylation correlate with changes in Cdk1/Clb2 histone H1 kinase activity. Wild type, *swe1Δ*, *mih1Δ* and *mih1Δ ptp1Δ* cells were grown in nocodazole (noc); or arrested with mating pheromone (αf; 100ng/ml) and released into the cell cycle at t=0 and either harvested at the time of peak Cdk1-Y19 phosphorylation (t=60' or 75' post-release) or incubated with latA for two hours. *GAL-SWE1* cells were grown in YEP+2% raffinose, arrested in nocodazole and then grown for ninety minutes after the addition of 2% galactose. Cells were harvested, lysed and blotted with the indicated antibodies (top panels), or Cdk1/Clb2 complexes were immunoprecipitated with anti-Clb2 antibody, and blotted with anti-Cdk1 and anti-Cdk1-P-tyr antibodies, or the Clb2-associated histone H1 kinase activity was assayed. The indicated ratios (average (±SEM) of three independent experiments) were calculated and normalized so that the ratio of synchronous mitotic wild type cells (t=60) is set to 1. (E) *mih1Δ rts1Δ* and *mih1Δ ptp1Δ rts1Δ* cells are inviable, and this inviability is rescued by *swe1Δ*. Ten-fold serial dilutions of the indicated strains were spotted on SC-URA or SC+FOA media and grown at 25°C. All strains harbour a *RTS1-CEN-URA3* plasmid and their dependence on *RTS1* was examined by selecting for loss of the plasmid using 5 fluoro-orotic acid (5-FOA), which counterselects for *URA3* cells.

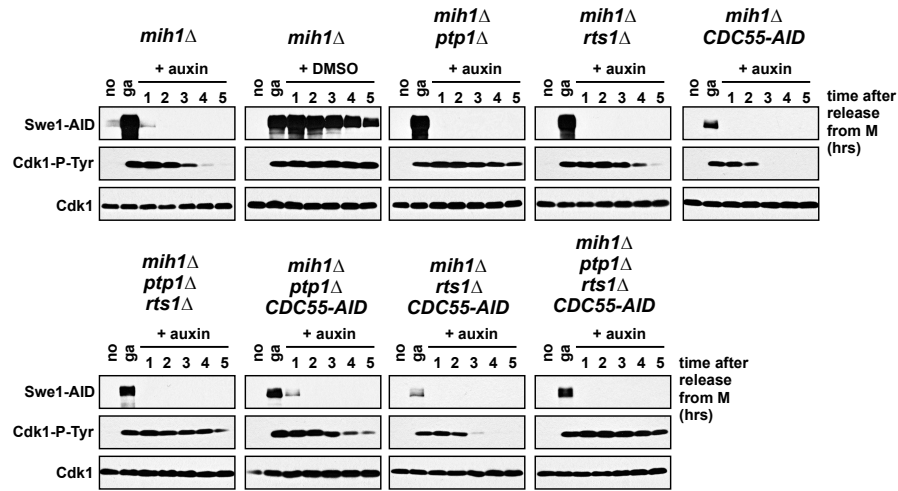
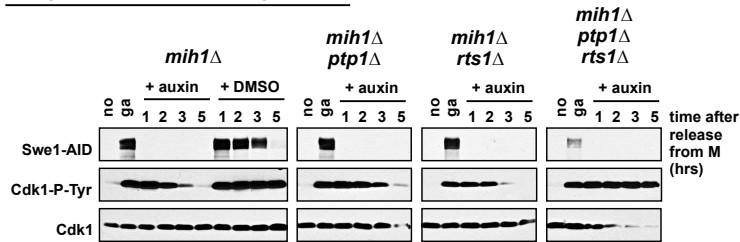
A + 25ng/ml alpha-factor during release**B** + 1μg/ml alpha-factor during release

Figure 2. *MIH1*, *PTP1* and *RTS1* redundantly regulate Cdk1-Y19 dephosphorylation.

The indicated strains were grown overnight in YEP + raffinose at 25°C, arrested in mitosis with nocodazole (noc), and then galactose was added to induce high levels of Swe1-AID and Cdk1-Y19 phosphorylation. After an hour of induction (gal), cells were released from the nocodazole arrest, dextrose was added to repress transcription of *GAL-SWE1-AID*, and auxin added to induce Tir1-dependent degradation of Swe1-AID and Cdc55-AID if present in the strain. Control *mih1Δ* cells were also released into media containing dextrose and vehicle (DMSO). α -factor was added 90 minutes after release at **(A)** 25 ng/ml or **(B)** 1 μ g/ml in order to re-arrest the cells in the subsequent G1. Cells were harvested at the indicated times for immunoblotting with the indicated antibodies.

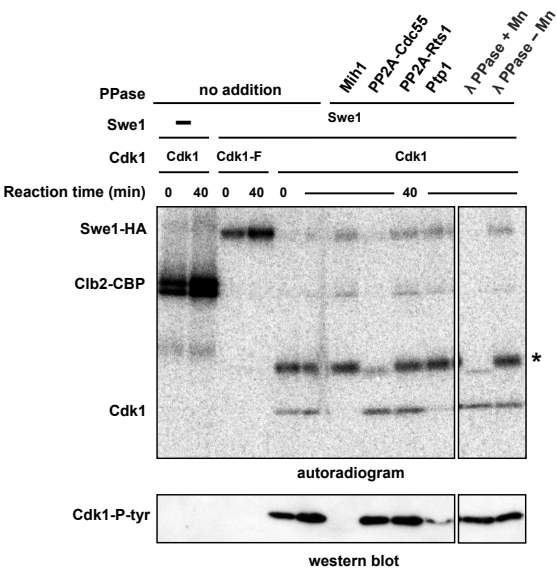


Figure 3. Mih1 and Ptp1 dephosphorylate Cdk1-Y19 *in vitro*. Purified Cdk1/Clb2-CBP was phosphorylated by bead-bound Swe1-HA in the presence of γ -[^{32}P]ATP. Following phosphorylation, Cdk1/Clb2-CBP was removed from the beads and mixed with purified phosphatases and an excess of unlabeled ATP to prevent re-phosphorylation of Cdk1 by γ -[^{32}P]ATP and Swe1-HA washed off the beads. Reactions were run on a polyacrylamide gel and either dried and exposed to phosphorimager screen, or transferred to nitrocellulose and immunoblotted with anti-Cdk1-phospho-Y19 antibody. In the absence of Swe1-HA, Clb2 is heavily phosphorylated by Cdk1. In the presence of Swe1-HA, Clb2 is not phosphorylated, showing Cdk1 is completely inhibited by Swe1-dependent phosphorylation of Y19. Swe1 does not phosphorylate Cdk1-F, but Cdk1-F phosphorylates Swe1 (Harvey *et al.* 2005). After incubation for 40 min at 25°C, purified Mih1 and Ptp1 (see Fig. S3) can dephosphorylate Cdk1-Y19 *in vitro*, but PP2A^{Cdc55}, PP2A^{Rts1}, nor lambda phosphatase (λ PPase) cannot. PP2A^{Cdc55} and λ PPase can effectively de-phosphorylate a fortuitous phosphorylated background protein (indicated with a *), providing a control indicating both PP2A^{Cdc55} and λ PPase are active.

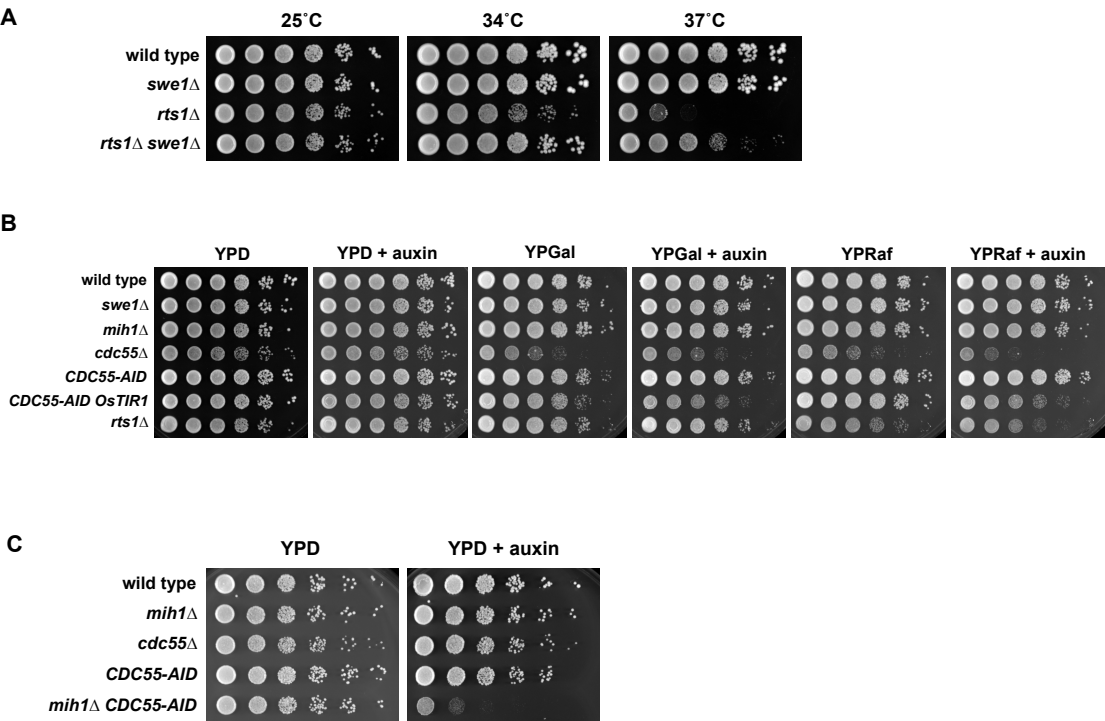


Figure S1. Additional analysis of *CDC55* and *RTS1*. (A) *rts1Δ* temperature sensitivity is partially suppressed by *swe1Δ*. Ten-fold serial dilutions of wild type, *swe1Δ*, *rts1Δ* and *rts1Δ swe1Δ* cells were spotted onto YPD plates and incubated at the indicated temperatures. (B) *cdc55Δ* and *CDC55-AID* grow poorly on non-fermentable carbon sources. Ten-fold serial dilutions of the indicated strains were spotted onto the indicated plates and incubated at 25 °C for two to three days. *cdc55Δ* grows poorly on YEP + 2% galactose (YPGal) and YEP + 2% raffinose (YPRaf) plates, and *CDC55-AID* cells, that also express *OsTIR1*, grow poorly on YPGal and YPRaf plates that contain 0.75mM auxin. (C) *mih1Δ CDC55-AID* cells are inviable on media containing auxin. Ten-fold serial dilutions of the indicated strains were spotted onto the indicated plates and incubated at 25 °C for two days.

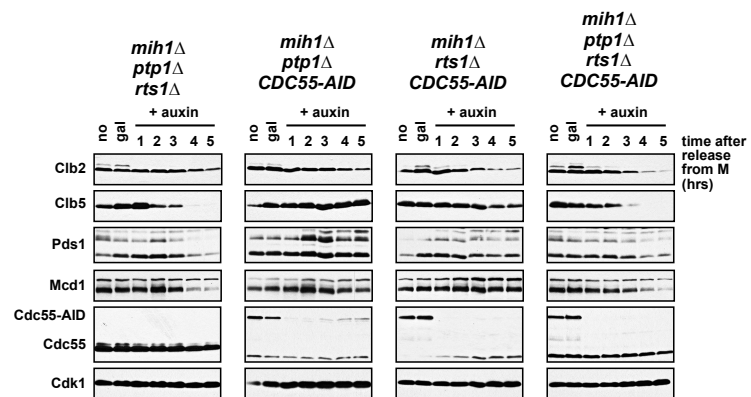
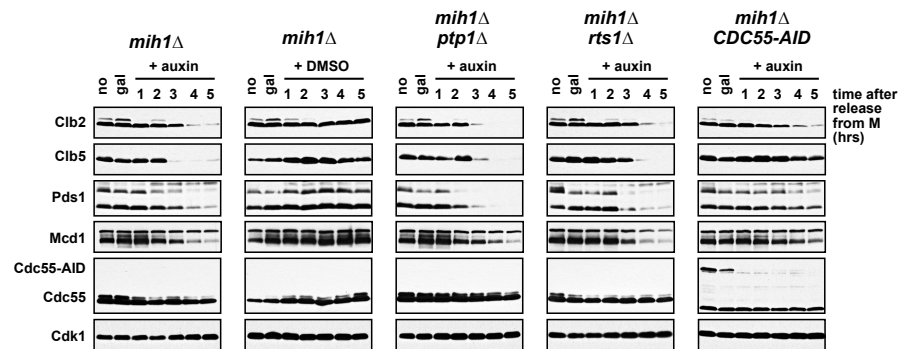
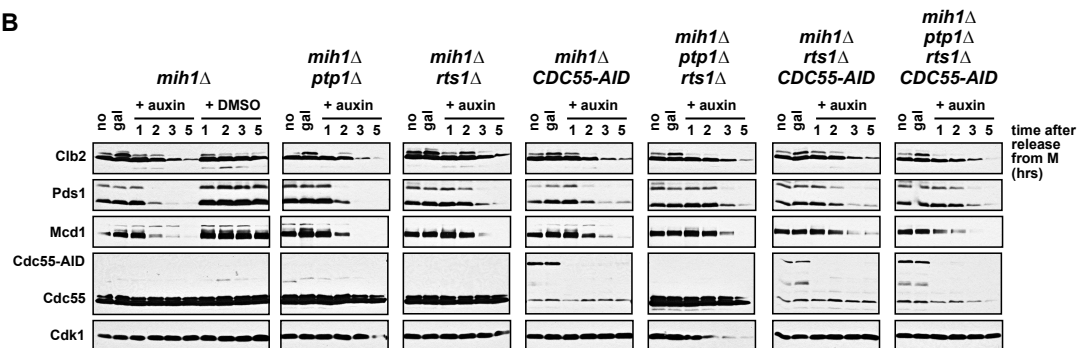
A**B**

Figure S2. *mih1* Δ *ptp1* Δ *rts1* Δ cells do not arrest the cell cycle in the *in vivo* phosphatase assay. (A) Samples from Fig. 2A were immunoblotted with the indicated antibodies. When 25ng/ml α -factor is added to the cultures after release from the nocodazole arrest, *CDC55-AID* strains arrest the cell cycle as determined by the stability of APC substrates (Clb2, Clb5 and Pds1) and degradation of full length Mcd1. **(B)** Samples from Fig. 2B (as well as additional samples from three strains) were immunoblotted with the indicated antibodies. When 1 μ g/ml α -factor is added to the cultures after release from the nocodazole arrest, all strains treated with auxin do not arrest the cell cycle.

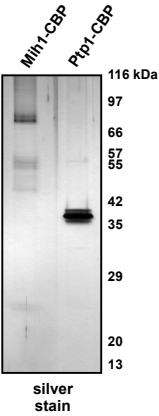


Figure S3. Purified phosphatases. Mih1-CBP and Ptp1-CBP were purified from cells harbouring *pGAL-MIH1-TAP-2 μ* or *pGAL-PTP1-TAP-2 μ* plasmids using a two-step TAP purification. PP2A-Cdc55 and PP2A-Rts1 complexes were purified from cells which contain an endogenously tagged *CDC55-TAP* or *RTS1-TAP*. 10% of each purification was separated by SDS-PAGE and silver stained.

Table 1. Complete list of strains

Strain	Genotype^a
ADR21	<i>MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100</i>
ADR22	<i>MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100</i>
ADR601	<i>MATa swe1Δ::TRP1</i>
ADR2562	<i>MATa pGAL-SWE1-HA-LEU2, bar1Δ</i>
ADR3173	<i>MATa swe1Δ::TRP1 bar1Δ</i>
ADR4006	<i>MATa bar1Δ</i>
ADR4031	<i>MATa swe1Δ::LEU2-pGAL-SWE1-HA mih1Δ::kanMX bar1Δ</i>
ADR4215	<i>MATa rts1Δ::kanMX</i>
ADR4217	<i>MATa kanMX-CDC28-Y19F</i>
ADR5297	<i>MATa RTS1-TAP-KITRPI bar1Δ</i>
ADR5465	<i>MATa CDC55-TAP-KITRPI bar1Δ</i>
ADR6320	<i>MATa CDC55-AID-kanMX bar1Δ</i>
ADR6385	<i>MATa CDC55-AID-kanMX leu2::pGPD1-OsTIR1-LEU2 bar1Δ</i>
ADR6876	<i>MATa cdc55Δ::hphMX bar1Δ</i>
ADR6929	<i>MATa rts1Δ::hphMX</i>
ADR7368	<i>MATa mih1Δ::hphMX CDC55-AID-kanMX leu2::pGPD1-OsTIR1-LEU2 bar1Δ</i>
ADR7447	<i>MATa mih1Δ::hphMX leu2::pGPD1-OsTIR1-LEU2 bar1Δ</i>
ADR7921	<i>MATa mih1Δ::hphMX CDC55-AID-kanMX swe1Δ::LEU2-pGAL-SWE1-AID-natMX trp1::pGPD1-OsTIR1-TRP1 SPC42-GFP-Sphis5⁺</i>

	<i>bar1Δ</i>
ADR7942	<i>MATa mih1Δ::hphMX ptp1Δ::CaURA3 bar1Δ</i>
ADR8041	<i>MATa mih1Δ::kanMX CDC55-AID-kanMX swe1Δ::LEU2-pGAL-SWE1-AID-natMX trp1::pGPD1-OsTIR1-TRP1 SPC42-GFP-Sphis5⁺ bar1Δ</i>
ADR8300	<i>MATa ptp1Δ::CaURA3</i>
ADR8304	<i>MATa mih1Δ::kanMX ptp1Δ::CaURA3 rts1Δ::hphMX CDC55-AID-kanMX swe1Δ::LEU2-pGAL-SWE1-AID-natMX trp1::pGPD1-OsTIR1-TRP1 SPC42-GFP-Sphis5⁺ bar1Δ</i>
ADR9027	<i>MATa rts1Δ::hphMX [RTS1-CEN-URA3 (pAR1286)]</i>
ADR9028	<i>MATa [RTS1-CEN-URA3 (pAR1286)]</i>
ADR9029	<i>MATa mih1Δ::LEU2 ptp1Δ::natMX [RTS1-CEN-URA3 (pAR1286)]</i>
ADR9030	<i>MATa mih1Δ::LEU2 rts1Δ::hphMX [RTS1-CEN-URA3 (pAR1286)]</i>
ADR9031	<i>MATa ptp1Δ::natMX rts1Δ::hphMX [RTS1-CEN-URA3 (pAR1286)]</i>
ADR9032	<i>MATa mih1Δ::LEU2 ptp1Δ:: natMX rts1Δ::kanMX swe1Δ::TRP1 [RTS1-CEN-URA3 (pAR1286)]</i>
ADR9034	<i>MATa mih1Δ::LEU2 ptp1Δ::natMX rts1Δ::hphMX [RTS1-CEN-URA3 (pAR1286)]</i>
ADR9422	<i>MATa mih1Δ::kanMX ptp1Δ::CaURA3 swe1Δ::LEU2-pGAL-SWE1-AID-natMX trp1::pGPD1-OsTIR1-TRP1 SPC42-GFP-Sphis5⁺ bar1Δ::hphMX</i>
ADR9423	<i>MATa mih1Δ::kanMX ptp1Δ::CaURA3 CDC55-AID-kanMX swe1Δ::LEU2-pGAL-SWE1-AID-natMX trp1::pGPD1-OsTIR1-TRP1</i>

	<i>SPC42-GFP-Sphis5⁺ bar1Δ</i>
ADR9461	<i>MATa ptp1Δ::CaURA3 bar1Δ</i>
ADR9475	<i>MATa mih1Δ::kanMX rts1Δ::hphMX swe1Δ::LEU2-pGAL-SWE1-AID-natMX trp1::pGPD1-OsTIR1-TRP1 SPC42-GFP-Sphis5⁺ bar1Δ::CaURA3</i>
ADR9477	<i>MATa mih1Δ::kanMX ptp1Δ::CaURA3 rts1Δ::hphMX swe1Δ::LEU2-pGAL-SWE1-AID-natMX trp1::pGPD1-OsTIR1-TRP1 SPC42-GFP-Sphis5⁺ bar1Δ::hphMX</i>
ADR9485	<i>MATa mih1Δ::kanMX swe1Δ::LEU2-pGAL-SWE1-AID-natMX trp1::pGPD1-OsTIR1-TRP1 SPC42-GFP-Sphis5⁺ bar1Δ::hphMX</i>
ADR9532	<i>MATa rts1Δ::kanMX swe1Δ::TRP1</i>
ADR9536	<i>MATa rts1Δ::kanMX</i>
ADR9539	<i>MATa swe1Δ::TRP1</i>

^a All strains are isogenic to W303-1a (ADR21) and W303-1b (ADR22).

Table 2. Strains used in each figure

Figure	Relevant genotype ^a	Strain number
1A	wild type	ADR4006
	<i>swe1Δ</i>	ADR3173
	<i>mih1Δ</i>	ADR7447
	<i>cdc55Δ</i>	ADR6876
	<i>rts1Δ</i>	ADR6929
	<i>ptp1Δ</i>	ADR8300
1B	wild type	ADR4006
	<i>mih1Δ</i>	ADR7747
	<i>ptp1Δ</i>	ADR9461
	<i>mih1Δ ptp1Δ</i>	ADR7942
1C	<i>mih1Δ</i>	ADR7747
	<i>mih1Δ ptp1Δ</i>	ADR7942
	wild type	ADR4006
	<i>GAL-SWE1</i>	ADR4031
1D	wild type	ADR4006
	<i>swe1Δ</i>	ADR3173

	<i>mih1Δ</i>	ADR7747
	<i>mih1Δ ptp1Δ</i>	ADR7942
	<i>GAL-SWE1</i>	ADR4031
1E	<i>[RTS1-CEN-URA3]</i>	ADR9028
	<i>rts1Δ [RTS1-CEN-URA3]</i>	ADR9027
	<i>mih1Δ ptp1Δ [RTS1-CEN-URA3]</i>	ADR9029
	<i>mih1Δ rts1Δ [RTS1-CEN-URA3]</i>	ADR9030
	<i>ptp1Δ rts1Δ [RTS1-CEN-URA3]</i>	ADR9031
	<i>mih1Δ ptp1Δ rts1Δ [RTS1-CEN-URA3]</i>	ADR9034
	<i>mih1Δ ptp1Δ rts1Δ swe1Δ [RTS1-CEN-URA3]</i>	ADR9032
2A	<i>mih1Δ pGAL-SWE1-AID</i>	ADR9485
	<i>mih1Δ ptp1Δ pGAL-SWE1-AID</i>	ADR9422
	<i>mih1Δ rts1Δ pGAL-SWE1-AID</i>	ADR9475
	<i>mih1Δ CDC55-AID pGAL-SWE1-AID</i>	ADR7921
	<i>mih1Δ ptp1Δ rts1Δ pGAL-SWE1-AID</i>	ADR9477
	<i>mih1Δ ptp1Δ CDC55-AID pGAL-SWE1-AID</i>	ADR9423
	<i>mih1Δ rts1Δ CDC55-AID pGAL-SWE1-AID</i>	ADR8041
	<i>mih1Δ ptp1Δ rts1Δ CDC55-AID pGAL-SWE1-AID</i>	ADR8304
2B	<i>mih1Δ GAL-SWE1-AID</i>	ADR9485

	<i>mih1Δ ptp1Δ GAL-SWE1-AID</i>	ADR9422
	<i>mih1Δ rts1Δ GAL-SWE1-AID</i>	ADR9475
	<i>mih1Δ ptp1Δ rts1Δ GAL-SWE1-AID</i>	ADR9477
3	<i>GAL-SWE1-HA</i>	ADR2562
	wild type	ADR4006
	<i>CDC55-TAP</i>	ADR5465
	<i>RTS1-TAP</i>	ADR5297
	<i>swe1Δ</i>	ADR601
	<i>CDC28-Y19F</i>	ADR4217
S1A	wild type	ADR22
	<i>swe1Δ</i>	ADR9539
	<i>rts1Δ</i>	ADR9536
	<i>rts1Δ swe1Δ</i>	ADR9532
S1B	Wild type	ADR4006
	<i>swe1Δ</i>	ADR601
	<i>mih1Δ</i>	ADR7747
	<i>cdc55Δ</i>	ADR6876
	<i>CDC55-AID</i>	ADR6320
	<i>CDC55-AID OsTIR1</i>	ADR6385

	<i>rts1Δ</i>	ADR4215
S1C	Wild type	ADR4006
	<i>mih1Δ</i>	ADR7747
	<i>cdc55Δ</i>	ADR6876
	<i>CDC55-AID OsTIR1</i>	ADR6385
	<i>mih1Δ CDC55-AID OsTIR1</i>	ADR7368
S2A	<i>mih1Δ GAL-SWE1-AID</i>	ADR9485
	<i>mih1Δ ptp1Δ GAL-SWE1-AID</i>	ADR9422
	<i>mih1Δ rts1Δ GAL-SWE1-AID</i>	ADR9475
	<i>mih1Δ CDC55-AID GAL-SWE1-AID</i>	ADR7921
	<i>mih1Δ ptp1Δ rts1Δ GAL-SWE1-AID</i>	ADR9477
	<i>mih1Δ ptp1Δ CDC55-AID GAL-SWE1-AID</i>	ADR9423
	<i>mih1Δ rts1Δ CDC55-AID GAL-SWE1-AID</i>	ADR8041
	<i>mih1Δ ptp1Δ rts1Δ CDC55-AID GAL-SWE1-AID</i>	ADR8304
S2B	<i>mih1Δ GAL-SWE1-AID</i>	ADR9485
	<i>mih1Δ ptp1Δ GAL-SWE1-AID</i>	ADR9422
	<i>mih1Δ rts1Δ GAL-SWE1-AID</i>	ADR9475
	<i>mih1Δ CDC55-AID GAL-SWE1-AID</i>	ADR7921
	<i>mih1Δ ptp1Δ rts1Δ GAL-SWE1-AID</i>	ADR9477

	<i>mih1Δ rts1Δ CDC55-AID GAL-SWE1-AID</i>	ADR8041
	<i>mih1Δ ptp1Δ rts1Δ CDC55-AID GAL-SWE1-AID</i>	ADR8304
S3	wild type	ADR4006
	<i>swe1Δ</i>	ADR601
	<i>GAL-SWE1-HA</i>	ADR2562
	<i>CDC28-Y19F</i>	ADR4217
	<i>CDC55-TAP</i>	ADR5465
	<i>RTS1-TAP</i>	ADR5297

^a See Supplemental Table 1 for complete genotype

Materials and Methods

Strain and Plasmid Construction

Supplemental Table 1 lists the strains used in this work and Supplemental Table 2 lists the strains used in each figure. All strains are derivatives of the W303 strain background (W303-1a), see Table S1 for complete genotype. All deletions and replacements were confirmed by immunoblotting, phenotype or PCR. Strains were constructed by genetic cross and transformation. The sequences of all primers used in this study are available upon request. Phusion polymerase (NEB) was used for all PCR reactions. The bacterial strains TG1 and DH5 α were used for amplification of DNA.

cdc55 Δ ::hphMX, *rts1 Δ ::hphMX*, *mih1 Δ ::hphMX* and *bar1 Δ ::hphMX* were constructed by amplifying *hphMX* off pAG32 (Goldstein and McCusker 1999) and deleting *CDC55*, *RTS1*, *MIH1* and *BAR1* respectively. *ptp1 Δ ::natMX* was constructed by amplifying *natMX* off pAG25 (Goldstein and McCusker 1999) and deleting *PTP1*. *mih1 Δ ::kanMX* and *rts1 Δ ::kanMX* was constructed by amplifying *kanMX* off *pFA6a-kanMX6* (Longtine *et al.* 1998) and deleting *MIH1* and *RTS1*. *ptp1 Δ ::CaURA3* was constructed by amplifying *CaURA3* off pAR747 {Larin:2015bp} and deleting *PTP1*. Unmarked *bar1 Δ* was constructed using pJGsst1 (J. Thorner, University of California, Berkeley, CA). *mih1 Δ ::LEU2* was constructed using pIP33 (P. Sorger, Harvard Medical School, Boston, MA). *swe1 Δ ::TRP1* strains were made by crossing JM449 (J. Minshull, DNA2.0, Menlo Park, CA) to the appropriate strains.

RTS1-CEN-URA3 was constructed by PCR amplifying *RTS1* with upstream and downstream regions (207bp and 195bp, respectively) and cloning the PCR fragment into pRS316 digested with BamH1/Xho1 to create pAR1286.

swe1 Δ ::LEU2-pGAL-SWE1-HA was constructed using pSwe1-41 (a gift of Bob

Booher, Onyx Pharmaceuticals, Richmond, CA; (Booher *et al.* 1993)). *swe1Δ::LEU2-pGAL-SWE1-AID-natMX* was constructed by amplifying *AID* and *natMX* off pAR1099 and integrating the resulting PCR product at *swe1Δ::LEU2-pGAL-SWE1-HA* to replace the HA tag with the AID tag. pAR1099 was created by cloning a PacI/SalI *natMX* fragment from pAG25 into pAID1 (Nishimura *et al.* 2009) digested with PacI/SalI. *CDC55-AID-kanMX* was constructed by amplifying *AID* and *kanMX* off pAID1 and integrating the resulting PCR product at *CDC55*. *leu2::pGPD1-OsTIR1-LEU2* and *trp1::pGPD1-OsTIR1-TRP1* were constructed by digesting pTIR4 and pTIR6 (Nishimura *et al.* 2009) with StuI and NdeI, respectively, to integrate at *LEU2* or *TRP1* (plasmids were gifts of T. Eng and D. Koshland, UC, Berkeley, Berkeley, CA).

The *2μ-pGAL-CLB2-TAP-URA3* (pAR546), *2μ-pGAL-MIH1-TAP-URA3* (pAR1327) and *2μ-pGAL-PTP1-TAP-URA3* (pAR1328) were created by PCR amplifying *CLB2*, *MIH1* and *PTP1*, respectively, and the resultant PCR product, designed to have overlapping homology, was co-transformed into yeast along with pRS-AB1234 (C. Carroll and D.O. Morgan, UC San Francisco, San Francisco, CA) cut with BamHI and HindIII. *CDC55-TAP-KITRPI* and *RTS1-TAP-KITRPI* strains were made using pBS1479 (Rigaut *et al.* 1999).

The construction of *kanMX-CDC28-Y19F* was described previously (Liang *et al.* 2013).

Physiology

Unless noted in the figure legend, cells were grown in yeast extract peptone media + 2% dextrose (YEPD) at 25°C or 30°C. Cells cycle arrests were performed with 10μg/mL nocodazole (Sigma-Aldrich; made as a 10mg/ml stock in DMSO) and 1μg/mL α-factor (Biosynthesis; made as a 10mg/ml stock in DMSO) for 3 hours. LatA was used at 2.5μM

(Sigma-Aldrich or Tocris Biosciences). Auxin (indole-3-acetic acid, Sigma-Aldrich; made as a 1M stock in DMSO) was used at 500 μ M in liquid and solid media. Plate-based viability assays were performed using a multi-pronged serial dilution fork (DAN-KAR).

Western Blots

These methods have been described previously (Rudner *et al.* 2005; Lianga *et al.* 2013). Yeast extracts for Western blotting were made by bead beating (multitube bead beater; Biospec) frozen cell pellets in 1X sample buffer (2% SDS, 80mM Tris-HCl pH6.8, 10% glycerol, 10mM EDTA, bromophenol blue, 5% 2-mercaptoethanol). Samples were normalized by cell concentration before harvesting.

Standard methods were used for polyacrylamide gel electrophoresis and protein transfer to nitrocellulose (Pall). Typically, samples were run on 12.5% polyacrylamide gels (120:1 acrylamide:bis, respectively, with no added SDS). Blots were stained with Ponceau S to confirm transfer and equal loading of samples, and then were blocked for 30 min in blocking buffer (4% nonfat dried milk (Carnation) in TBST (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20). All antibodies were incubated overnight at 4°C. Unless noted, primary antibodies were used in TBS-T with 4% fat free milk powder, 5% glycerol and 0.02 % NaN₃. An autoclaved solution of 5% milk was used to make the 4% milk dilution buffer to increase the longevity of the antibody solution. After washing with TBST, the blots were incubated with horseradish peroxidase-conjugated anti-rabbit antibodies (Bio-Rad) at a 1:5,000 dilution in blocking buffer for 30 min at 25°C, washed again, incubated incubated in Western Lightning Plus-ECL (PerkinElmer). Signal detection was on HyBlot CL (Harvard Scientific) autoradiography film.

The use and production of 12CA5 ascites (BabCO), anti-Swe1, anti-Clb2, anti-Pds1,

anti-Clb5, anti-Mcd1, anti-Cdk1, anti-Cdc2-Y15 (Cell Signaling) antibodies have been described previously (Kellogg and Murray 1995; Lianga *et al.* 2013). Anti-Cdc55 and anti-Rts1 rabbit polyclonal antibodies were used at 1:1000 and generated as follows: 1mg of GST-Cdc55 (aa322-462) and GST-Rts1 (aa1-150) was injected into rabbits every 4 weeks for 8 to 16 weeks (uOttawa animal facility). Rabbit serum was harvested, clarified by centrifugation and loaded on Affigel-10 (Bio-rad) columns coupled to purified malE-Cdc55 (aa322-462) and malE-Rts1 (aa1-150), respectively. Antibody was eluted from Affigel columns with either 100mM triethylamine pH 11.5 or 100mM glycine pH 2.3. The triethylamine and glycine elutions were neutralized, dialyzed in PBS + 50% glycerol and stored at -80°C.

GST-Cdc55 and GST-Rts1 were expressed from pAR739 and pAR740, respectively, in which Cdc55 (aa322-462) and Rts1 (aa1-150) were cloned as BamH1/EcoR1 fragments into pGEX-6P. malE-Cdc55 and malE-Rts1 were expressed from pAR1115 and pAR1116, respectively, in which Cdc55 (aa322-462) and Rts1 (aa1-150) were cloned as BamH1/Sal1 fragments into mMAL-c2 (NEB).

Immunoprecipitation and *in vitro* histone H1 kinase assays

These methods have been described previously (Rudner and Murray 2000). Cell pellets were lysed by mechanical disruption (Biospec mini beadbeater-16; 2 pulses of 20'' with a 5' incubation on ice between pulses) in APC lysis buffer (50 mM Hepes-KOH pH 7.8, 700 mM NaCl, 150 mM NaF, 150 mM Na-β-glycerophosphate pH 8.3, 1 mM EDTA, 1 mM EGTA, 5% glycerol, 0.25% NP-40, 1 mM DTT, 1 mM PMSF, 1 mM Na₃VO₄, 1 mM benzamidine, and leupeptin, bestatin, pepstatin A and chymostatin all at 1 mM), lysates clarified by centrifugation (5' at top speed in a microfuge) and Clb2 complexes isolated by

immunoprecipitation using 0.5 μ l of anti-Clb2 antibody and proteinA coupled dynabeads (Invitrogen). Beads were washed three times in APC lysis buffer, split in half into fresh tubes during the third wash, and washed one time in kinase buffer (20mM Tris-HCl, pH 8.0, 50mM NaCl, 5mM MgCl₂, 0.01% NP-40, 1mM DTT). One set of beads was incubated in 15 μ l kinase buffer containing 2.5 μ g histone H1 (Upstate Biotechnology), 0.01 mM ATP and 1 μ Ci γ -[³²P]ATP for 20' at 25°C and the reaction was stopped by addition of 15 μ l 2X sample buffer. Reactions were analyzed by PAGE, dried and exposed to phosphorimager screen and the phosphorylation of histone H1 was quantified on a Typhoon Trio Phosphorimager and ImageQuant software (GE). Cdk1/Clb2 complexes were eluted from the other set of beads with 1X sample buffer, separated by PAGE, immunoblotted with anti-Cdk1 and anti-Cdc2-Y15 (Cell Signaling) antibodies, and the amount of Cdk1 and Cdk1-P-tyr in each IP was quantified using a ImageQuant LAS4000 imager and ImageQuant software (GE).

***In vivo* Cdk1-Y19 phosphatase assay**

Cultures were grown overnight at 25°C in YEP + 2% raffinose and arrested in mitosis using nocodazole (Sigma-Aldrich) for 2.5 hrs at 25°C. Galactose (2%) was then added while cultures were still arrested for 30 min. Cells were harvested, and washed in YPD + 500 μ M auxin (or DMSO vehicle) to release from the nocodazole arrest, to repress transcription from the *pGAL* promoter and activate Tir1/SCF-dependent degradation of AID-tagged proteins. α -factor was added at t=1 hr following release to capture cells in G1. Samples were taken every hour min for 5 hrs and analyzed by western blot.

***In vitro* phosphatase assay**

GAL-SWE1-TAP (ADR2562) was grown overnight at 25°C in YEP + 2% raffinose and induced with 2% galactose for 6 hrs. Cultures were harvested, washed with 50mM Hepes-KOH pH7.8, and 2 ml of APC lysis buffer was added per gram of pellet and lysed by bead beating (BioSpec) with acid washed glass beads (BioSpec). Lysate was clarified by centrifugation at 16,000X g for 10 min at 4°C. 1µl of 12CA5 anti-HA (BabCO) was incubated with the lysate on ice for 10 min, 5µl of protein G-coupled magnetic beads (Invitrogen) was added per 100µl of lysate and incubated for 1 hr at 4°C with rotation. Beads were washed 3X with APC lysis buffer and resuspended in Kinase buffer (20 mM HEPES pH 7.5 KOH, 5 mM MgCl₂, 5% glycerol).

Cdk1/Clb2-CBP, Cdk1-F/Clb2-CBP, Mih1-CBP and Ptp1-CBP were purified from cells containing pAR546 (*2µ-pGAL-CLB2-TAP*), pAR1327 (*2µ-pGAL-MIH1-TAP*) and pAR1328 (*2µ-pGAL-PTP1-TAP*), respectively, after induction by growth in galactose. Cdc55-CBP and Rts1-CBP complexes were purified from asynchronously growing *CDC55-TAP* (ADR5465) or *RTS1-TAP* (ADR5297) cells.

Protein complexes were purified as described previously (Rudner *et al.* 2005; Lianga *et al.* 2013). Harvested cells were lysed in APC lysis buffer by mechanical disruption with 0.5mm glass beads (Biospec mini beadbeater-16 for small scale purification (1-5 grams; 2 pulses of 20'' with a 5' incubation on ice between pulses), or Biospec blender beadbeater for large scale scale purification (5-20 grams; 10 pulses of 30'' with 30'' incubation in an ice bath)). Lysate was clarified by centrifugation (microfuge for 10' or Sorval SA-600 rotor at 15,000 rpm for 20'), and incubated with 100-500µl of a slurry of IgG-sepharose (GE) for four hours. Beads were pelleted at 500g, washed one time in lysis buffer, and transferred into a 3 ml column (Bio-rad bio-spin disposable chromatography column), and washed

extensively with lysis buffer and TEV cleavage buffer (10mM Tris-HCl pH 8.0, 150mM NaCl, 0.5mM EDTA, 5% glycerol, 0.1% NP-40, and added just before use 1mM DTT, 1mM PMSF). Beads were incubated overnight at 4°C in an equal volume of TEV cleavage buffer and 10-20µl of TEV protease. After cleavage the column was drained and the supernatant was supplemented with 5mM CaCl₂ and 10mM 2-mercaptoethanol, mixed with 100µl of calmodulin-Sepharose in a new 3ml column and incubated for 1-2 hours at 4°C. The resin was then washed extensively in CaM binding buffer (10mM Tris-HCl pH 8.0, 150mM NaCl, 0.5mM EDTA, 5% glycerol, 0.1% NP-40, 1mM MgCl₂, 2mM CaCl₂ and added just before use 10mM 2-mercaptoethanol, 1mM PMSF), and the protein complexes eluted in CaM elution buffer (CaM binding buffer in which the CaCl₂ is replaced with 10mM EGTA) either in batch or in 200µl fractions. Fractions were analyzed by gel electrophoresis followed by silver staining, and for Cdk1/Clb2-CBP complexes, tested by *in vitro* phosphorylation of histone H1 in the presence of γ -[³²P]-ATP.

Purified Cdk1/Clb2-CBP and Cdk1-F/Clb2-CBP complexes were incubated with purified Swe1 (on beads) in kinase buffer with 1 mM DTT, 0.001 mM ATP, 1µCi of γ -[³²P]-ATP (Perkin Elmer) for 60 min at room temperature. Beads were removed and the phosphorylated Cdk1 complexes were mixed with purified phosphatases or lambda phosphatase (NEB), and 1mM ATP. Lambda phosphatase was inhibited by removing MnCl₂ from the reaction. Reactions were incubated for 40 minutes at room temperature and stopped by the addition of 2X sample buffer. Samples were analyzed by PAGE, dried and exposed to phosphorimager screen, and imaged using a Typhoon Trio Phosphorimager (GE).

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Appendix 1

This section of the thesis incorporates data from the *TRNTI/CCAI* project (Chapters 2 and 3 of this thesis) not intended for publication.

A1.1. Results

A1.1.1. 4E-BP eIF4E interaction

No change in migration due to phosphorylation of Eap1 and Caf20 was observed in *cca1-V188S* at permissive and semi-permissive temperatures (Figure A1.1), indicating that loss of Cca1 function does not change the phosphorylation state of the 4E-BPs as a means of regulating translation in response to stress. Furthermore, no change in Eap1 or Caf20 binding to Cdc33 (yeast eIF4E) was detected in *cca1-V188S* under conditions tested (Figure A1.2). More work, including positive controls, is needed to further support this conclusion.

A1.1.2. Over expression of *EAP1*

Because of the synthetic interaction of *eap1Δ* and *cca1-V188S* / *TRNT1* mutants, we tested if over expression of *EAP1* could suppress the growth phenotype. In *cca1-V188S*, the addition of *EAP1* on a high copy number plasmid did not suppress the phenotype, as seen in *cca1-V188S* 2μ-empty and *cca1-V188S* 2μ-*EAP1* having the same growth on either carbon source at all temperatures (Figure A1.3). The *eap1Δ* 2μ-*EAP1* was able to suppress the growth defect of *eap1Δ* 2μ-empty, indicating Eap1 was being expressed (Figure A.1.3). The presence of *EAP1* on a the expression plasmid may result in some growth defects, as observed in wild type 2μ-empty having better growth than wild type 2μ-EAP1 at 32.5°C on glycerol (Figure A.1.3). High copy number plasmids provide copy number of 20 to 100 copies per cell, it should be noted that this dosage could also be too high in some cases.

Interestingly, at 36.5°C on dextrose and 32.5°C on glycerol, *cca1-V188S eap1Δ* 2μ-*EAP1* grew better than *cca1-V188S eap1Δ* 2μ-empty (Figure A.1.3). This was also observed for *TRNT1* wild type *eap1Δ* 2μ-*EAP1* compared to *TRNT1* wild type *eap1Δ* 2μ-empty

Figure A1.1.

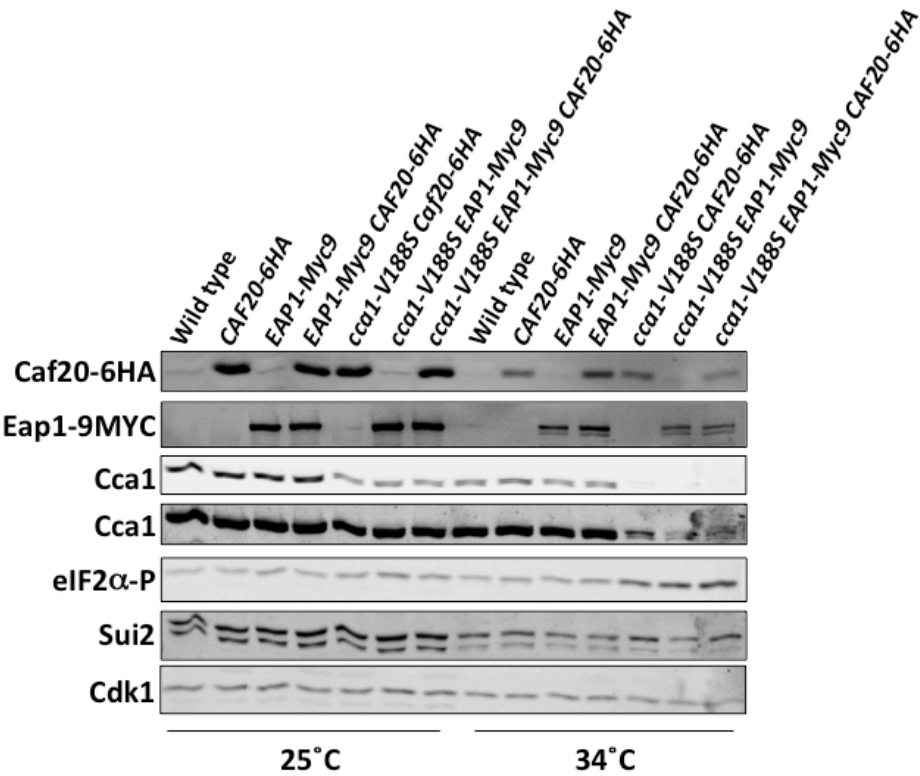
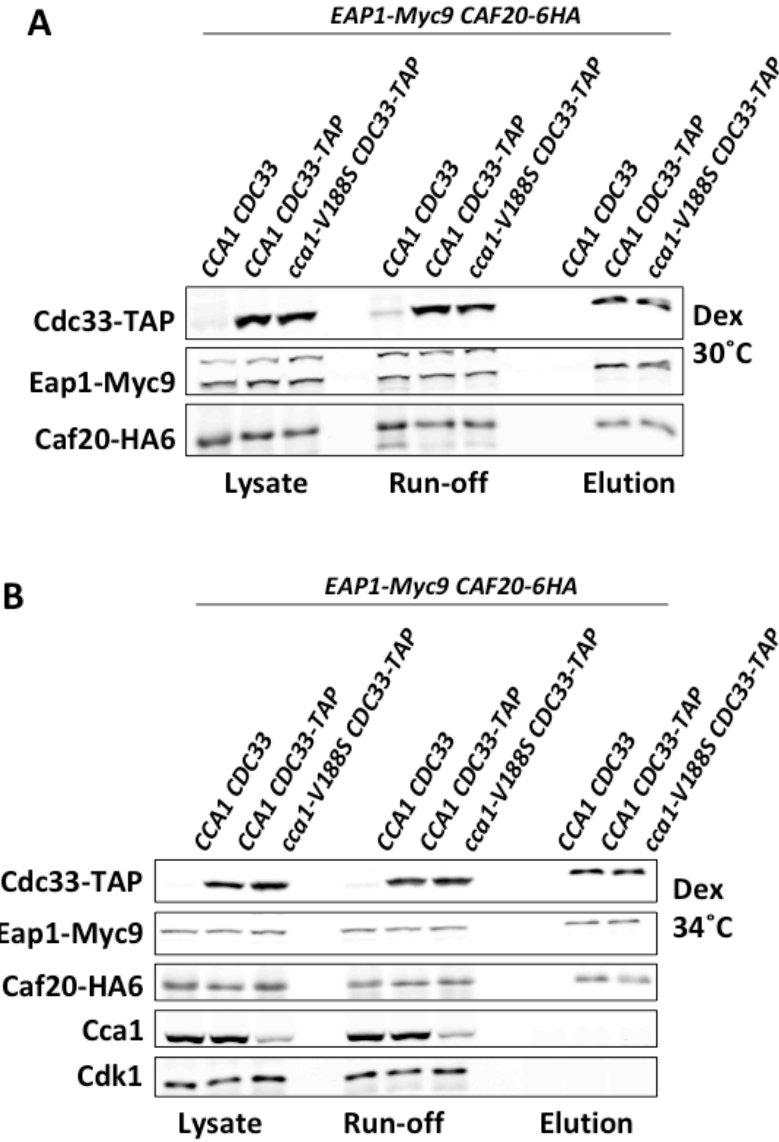


Figure A1.1. *cca1-V188S* mutation did not result in a difference in phosphorylation of Eap1 or Caf20 based on size shift. Wild type (ADR22), *CAF20-HA* (EKK287), *EAP1-Myc9* (EKK289), *EAP1-Myc9 CAF20-HA* (EKK300), *cca1-V188S CAF20-HA* (EKK312), *cca1-V188S EAP1-Myc9* (EKK310), and *cca1-V188S EAP1-Myc9 CAF20-HA* (EKK302) were grown overnight, diluted and grown at the indicated temperatures for 3 hrs, and harvested. Samples were mechanically lysed in 1X sample buffer and analyzed by western blot with the indicated antibodies.

Figure A1.2.



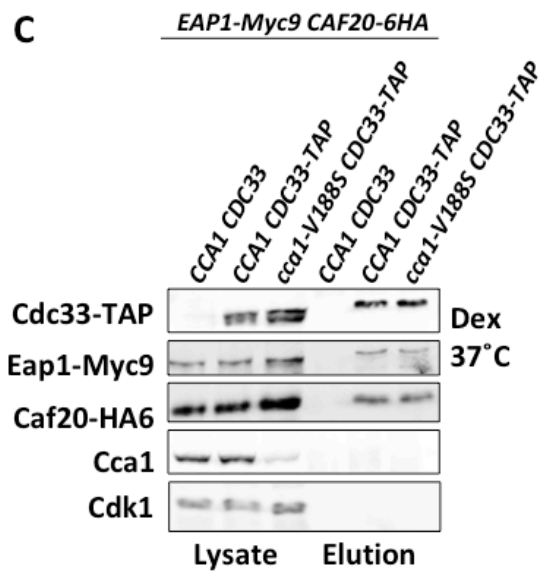


Figure A1.2. *cca1-V188S* did not change Eap1 or Caf20 binding to eIF4E (*CDC33*). *EAP1-Myc9 CAF20-HA* (EKK300), *EAP1-Myc9 CAF20-HA CDC33-TAP* (EKK437), and *cca1-V188S EAP1-Myc9 CAF20-HA CDC33-TAP* (EKK442) were grown between 1.5 to 2.5×10^7 cells/mL at 25°C overnight then transferred to (A) 30°C, (B) 34°C, or (C) 37°C for one hour before cells were harvested and lysed. Lysate was incubated with IgG dynabeads overnight at 4°C, washed and eluted with sample buffer prior to western blot analysis with the indicated antibodies.

Figure A1.3.

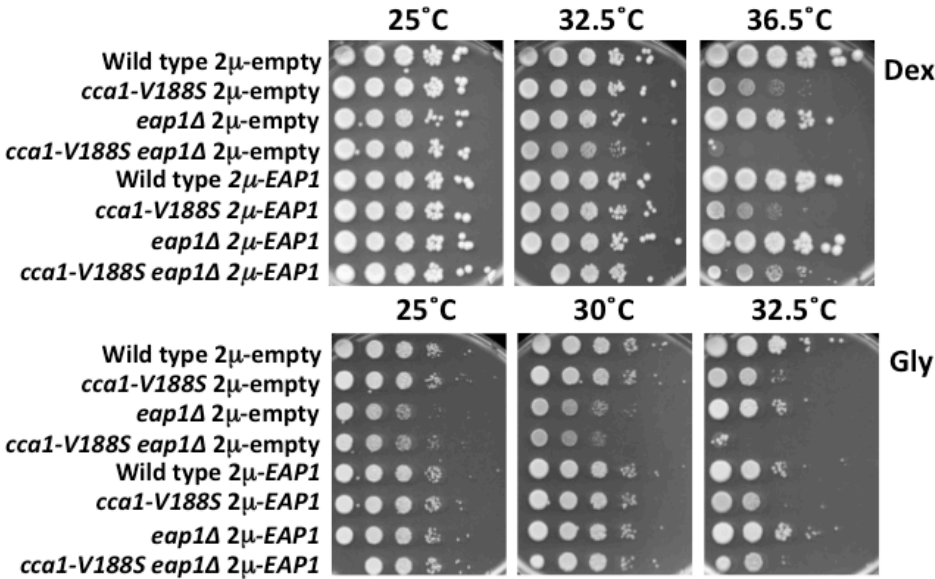


Figure A1.3. Over expression of *EAP1* in an *eap1*Δ was suppressive to *cca1-V188S*.

Ten-fold serial dilutions of wild type 2μ-empty (EKK481), *cca1-V188S* 2μ-empty (EKK483), *eap1*Δ 2μ-empty (EKK485), *cca1-V188S eap1*Δ 2μ-empty (EKK487), wild type 2μ-*EAP1* (EKK473), *cca1-V188S* 2μ-*EAP1* (EKK475), *eap1*Δ 2μ-*EAP1* (EKK477), and *cca1-V188S eap1*Δ 2μ-*EAP1* (EKK479) were grown overnight at 25°C to 2 to 4 x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates and grown at the indicated temperatures for 2 to 4 days.

(A1.4A) and *TRNT1-L166S eap1Δ 2μ-EAP1* compared to *TRNT1-L166S eap1Δ 2μ-empty* (A1.4B). Therefore, although over expression of *EAP1* did not suppress the mutant growth phenotype, over expression of *EAP1* in the *TRNT1/CCA1* mutants when *EAP1* was deleted resulted in better growth than *TRNT1/CCA1* mutants alone. Dosage is likely very important when the amount of Eap1 in a cell may change the amount bound to eIF4E or change the proportion activated or inactivated by phosphorylation state. We show that a moderate increase in amount of Eap1 (high expression plasmid in a delete background) was beneficial to *TRNT1/CCA1* mutants, where lack of Eap1 (*eap1Δ*) or too much Eap1 (*EAP1* and high expression plasmid of *EAP1*) was detrimental or provided no suppression.

A1.1.3. Decreasing TOR signaling

Interestingly, the predominantly cytoplasmic-localized Cca1 (2 % mitochondrial) re-localizes to the mitochondria when exposed to increasing doses of rapamycin (48 % mitochondrial with rapamycin); this is in non-respiratory requiring conditions (CYCLOPS, Koh et al., 2015). Many other mitochondrial proteins also showed this effect (CYCLOPS, Koh et al., 2015), which is consistent with observations that caloric restriction increases mitochondrial respiration, protein synthesis, and amino acid catabolism (Johnson et al., 2014). This occurs independent of carbon (respiration or non-respiration) sources, and is proposed to inhibit cytoplasmic anabolism preventing cellular proliferation in times of nutrient stress (Johnson et al., 2014). Therefore, I looked at the affect of rapamycin on *TRNT1* and *cca1* mutants. *cca1-V188S* and *TRNT1* wild type were more sensitive rapamycin than *CCA1* wild type, but *TRNT1-L166S* showed increased resistance to rapamycin compared to wild type and similar to petite strains (Figure A1.5A). This is consistent with

Figure A1.4.

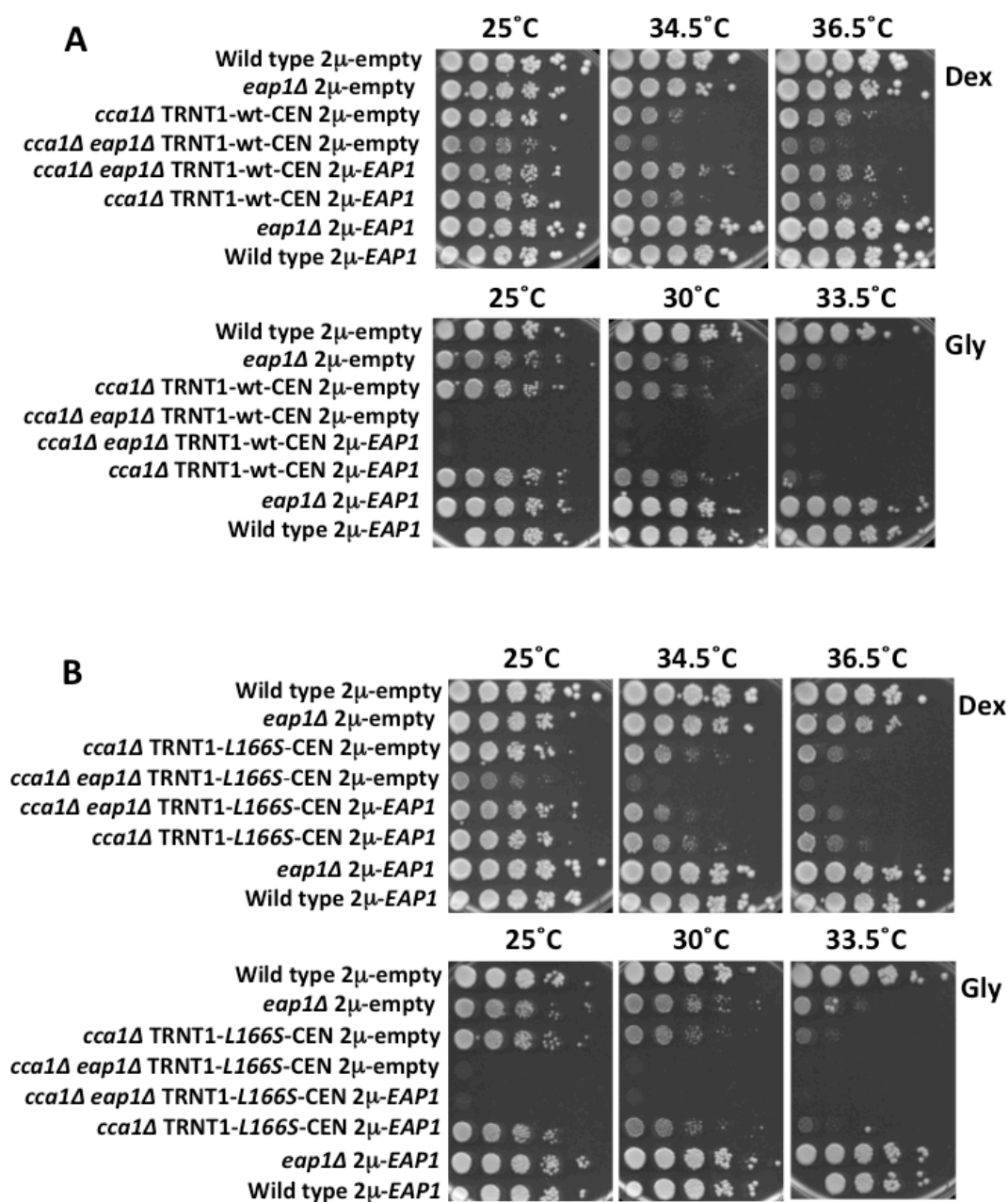


Figure A1.4. Over expression of *EAP1* in an *eap1*Δ was suppressive to *TRNT1* wild type and *TRNT1-L166S*

(A) Ten-fold serial dilutions of wild type 2μ-empty (EKK481), *eap1*Δ 2μ-empty (EKK485), *cca1*Δ *TRNT1*-wt-CEN 2μ-empty (EKK489), *cca1*Δ *eap1*Δ *TRNT1*-wt-CEN 2μ-empty (not saved, too sick), *cca1*Δ *eap1*Δ *TRNT1*-wt-CEN 2μ-*EAP1* (EKK497), *cca1*Δ *TRNT1*-wt-CEN 2μ-*EAP1* (EKK493), *eap1*Δ 2μ-*EAP1* (EKK477), and wild type 2μ-*EAP1* (EKK473) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates and grown at the indicated temperatures for 2 to 4 days.

(B) Ten-fold serial dilutions of wild type 2μ-empty (EKK481), *eap1*Δ 2μ-empty (EKK485), *cca1*Δ *TRNT1-L166S*-CEN 2μ-empty (EKK491), *cca1*Δ *eap1*Δ *TRNT1-L166S*-CEN 2μ-empty, *cca1*Δ *eap1*Δ *TRNT1-L166S*-CEN 2μ-*EAP1* (EKK499), *cca1*Δ *TRNT1-L166S*-CEN 2μ-*EAP1* (EKK495), *eap1*Δ 2μ-*EAP1* (EKK477), and wild type 2μ-*EAP1* (EKK473) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates and grown at the indicated temperatures for 2 to 4 days.

Figure A1.5.

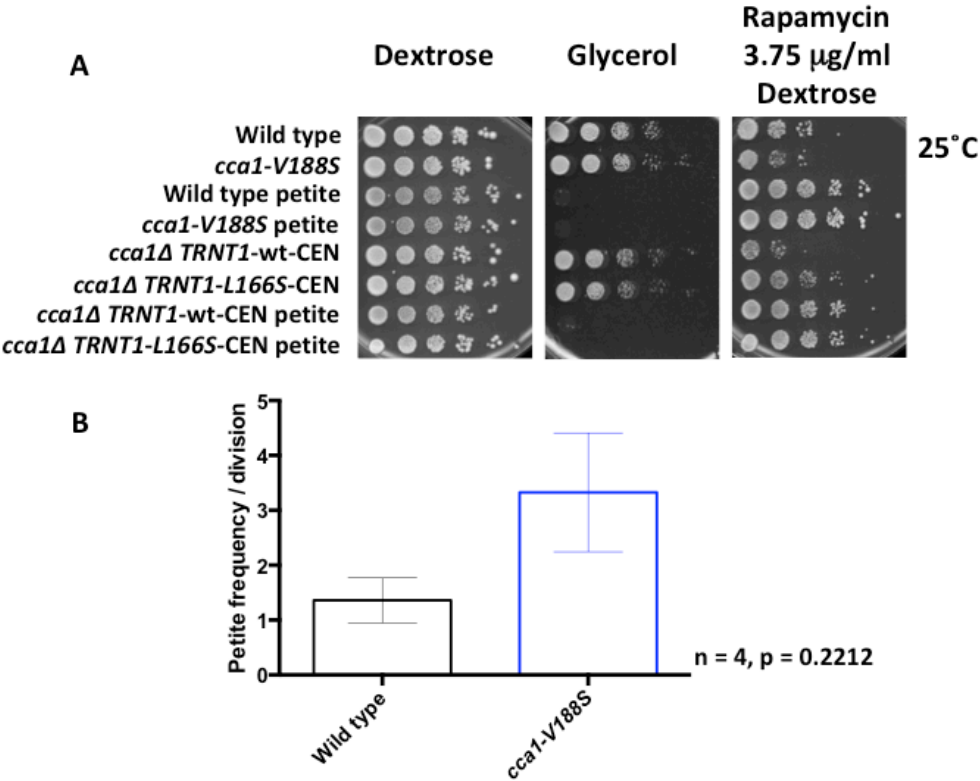


Figure A1.5. Rapamycin resistance was not due to increased frequency of petite cells in *cca1-V188S*.

(A) Wild type (ADR4006), *cca1-V188S* (EKK148), wild type petite (EKK275), *cca1-V188S* petite (EKK273), *cca1Δ TRNT1*-wt-CEN (EKK100), *cca1Δ TRNT1-L166S*-CEN (EKK334), *cca1Δ TRNT1*-wt-CEN petite (EKK277), *cca1Δ TRNT1-L166S*-CEN petite (EKK346) were grown overnight at 25°C to 2 to 4x10⁷ cells/ml, diluted to 1.85 x10⁷ cells/mL and spotted to YEP dextrose (Dex), glycerol (Gly), or dextrose containing 3.75 mg/mL plates and grown at 25°C for 2 to 4 days.

(B) Wild type (ADR4006) and *cca1-V188S* (EKK130) were grown overnight in YEP glycerol at 25°C to 2 to 4x10⁷ cells/mL and samples taken for plate counts. Cultures were resuspended in YEP dextrose for 8 hours at 30°C, doubling approximately 3 times and samples were taken for plate counts. Plate count samples were measured by OD, diluted to have approximately 300 colonies per plate and spread on YEP dextrose or YEP glycerol plates, grown at 30°C for 2 days. Colony forming units (cfu) were counted by scanning the plates on a flat-bed scanner and counting single colonies using ImageJ software. Data is of four replicates with error bars representing SEM.

rapamycin increasing mitochondrial biogenesis and impaired *TRNT1/CCA1* would limit mitochondrial translation, resulting in a growth defect.

The suppression of the *TRNT1-L166S* may have been caused by the up-regulation of the Pleiotropic Drug Resistance pathway. The PDR pathway is upregulated in response to drugs, heat shock, and mitochondrial dysfunction to enable tolerance to a wide range of toxic agents and anti-fungal drugs in yeast (Hallstrom et al., 2000, Balzi et al., 1995, Prasad et al., 2012, Shahi et al., 2010). This is consistent the petite strains analyzed having increased resistance to rapamycin compared to both wild type and their respective non-petite strains (Figure A1.5A). Note: no significant increase in petite frequency occurs in *cca1-V188S* at 30°C (Figure A1.5.B), indicating that the respiratory deficiency observed is due to individual deficient mitochondrial function and not an increase in petite formation (loss of mtDNA) in the population.

eap1Δ also results in increased resistance to rapamycin (Cosentino et al., 2000). The double mutant of *eap1Δ cca1-V188S* does not retain the rapamycin resistance observed in *eap1Δ* alone, (Figure A1.6.A). Rapamycin is known to result in the phosphorylation of Eap1 and eIF2α to regulate translation (Cherkasova & Hinnebusch, 2003; Gingras, et al., 1998). In our analysis, the rapamycin resistance is further increased with *sui2-S51A* is combined with *eap1Δ* (*eap1Δ* compared to *sui2-S51A eap1Δ*), and this resistance is kept in *cca1-V188S sui2-S51A eap1Δ* but not *cca1-V188S eap1Δ* or *cca1-V188S sui2-S51A* alone (Figure A1.6.B). This suggests that deregulation of translation by *eap1Δ* and *sui2-S51A* made cells less sensitive to rapamycin, consistent with the regulation of Eap1 by TORC1.

Figure A1.6.

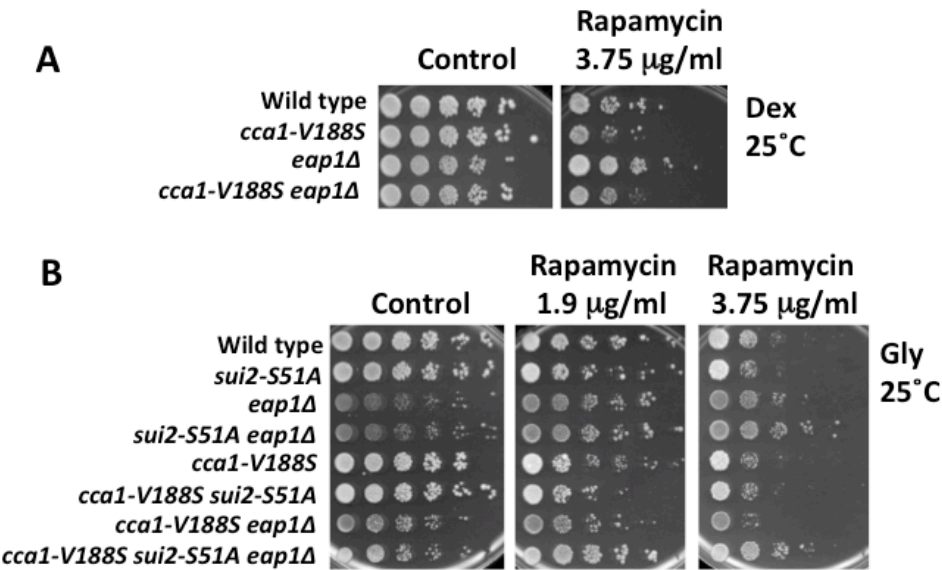


Figure A1.6. *cca1-V188S* resistance to rapamycin in petite condition

(A) Rapamycin resistance from *eap1Δ* is lost in *cca1-V188S eap1Δ*. Wild type (ADR4006), *cca1-V188S* (EKK130), *eap1Δ* (EKK238), and *cca1-V188S eap1Δ* (EKK240) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or dextrose containing 3.75 mg/mL plates and grown at 25°C for 2 to 4 days.

(B) Resistance to rapamycin by *eap1Δ* was increased in *eap1Δ sui2-S15A* and this increased resistance was maintained in *cca1-V188S eap1Δ sui2-S51A*. Wild type (ADR4006), *sui2-S51A* (EKK144), *eap1Δ* (EKK238), *sui2-S51A eap1Δ* (EKK384), *cca1-V188S* (EKK130), *cca1-V188S sui2-S51A* (EKK247), *cca1-V188S eap1Δ* (EKK240), and *cca1-V188S sui2-S51A eap1Δ* (EKK382) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex), glycerol (Gly), or dextrose or glycerol containing 1.9 or 3.75 mg/mL plates and grown at 25°C for 2 to 4 days.

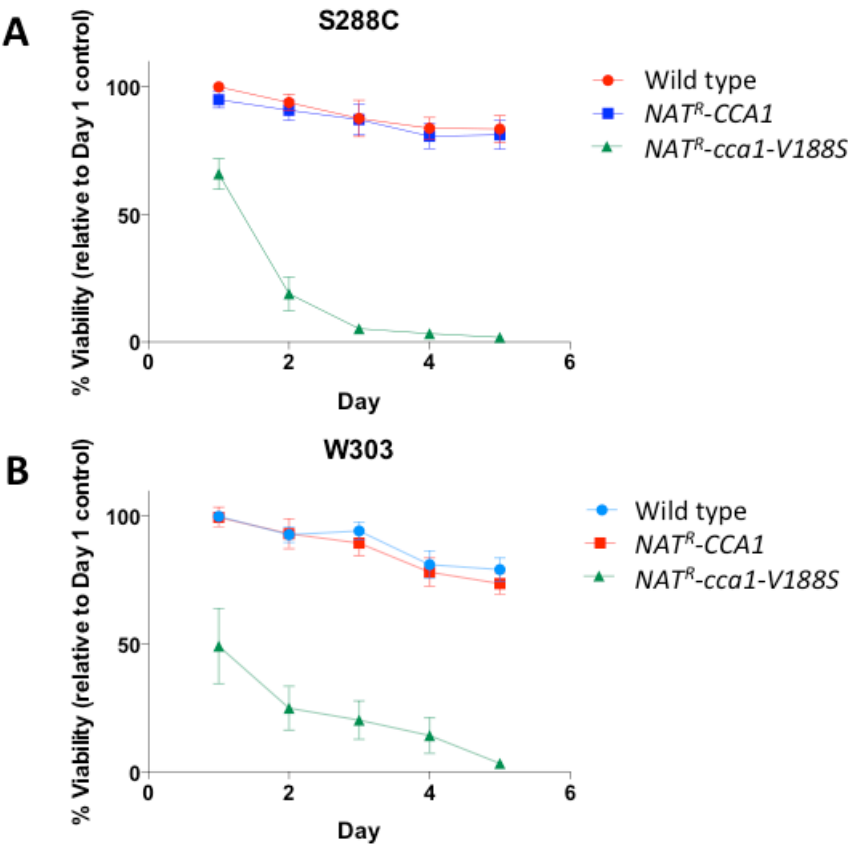
A1.1.4. Viability phenotype identified through SGA troubleshooting.

The Synthetic Genetic Array was attempted three times using *NAT^R-cca1-VI88S*, but due to viability issues most of the crosses presented as false positives. The SGA screen was not undertaken for three reasons: 1) The use of Geneticin (G418, an aminoglycoside which relaxes the fidelity of bacterial and mitochondrial translation) as a selection marker may lead to an increase in false positives when assessing a potential mitochondrial translation mutant (Hobbie et al., 2008); and 2) concerns about variable plasmid loss when a sheltering plasmid was used; and 3) the *cca1-VI88S* strains has reduced viability when as it approaches saturation in both the Y7096 (S288C) (Figure A1.7A) and W303 (Figure A1.7B) backgrounds.

Yeast cells regularly enter stationary phase (measured as chronological lifespan, non-dividing cells) where levels of one or more core nutrients (carbon, nitrogen, phosphate, or sulphur) result in starvation, prompting the yeast to enter G₀ (Gray et al., 2004; Werner-Washburne et al., 1993). Cells that are starved in an unnatural way (auxotrophic versus carbon) fail to arrest properly in G₀ and rapidly lose viability (Saldanha et al., 2004). Additionally, functional mitochondria are required for the diauxic shift from fermentation to respiration as carbon sources are depleted, and petite cells have decreased viability at this phase (Fabrizio et al., 2010; Harris et al., 2012). In both S288C and W303, the *cca1-VI88S* strains had markedly reduced viability, prior to cultures reaching saturation. This viability defect was fully rescued by the addition of the *CCA1-CEN-URA3* plasmid (Figure A1.7C).

Given the viability defect observed in S288C at 30°C with glutamate for a nitrogen source, further work was done to assess the viability phenotype in W303 at permissive temperature (25°C) with ammonium sulfate, a better nitrogen source. As observed

Figure A1.7.



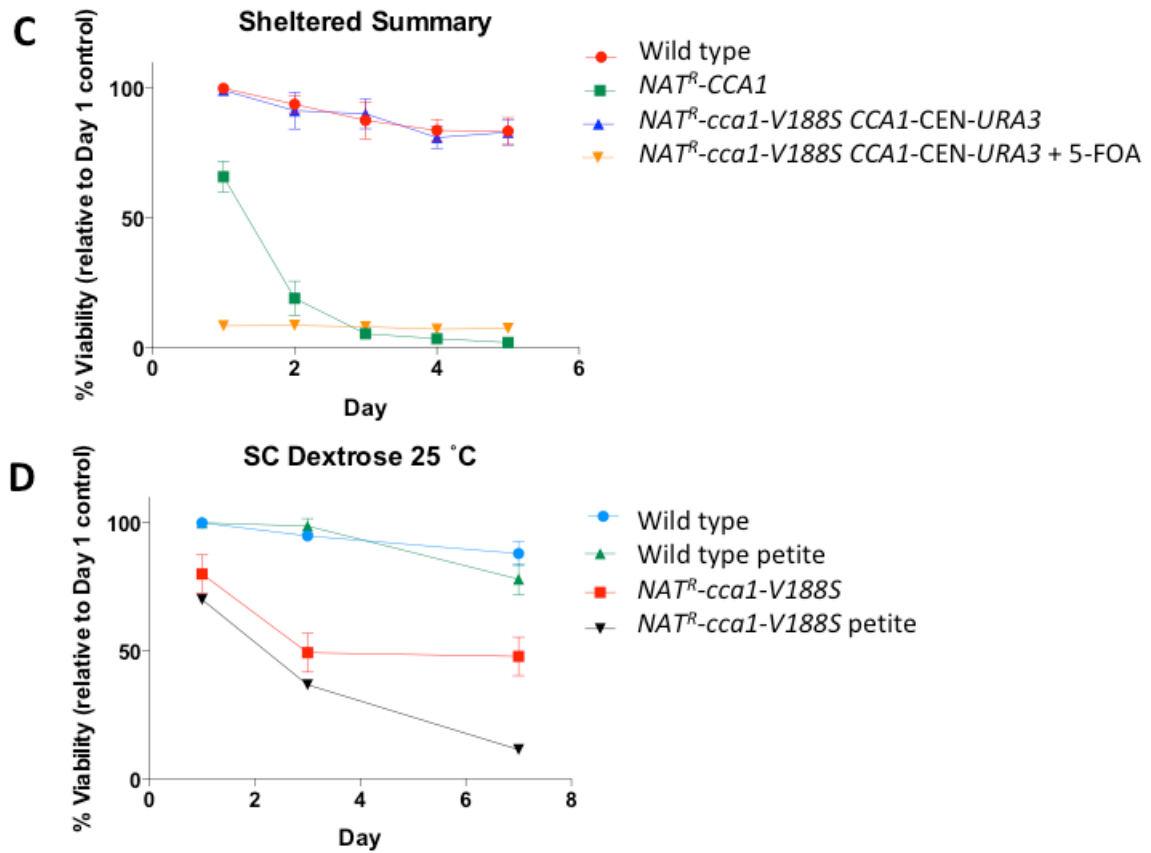


Figure A1.7. *cca1-V188S* had a viability defect prior to the diauxic shift

(A) *cca1-V188S* has a viability defect when grown under conditions for the Synthetic Genetic Array. Wild type (ADR9360), *NAT^R-CCA1* (EKK138), and *NAT^R-cca1-V188S* (ADR9214) were grown to 1 to 2×10^7 cells/mL in SC with MSG (monosodium glutamate) and dextrose at 30°C overnight and samples taken as t=0. Cultures were grown for 1, 2, 3, 4, and 5 days at 30°C and samples taken. These strains are in the Y7092 (S288C Synthetic Genetic Array query strain). As samples were being assessed for issues proceeding through the Synthetic Genetic Array protocol. Samples were measured by OD for concentration and plated at approximately 300 colonies per plate on YEP dextrose. Colony forming units (cfu) were counted by scanning the plates on a flat-bed scanner and counting single colonies using ImageJ software. Data is of three replicates with error bars representing SEM.

(B) *cca1-V188S* had a viability defect in W303. Wild type (ADR4006), *NatR-CCA1* (EKK132), and *NatR-cca1-V188S* (EKK130) were grown to 1 to 2×10^7 cells/mL in SC with MSG and dextrose at 30°C overnight and samples taken as t=0. Cultures were grown for 1, 2, 3, 4, and 5 days at 30°C and samples taken. These strains are in the W303 background used for the majority of data presented in this thesis. Viability was assessed as described in (A).

(C) The viability defect of *cca1-V188S* was rescued by the presence of wild type *CCA1* on a plasmid. Wild type (ADR9360), *NAT^R-cca1-V188S* (ADR9214), *NAT^R-cca1-V188S CCA1-CEN-URA3* (EKK308) were grown to 1 to 2×10^7 cells/mL in SC with MSG and dextrose at 30°C overnight and samples taken as t=0. Cultures were grown for 1, 2, 3, 4, and 5 days at 30°C and samples taken. These strains are in the Y7092 (S288C Synthetic Genetic Array query strain). Samples were measured by OD for concentration and plated at approximately 300 colonies per plate on YEP dextrose or YEP dextrose containing 5-FOA to prevent *CCA1-CEN-URA3* from sheltering *NAT^R-cca1-V188S*. Viability was assessed as described in (A).

(D) The viability defect of *cca1-V188S* is prior to the diauxic shift. Wild type (ADR4006), wild type petite (EKK275), *cca1-V188S* (EKK130), and *cca1-V188S* petite (EKK273) were grown to 1 to 2×10^7 cells/mL in SC with ammonium sulfate and dextrose at 25°C overnight and samples taken as t=0. Cultures were grown for 1, 3, 5, and 7 days at 25°C and samples taken. These strains are grown in the W303 background with ammonium sulfate as a nitrogen source instead of MSG, which is used for the Synthetic Genetic Array; ammonium sulfate is a better nitrogen source for yeast. Viability was assessed as described in (A).

previously in W303 at 30°C (Figure A1.7B), although taking longer, the *cca1-V188S* mutant decreased in viability prior to reaching saturation (between day 3 to 7 when grown at 25°C) (Figure A1.7C). Wild type petite cells did not have a loss of viability at the point, prior to the diauxic shift, indicating that the *cca1-V188S* phenotype was not due to inability to progress through the diauxic shift, which would also occur in petite cells (Figure A1.7.D). Additionally, making *cca1-V188S* petite reduced viability compared to wild type petite, indicating that mitochondrial function was not responsible for the decrease in viability observed in *cca1-V188S* (Figure A1.7.D).

A1.1.5. Double *TRNT1* mutations in *cis* and *trans*

tRNA-NTs (tRNA nucleotidyl transferase, *TRNT1* and *CCA1*) contain an amino-terminal head, neck, body, and C-terminal tail domains with the single active site responsible for both C and A addition contained in the highly conserved head domain and consisting of five motifs (A to E) (Figure 3.1). The *L166S* and *I223T* mutations are both found within the CCA-adding enzymatic region of *TRNT1*, with *L166S* in the C-motif and *I223T* between the D and E motifs. The phenotype of *TRNT1-L166S* results from poor stability of the protein and the *TRNT1-I223T* from both poor stability and reduced catalytic function (Leibovitch et al., 2018).

The C-terminal body (*TRNT1-I326T*) and tail domains are proposed to interact with the tRNA, evidenced by foot-printing and phosphate modification interference data (Li et al., 2002; Zhu & Deutscher, 1987). Other studies involving purification of tRNA-NTs have reported the presence of dimers (103 kDa) and oligomers (780 kDa), leaving the possibility open of tRNA-NTs open (Yakunin et al., 2004). It is possible that the *TRNT1-I326T*

mutation results in increased or decreased interaction of TRNT1 proteins, which while detrimental in humans may be beneficial in yeast.

Mutations in *TRNT1* cause both gain of function (*TRNT1-I326T*, *R99W*, *K107R*) and loss of function (all other mutations) relative to *TRNT1* wild type. To determine if the *TRNT1-I326T* mutation could rescue the loss of function mutations, strains were made with the *TRNT1-I326T* and *L166S* mutations in either *cis* or *trans*. The *TRNT1-I326T* mutation increased growth when combined with the *TRNT1-L166S* mutation compared to the *TRNT1-L166S* mutation alone, but not to the full extent of *TRNT1-I326T* alone (Figure A1.8); this was also observed for the *TRNT1-I223T* mutation (Figure A1.8). This suggests that the gain of function associated with the *I326T* mutation is separate from the loss of function of the *L166S* and *I223T* mutations. Analysis of the *I326T* and γ *L166S* mutations in *trans* showed that the presence of a *TRNT1-I326T* was all that was necessary for gain of function, although stoichiometry was not controlled given that the two genes were on separate CEN plasmids and copy number could have drifted (Figure A1.9.A). A slight synthetic affect from increasing plasmid number was observed, but was outweighed by the presence of the *TRNT1-I326T* mutation adding benefit to other mutations (Figure A1.9.B).

Figure A1.8.

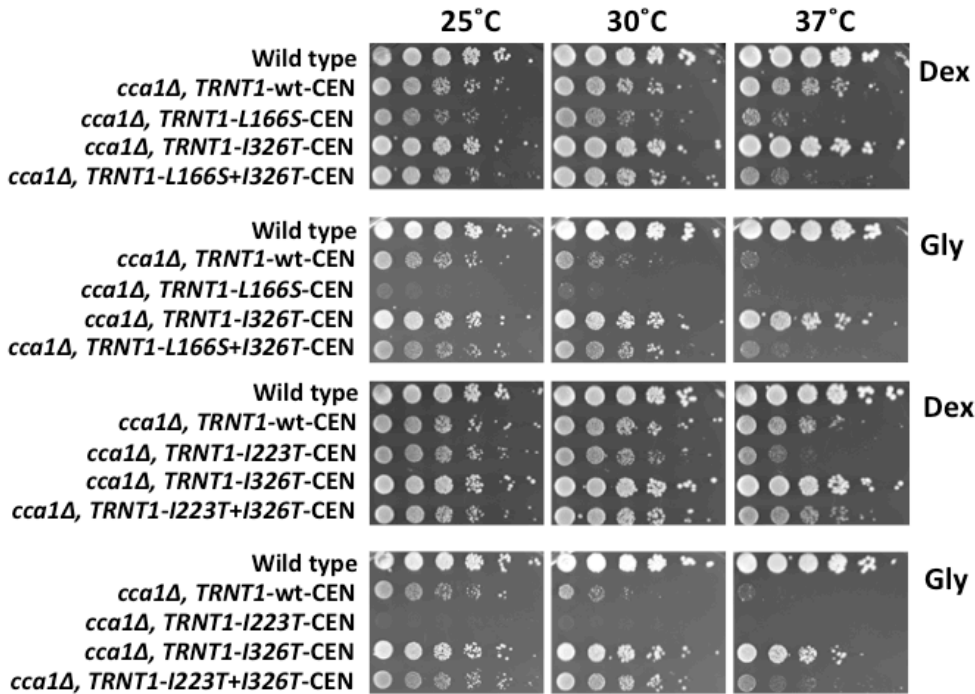


Figure A1.8. In *cis*, *TRNT1-I326T* only partially suppressed the phenotype due to the presence of the L166S or I223T mutations. Wild type (ADR4006), *cca1Δ TRNT1*-wt-CEN (ADR6791), *cca1Δ TRNT1-L166S*-CEN (ADR6935), *cca1Δ TRNT1-I223T*-CEN (ADR 6939), *cca1Δ TRNT1-L166S+I326T*-CEN (ADR9216), and *cca1Δ TRNT1-I223T+I326T*-CEN (ADR9220) were grown overnight at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to YEP dextrose (Dex) or glycerol (Gly) plates and grown for 2 to 4 days at the temperatures indicated.

Figure A1.9.

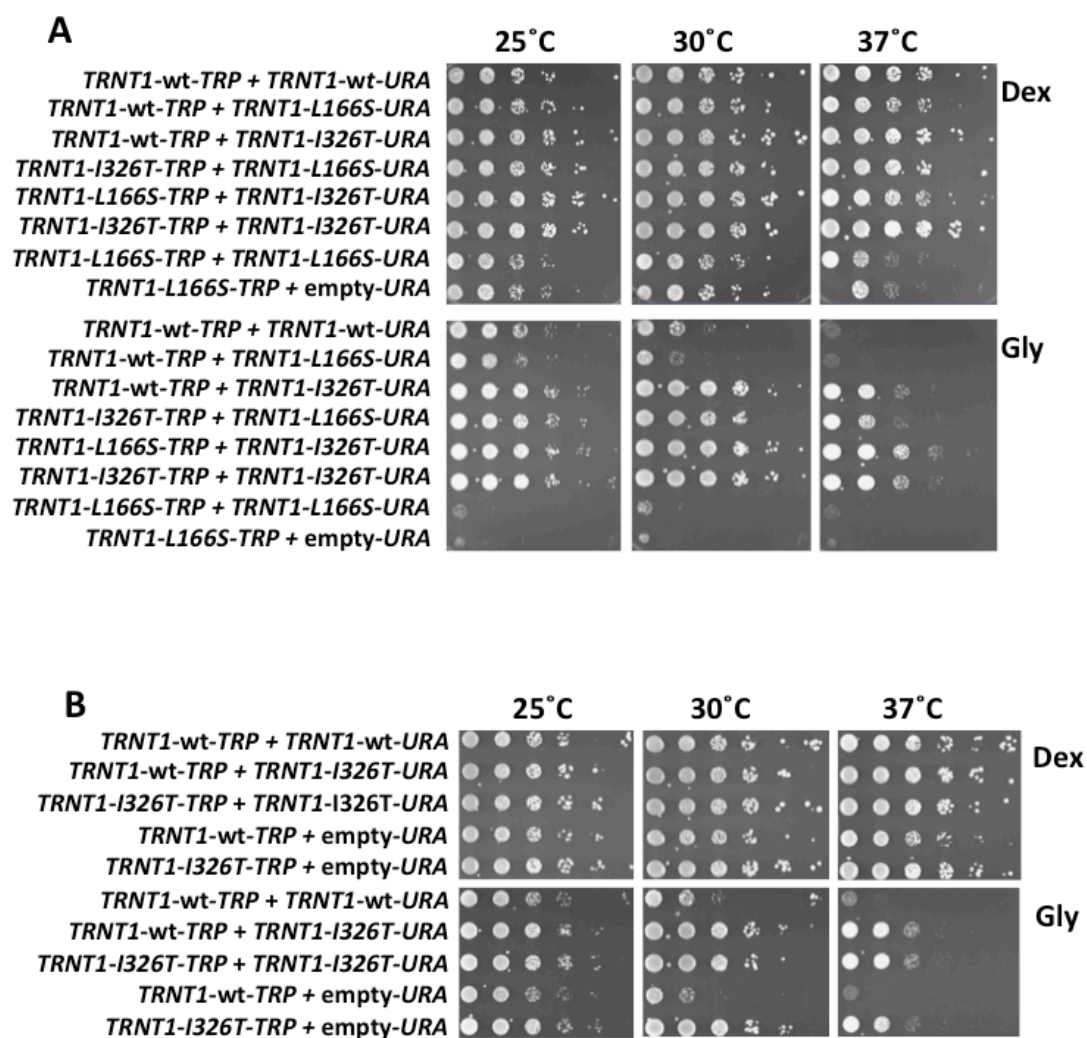


Figure A1.9. *TRNT1* mutants in trans.

(A) In trans, *TRNT1-I326T* suppresses the phenotype of *TRNT1-L166S*. *cca1Δ TRNT1-wt-TRP* and *TRNT1-wt-URA* (ADR6791 + pAR1200), *cca1Δ TRNT1-wt-TRP* and *TRNT1-L166S-URA* (ADR6791 + pAR1203), *cca1Δ TRNT1-wt-TRP* and *TRNT1-I326T-URA* (ADR6791 + pAR1206), *cca1Δ TRNT1-I326T-TRP* and *TRNT1-L166S-URA* (ADR6941 + pAR1203), *cca1Δ TRNT1-L166S-TRP* and *TRNT1-I326T-URA* (ADR6935 + pAR1206), *cca1Δ TRNT1-I326T-TRP* and *TRNT1-I326T-URA* (ADR6941 + pAR1206), *cca1Δ TRNT1-L166S-TRP* and *TRNT1-L166S-URA* (ADR6935 + pAR1203), *cca1Δ TRNT1-L166S-TRP* and empty-*URA* (ADR6935 + pAR358), were grown overnight in SC-trp-ura dextrose at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to SC-trp-ura dextrose (Dex) or glycerol (Gly) plates and grown for 2 to 4 days at the temperatures indicated.

(B) The suppression by *TRNT1-I326T* is not due to dosage. *cca1Δ TRNT1-wt-TRP* and *TRNT1-wt-URA* (ADR6791 + pAR1200), *cca1Δ TRNT1-wt-TRP* and *TRNT1-I326T-URA* (ADR6791 + pAR1206), *cca1Δ TRNT1-I326T-TRP* and *TRNT1-I326T-URA* (ADR6941 + pAR1206), *cca1Δ TRNT1-wt-TRP* and empty-*URA* (ADR6791 + pAR358), *cca1Δ TRNT1-I326T-TRP* and empty-*URA* (ADR6941 + pAR358) were grown overnight in SC-trp-ura dextrose at 25°C to 2 to 4x10⁷ cells/mL, diluted to 1.85x10⁷ cells/mL and spotted to SC-trp-ura dextrose (Dex) or glycerol (Gly) plates and grown for 2 to 4 days at the temperatures indicated.

Table A1.1. Strains used in Appendix 1

Strains used in this study are derivatives of W303-1a (*ura3-1 leu2-3,112 trp1-1 his3-11 ase2-1 can1-100*), Y7092 (*MATa can1Δ::STE2pr-Sp_his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*), or Y7096 from S288C (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) as indicated.

Strain	MAT	Background	Genotype
ADR22	α	W303-1a	wild type
ADR4006	a	W303-1a	wild type <i>bar1Δ</i>
ADR6791	α	W303-1a	<i>cca1Δ::HYG^R pADHI-TRNT1-CEN-TRP1</i>
ADR6793	α	W303-1a	<i>cca1Δ::HYG^R pADHI-TRNT1-T154I-CEN-TRP1</i>
ADR6834	α	W303-1a	<i>pADHI-TRNT1-CEN-URA3</i>
ADR6836	α	W303-1a	<i>pADHI-CCA1-CEN-URA3</i>
ADR6933	α	W303-1a	<i>cca1Δ::HYG^R pADHI-TRNT1-M158V-CEN-TRP1</i>
ADR6935	α	W303-1a	<i>cca1Δ::HYG^R pADHI-TRNT1-L166S-CEN-TRP1</i>
ADR6937	α	W303-1a	<i>cca1Δ::HYG^R pADHI-TRNT1-R190I-CEN-TRP1</i>
ADR6339	α	W303-1a	<i>cca1Δ::HYG^R pADHI-TRNT1-I223T-CEN-TRP1</i>
ADR6941	α	W303-1a	<i>cca1Δ::HYG^R pADHI-TRNT1-I326T-CEN-TRP1</i>
EKK9214	α	Y7096	<i>NAT^R-cca1-V188S</i>
ADR9216	α	W303-1a	<i>cca1Δ::HYG^R pADHI-TRNT1-L166S-I326T-CEN-TRP1</i>
ADR9220	α	W303-1a	<i>cca1Δ::HYG^R pADHI-TRNT1-I223T-I326T-CEN-TRP1</i>
ADR9360	α	Y7096	wild type

EKK002	a	W303-1a	<i>ccal-1 (ccal-ts-352)</i>
EKK100	α	W303-1a	<i>ccalΔ::HYG^R pADHI-TRNTI-CEN-TRP1</i>
EKK130	α	W303-1a	<i>NAT^R-ccal-V188S</i>
EKK132	α	W303-1a	<i>NAT^R-CCAI</i>
EKK138	α	Y7096	<i>NAT^R-CCAI</i>
EKK144	a	W303-1a	<i>NAT^R-sui2-S51A</i>
EKK148	a	W303-1a	<i>NAT^R-ccal-V188S</i>
EKK238	α	W303-1a	<i>eap1Δ::KAN^R</i>
EKK240	α	W303-1a	<i>NAT^R-ccal-V188S eap1Δ::KAN^R</i>
EKK247	α	W303-1a	<i>HYG^R-ccal-V188S NAT^R-sui2-S51A</i>
EKK262	a	Y7096	<i>fkrb12Δ::KAN^R</i>
EKK263	α	Y7096	<i>fkrb12Δ::KAN^R NAT^R-ccal-V188S</i>
EKK273	α	W303-1a	<i>NAT^R-ccal-V188S petite</i>
EKK275	α	W303-1a	wild type petite
EKK277	α	W303-1a	<i>ccalΔ::HYG^R pADHI-TRNTI-CEN-TRP1petite</i>
EKK287	α	W303-1a	<i>CAF20-HA-KAN^R</i>
EKK289	a	W303-1a	<i>EAP1-myc-HIS3</i>
EKK300	α	W303-1a	<i>EAP1-myc-HIS3 CAF20-HA-KAN^R</i>
EKK308	α	W303-1a	<i>NAT^R-ccal-V188S CCAI-CEN-TRP</i>
EKK310	α	W303-1a	<i>NAT^R-ccal-V188S EAP1-myc-HIS3</i>
EKK312	α	W303-1a	<i>NAT^R-ccal-V188S CAF20-6HA-KAN^R</i>
EKK334	α	W303-1a	<i>ccalΔ::HygR pADHI-TRNTI-L166S-CEN-TRP1</i>
EKK382	a	W303-1a	<i>HYG^R-ccal-V188S NatR-sui2-S51A eap1Δ::KAN^R</i>

EKK384	a	W303-1a	<i>NAT^R-sui2-S51A eap1Δ::KAN^R</i>
EKK437	α	W303-1a	<i>EAP1-myc-HIS3 CAF20-HA-KAN^R CDC33-TAP-TRP1</i>
EKK442	α	W303-1a	<i>NAT^R-ccal-V188S EAP1-myc-HIS3 CAF20-HA-KAN^R CDC33-TAP-TRP1</i>
EKK473	α	W303-1a	<i>EAP1-2μ-LEU2 bar1Δ</i>
EKK475	α	W303-1a	<i>NAT^R-ccal-V188S EAP1-2μ-LEU2</i>
EKK477	α	W303-1a	<i>eap1Δ::KAN^R EAP1-2μ-LEU2</i>
EKK479	α	W303-1a	<i>NAT^R-ccal-V188S eap1Δ::KAN^R EAP1-2μ-LEU2</i>
EKK481	α	W303-1a	<i>empty-2μ-LEU2 bar1Δ</i>
EKK483	α	W303-1a	<i>NAT^R-ccal-V188S empty-2μ-LEU2</i>
EKK485	α	W303-1a	<i>eap1Δ::KAN^R empty-2μ-LEU2</i>
EKK487	α	W303-1a	<i>NAT^R-ccal-V188S eap1Δ::KAN^R empty-2μ-LEU2</i>
EKK489	α	W303-1a	<i>ccalΔ::HYG^R pADHI-TRNT1-CEN-TRP1 empty-2μ-LEU2</i>
EKK491	α	W303-1a	<i>ccalΔ::HYG^R pADHI-TRNT1-L166S-CEN-TRP1 empty-2μ-LEU2</i>
EKK493	α	W303-1a	<i>ccalΔ::HYG^R pADHI-TRNT1-CEN-TRP1 EAP1-2μ-LEU2</i>
EKK495	α	W303-1a	<i>ccalΔ::HYG^R pADHI-TRNT1-L166S-CEN-TRP1 EAP1-2μ-LEU2</i>
EKK497	α	W303-1a	<i>ccalΔ::HYG^R eap1Δ::KAN^R pADHI-TRNT1-CEN-TRP1 EAP1-2μ-LEU2</i>

EKK499	α	W303-1a	<i>cca1</i> Δ :: <i>HYG</i> ^R <i>eap1</i> Δ :: <i>KAN</i> ^R <i>pADHI-TRNT1-L166S</i> - <i>CEN-TRP1 EAPI-2μ-LEU2</i>
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A1.2. Additional methods

A1.2.1. Yeast strain construction

The *EAP1-myc13-HIS3* and *CAF20-HA-KAN^R* were made using PCR-targeted recombination using pFA6a-13Myc-*HIS3* (Longine et al., 1998) and gene specific primers. The *CDC33-TAP-KITRPI* allele was made using PCR-targeting recombination with pBS1479 (Rigaut et al., 1999) and *CDC33* specific primers. Strains were then crossed with the *natMX-cca1-VI88S* strain to make the appropriate strains for analysis.

A1.2.2. IgG Dynabead preparation

M270 epoxy dynabeads (Invitrogen) were resuspended in dry DMF (Fisher) at a concentration of 2×10^9 beads/mL. Using a magnet, DMF was removed and beads were washed in 0.1M sodium phosphate buffer, pH 7.4 at 1×10^9 beads/mL. Beads were vortexed, washed again, and incubated at 25°C for 10 min with end-over-end rotation. Beads were resuspended in 0.1M sodium phosphate buffer at 3×10^9 beads/mL, vortexed, and an equal volume of 1 mg/mL rabbit IgG (Sigma-Aldrich) was added. IgG was made fresh in 0.1M sodium phosphate buffer. Beads were vortexed, an equal volume of 3M ammonium sulfate was added, and beads were incubated overnight at 37°C with end-over-end rotation. Beads were washed quickly with an equal volume sodium phosphate buffer at 25°C, followed by two washes with 10 min incubations. Beads were washed in sodium phosphate buffer with 1% Triton-X-100 for 10 min at 37°C with rotation, followed by a quick wash with equal volume sodium phosphate buffer at 25°C, and two washes with 10 min incubations. IgG, beads were washed four times with 0.1M citric acid, pH 3.1 (at a volume of 1×10^8 beads/mL). Finally, beads were washed with an equal volume sodium phosphate buffer,

followed by three washes with 10 min incubations, and stored at a final concentration of 1×10^9 beads/mL. This work was done by Dr. Elizabeth Williams and Dr. Adam Rudner.

A1.2.3. Cdc33-TAP/Eap1-HA/Caf20-Myc purification

Cells were grown overnight in 50 to 150mL cultures in YEP dextrose at 25°C to a concentration of 0.5 to 1.0×10^7 cells/mL. Cultures were then grown to 1.5 to 2.5×10^7 cells/mL at 30°C, 34°C, or 37°C. Harvested cells were lysed in low salt TAP lysis buffer (100mM Hepes-KOH, pH 8.5, 20mM Mg(OAc)₂, 10mM EGTA, 0.1mM EDTA, 0.4% NP-40, 150 mM NaOAc, 1mM DTT, 1mM PMSF, 1mM Na₃VO₄, 1mM benzamidine, and leupeptin, bestatin, pepstatin A and chymostatin all at 1mM) using glass beads with 2 x 30 second bead beating with a 5 min rest on ice between. Lysate was clarified by spinning 10 min at 14.5 rcf at 4°C. Protein concentration was normalized according to the Bradford method (Bradford, 1976). The clarified lysate was incubated with IgG-coupled magnetic dynabeads for overnight at 4°C. After binding, beads were washed three times with TAP wash buffer (100mM Hepes-KOH, pH 7.6, 20mM Mg(OAc)₂, 10mM EGTA, 0.1mM EDTA, 0.4% NP-40, 150 mM NaOAc, 1mM DTT, 1mM PMSF,). Samples were boiled off the beads in 1X sample buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 125mM Tris-HCl pH 6.8, 1mM PMSF), run on a polyacrylamide gel, transferred and probed for proteins as described above.

A1.2.4. Viability in liquid culture

For viability assays in liquid culture, exponentially growing cells were grown to a concentration of 1 to 2×10^7 cells/mL in complete medium (YEP dextrose) for $t=0$, samples taken and diluted 1000X and/or 10000X into sterile water; 200μL of the cultures were

plated. Cultures were further grown at 25°C or 30°C for up to 10 days. Cfu were counted by scanning the plates on a flat-bed scanner and counting single colonies using ImageJ software. Single plate images were cropped to include only the plate area, background was removed with a rolling ball radius of 50.0 pixels, threshold was adjusted to best separate individual colonies, the image was converted to binary and a watershed adjustment was performed to separate touching colonies into individual. Particles were analyzed with a pixel size of 0.2 to infinity and circularity of 0.70 to 1.00. Viability was determined relative to viability at t=0 by counting the number of colony forming units (cfu) on the plate containing 100<cfu<4000 and multiplying by the appropriate dilution factor.

A1.2.5. Petite frequency determination

Exponentially growing cells were grown to a concentration of 1 to 2×10^7 cells/mL in complete medium with non-fermentable carbon source (YEP glycerol) at 30°C. Samples were re-suspended in complete media with fermentable carbon (YEP dextrose) for 5 hours, measuring cell concentrations prior and at 8 hours to determine doublings, approximately 5 doublings. Aliquots were diluted by 1/10 dilution with sterile water until at approximately 1200 cells/mL; 300 cells were plated on complete media with dextrose or glycerol, grown for 2 to 3 days as required and counted using ImageJ software. Colony forming units (cfu) were counted by scanning the plates on a flat-bed scanner and counting single colonies using ImageJ software. Single plate images were cropped to include only the plate area, background was removed with a rolling ball radius of 50.0 pixels, threshold was adjusted to best separate individual colonies, the image was converted to binary and a watershed adjustment was performed to separate touching colonies into individual. Particles were analyzed with a pixel size of 0.2 to infinity and circularity of 0.70 to 1.00. Viability was

determined relative to viability at $t=0$ by counting the number of cfu on the plate containing $100 < \text{cfu} < 4000$ and multiplying by the appropriate dilution factor. Petite frequency was determined as the percentage of colonies unable to grow on non-fermentable carbon compared to fermentable carbon per doubling.

Appendix 2

This section of the thesis incorporates unpublished data regarding Cdk1 phospho regulation (Kennedy et al., 2016) presented in Chapter 4 of this thesis. This was a separate project and was accomplished while the *TRNT1/CCAI* project was in early stages.

Unpublished data from Michael Dysart involving this project was summarized in his Honours thesis, Spring 2015.

A2.1. Results

A2.1.1. Dephosphorylation of Cdk1 Y19 by alternative phosphatases

In budding yeast, although Mih1 (Cdc25 yeast homolog) is the only known Cdk1 phosphatase, deletion of *MIH1* exhibits only mild defects in mitotic entry and anaphase initiation and dephosphorylation of Cdk1 still occurs, although delayed, and correlates with the initiation of anaphase (Liang et al., 2013; Pal et al., 2008). I tested if PP2A^{Cdc55} and/or PP2A^{Rts1} are redundant Cdk1 phosphatases because *mih1Δ cdc55Δ* and *mih1Δ rts1Δ* cells are both inviable, and this inviability is rescued by deletion of *SWE1* (Pal et al., 2008). *cdc55Δ* and *rts1Δ* cells, like *mih1Δ* cells, also had elevated levels of Y19 phosphorylation on Cdk1. Interestingly, Ptp1, Ltp1, and Ppz1 also had elevated levels of Cdk1 Y19 in asynchronous cultures, but not to the extent of *cdc55Δ*, *rts1Δ*, or *mih1Δ* cells in a mitosis arrest (Figure A2.1).

A2.1.2. The roles of Rrd1 and Rrd2 in Cdk1-Y19 dephosphorylation

Two small regulatory proteins (Rrd1 and Rrd2, yeast homologs to mammalian PTPA) were found to regulate the catalytic subunit of PP2A and were proposed to be required PP2A and PP2A-like complexes substrate specificity between tyrosine or serine/threonine (Fellner et al., 2003). Deletion of either *RRD1* or *RRD2* resulted in increased phosphorylation of Cdk1 on Y19 in asynchronous cultures but not when arrested in nocodazole (G1 arrest) (Figure A2.1). This is consistent with Rrd1 and Rrd2 having a role in the regulation of Cdk1-Y19. Addition of Rrd1 and/or Rrd2 to *in vitro* dephosphorylation experiments did not allow for PP2A^{Cdc55} or PP2A^{Rts1} dephosphorylation of Cdk1-Y19 (Figure A2.2). Previously, PP2A^{Cdc55} or PP2A^{Rts1} were isolated by Cdc55-TAP or Rts1-TAP tagged purifications. Alternatively, Tpd3 (a scaffold subunit of PP2A)

Figure A2.1.

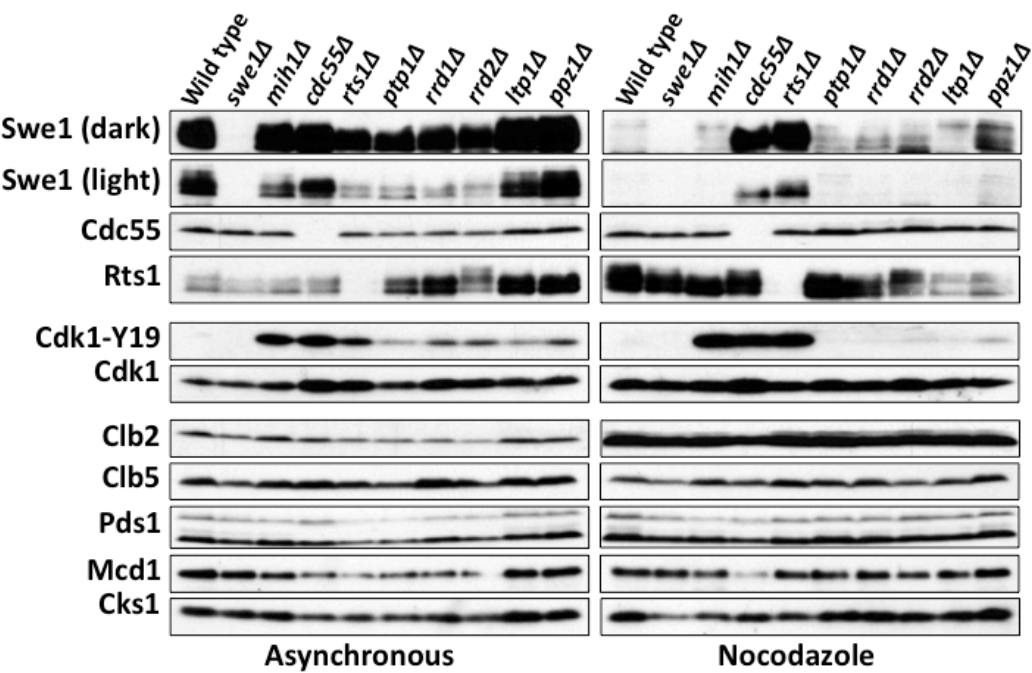


Figure A2.1. Phosphorylation of Cdk1-Y19 was increased by deletion of phosphatases in addition to known *MIH1*. Wild type (ADR4006), *swe1Δ* (ADR3173), *mih1Δ* (ADR7445), *cdc55Δ* (ADR6876), *rts1Δ* (ADR6929), *ptp1Δ* (ADR8300), *rrd1Δ* (ADR8279), *rrd2Δ* (ADR8282), *ltp1Δ* (ADR7960), and *ppz1Δ* (ADR7550) cells were arrested in mitosis with nocodazole, harvested, lysed and blotted with the indicated antibodies. Note the increased level of tyrosine phosphorylated Cdk1 in *mih1Δ*, *cdc55Δ*, and *rts1Δ* cells.

Figure A2.2.

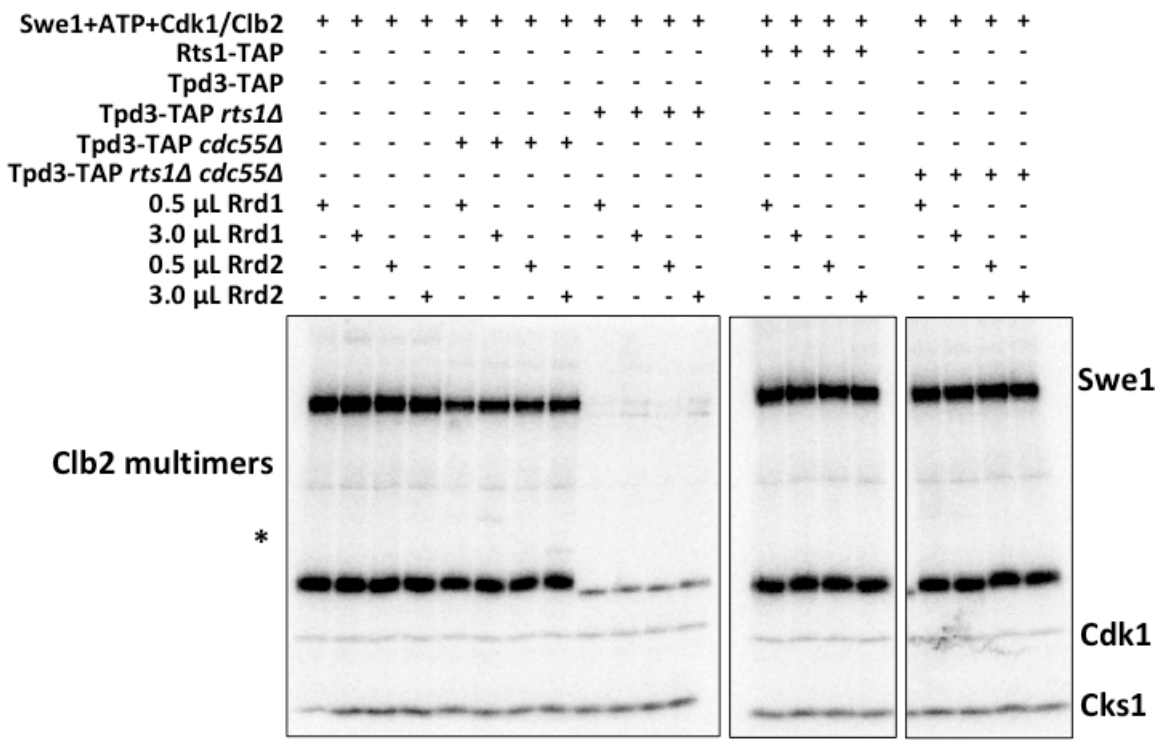


Figure A2.2. Different purification of PP2A^{Cdc55} or PP2A^{Rts1} nor addition of Rrd1 or Rrd2 enabled dephosphorylation of Cdk1 by PP2A. Purified Cdk1/Clb2-CBP was phosphorylated by bead-bound Swe1-HA in the presence of γ -[³²P]ATP. Following phosphorylation, Cdk1/Clb2-CBP was removed from the beads and mixed with purified phosphatases and an excess of unlabeled ATP to prevent re-phosphorylation of Cdk1 by γ -[³²P]ATP and Swe1-HA that was washed off the beads. Reactions were run on a polyacrylamide gel and either dried and exposed to a phospho-imager screen. After incubation for 40 min at 25°C, purified Cdc55 (from Tpd3-TAP), Rts1 (from Tpd3-TAP), and Rts1-TAP cannot dephosphorylate Cdk1-Y19 *in vitro*. PP2A^{Cdc55} and PP2A^{Rts1} can effectively dephosphorylate a fortuitous phosphorylated background protein (indicated with an asterisk), providing a control indicating that both PP2A^{Cdc55} and PP2A^{Rts1} are active. Addition of purified Rrd1 or Rrd2 did not change the *in vitro* activity. Purified Cdk1/Clb2 was used to phosphorylate purified Rts1 *in vitro*.

TAP-tagged purifications of PP2A (wild type, Tpd3-TAP *cdc55*Δ, Tpd3-TAP *rts1*Δ, and Tpd3-TAP *cdc55*Δ *rts1*Δ) were isolated with increase addition of the TAP tag to Cdc55 or Rts1 impacted activity. No change in Cdc55 (Tpd3-TAP *rts1*Δ) activity was detected and less activity was observed with Rts1 was isolated using Tpd3-TAP, likely due to loss of Rts1 in the purification (Figure A2.2). Purifications of Tpd3-TAP PP2A complexes, Rrd1, and Rrd2 were done by an honours student Michael Dysart whom I assisted in supervising and are presented in his honours thesis, Spring 2015.

A2.1.3. The phosphorylation state of Rts1

In addition to the Rts1 results presented in our publication (Kennedy et al., 2016), I also investigated the phosphorylation state of Rts1. I found that Rts1 was phosphorylated *in vivo* during mitosis and *in vitro* by Cdk1, a modification not previously shown for Rts1 (Figure A2.3). Mass spectrometry analysis identified four Cdk1 phosphorylation sites on both Cdc55 and Rts1 and further work into Cdc55 phosphorylation and the impact on cell cycle will be studied by other students in Dr. Rudner's lab (data not shown, Adam Rudner).

A2.1.4. The end-point phenotype of cells lacking Mih1 and Cdc55 or Rts1

Since double mutants between *mih1*Δ and either *cdc55*Δ or *rts1*Δ are lethal, I wanted to determine what is the terminal phenotype of cells lacking *MIH1* and either *CDC55* and/or *RTS1*. To do this, I created *CDC55-AID* and *RTS1-AID* strains that rapidly degrade Cdc55 and Rts1 when grown in the presence of auxin following an arrest and release into G1 (Figure A2.4). I have shown that *mih1*Δ *CDC55-AID* cells, but not *mih1*Δ *RTS1-AID* cells, arrest in mitosis with high levels of tyrosine phosphorylated Cdk1 and stable APC substrates (Figure A2.4). Cells lacking Mih1 and Rts1 do not arrest and Cdk1 is dephosphorylated

Figure A2.3.

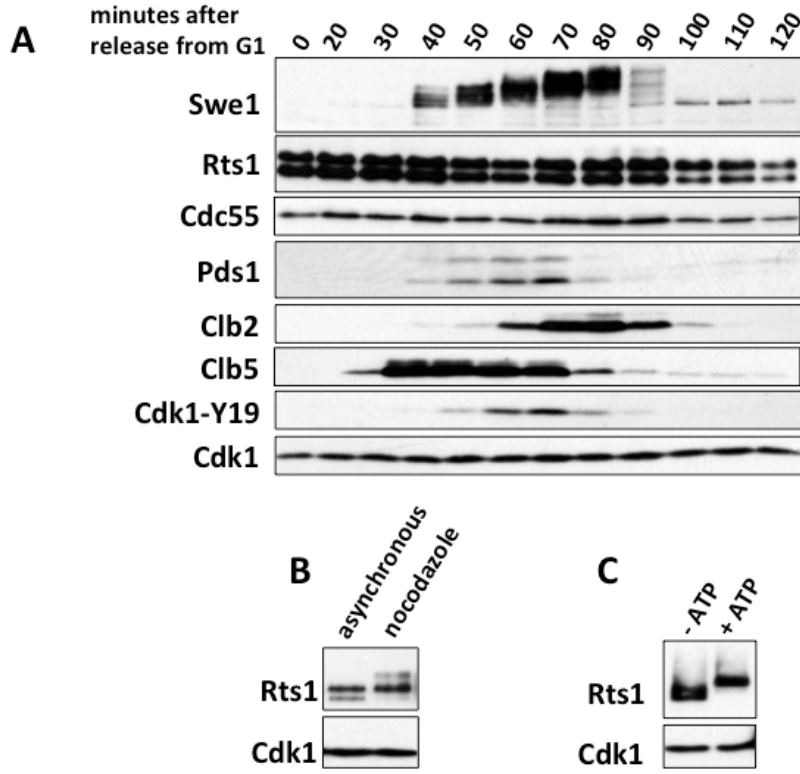


Figure A2.3. Rts1 is phosphorylated *in vivo* during mitosis and *in vitro* by Cdk1. (A) Wild type (ADR4006) cells were arrested in G1 with mating pheromone, released into the cell cycle and re-arrested in G1 by re-addition of pheromone. Lysates were blotted with the indicated antibodies. Note that a slower migrating form of Rts1 is present during the times of peak Clb2 protein (70-90 minutes). **(B)** Asynchronous and nocodazole arrested cells (ADR4006) were harvested, lysed and blotted with the indicated antibodies. Note that during a mitotic arrest Rts1 shifts to a slow migrating form. **(C)** Purified Cdk1/Clb2 was used to phosphorylate purified Rts1 *in vitro*. In reactions containing ATP, Rts1 is quantitatively phosphorylated.

Figure A2.4.

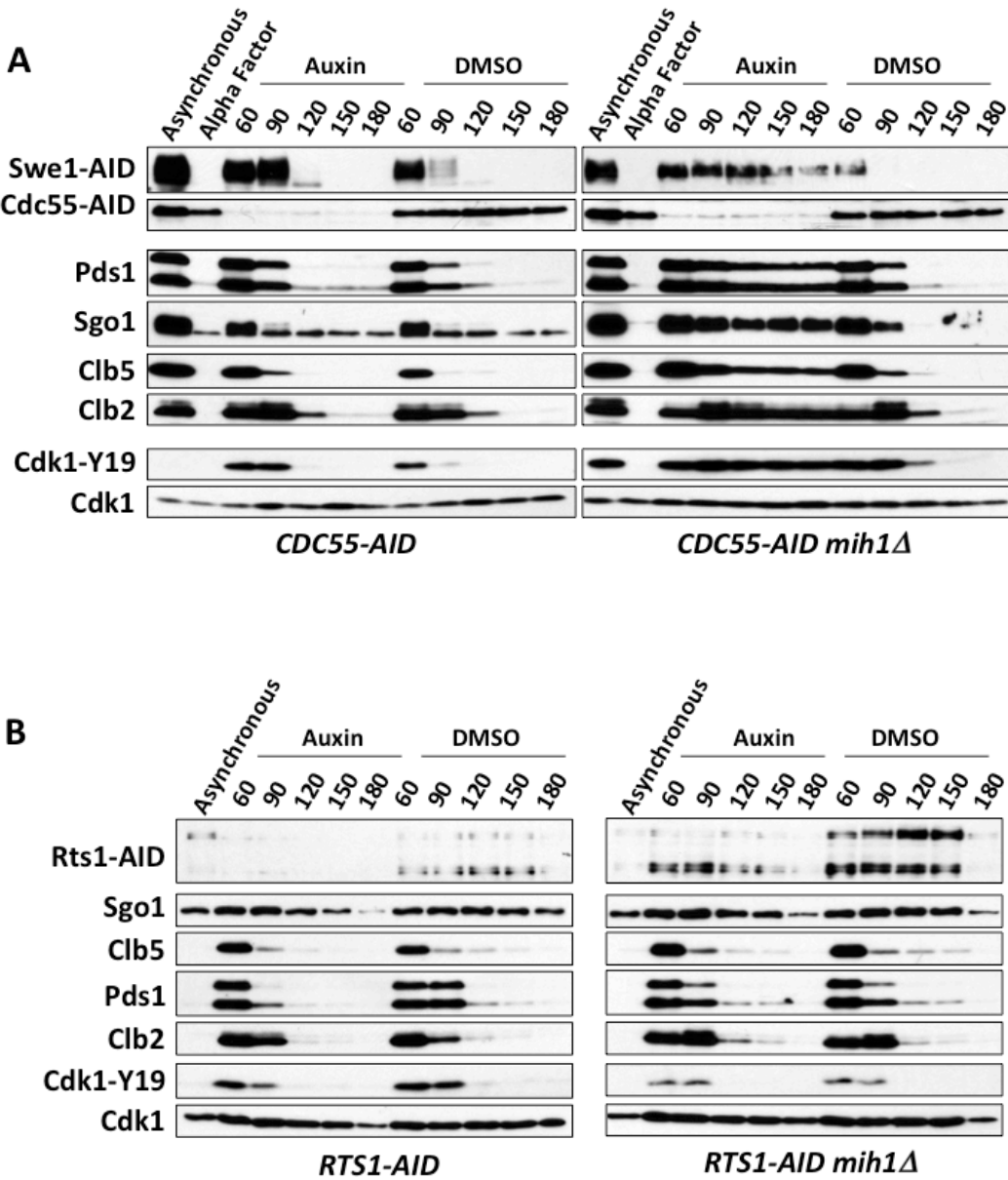


Figure A2.4. Terminal phenotypes of *mih1*Δ *CDC55-AID* and *mih1*Δ *RTS1-AID*. *CDC55-AID TIR1* (ADR6385), *CDC55-AID TIR1 mih1*Δ (ADR7368), and *RTS1-AID TIR1* (ADR7519), and *RTS1-AID TIR1 mih1*Δ (ADR7471) were grown YEP + raffinose (asyn), arrested in G1, then cultures were split and dextrose +/- auxin was added to repress transcription of and to induce Tir1-dependent degradation of the AID-tagged proteins. Cells were harvested at the indicated times for immunoblotting. **(A)** Cells lacking Mih1 and Cdc55 arrest in mitosis with stable APC substrates and with high tyrosine phosphorylated Cdk1, likely due to presence of Cdc55 phosphorylating and activating Swe1. Cdc55-AID was rapidly degraded following auxin treatment. **(B)** Cells lacking Mih1 and Rts1 do not arrest and Cdk1 is dephosphorylated indicating the presence of an additional phosphatase.

indicating the presence of an additional phosphatase or that sufficient Rts1-AID protein remains after auxin treatment. The Cdc55-AID strain maintains high Cdk1-Y19 levels due to an arrest prior to the metaphase/ anaphase transition due to lack of Swe1 regulation by Cdc55, not allowing cells to progress to a cell cycle point where Cdk1 could be dephosphorylated (Harvey et al., 2011; Pal et al., 2008). Strains were made containing Mih1-AID with either *cdc55Δ* or *rts1Δ* but the Mih1-AID was not efficiently degraded either due to loss of the tag allowing protein low levels of protein adequate to dephosphorylate Cdk1, or inefficient degradation due to mis-folding of the AID tag.

A2.1.5. Over expression of Rts1 in nocodazole arrested cells

To assess if Rts1 had any other roles in the metaphase to anaphase transition, cells over expressing Rts1 from a Gal promoter on a plasmid were arrested in G1 with nocodazole and held arrested followed by over expression of Rts1. Over expression of Rts1 was found to promote the degradation of Mcd1 (Figure A.2.5). Mcd1 is a component of the cohesin complex (holding sister chromatids together) which is degraded by Esp1 (separase) to allow separation of sister chromatids at the mitotic spindle (Uhlmann et al., 1999; Uhlmann et al., 2000). This is interesting, as the other PP2A complex in yeast, PP2A^{Cdc55} is found to dephosphorylate Mcd1 and prevent its cleavage by Esp1 (Liang et al., 2018; Morgan et al., 2012). Additionally, it was shown that in *cdc55Δ* cells, degradation of Mcd1 occurred prematurely and Cdk1 was shown to activate Esp1 (Clift et al., 2009; Liang et al., 2018). This may simply be that over expression of *RTS1* saturates the available PP2A complexes as PP2A^{Rts1}, thus reducing the ability of the cell to form PP2A^{Cdc55} and mimicking a *cdc55Δ*.

Figure A2.5.

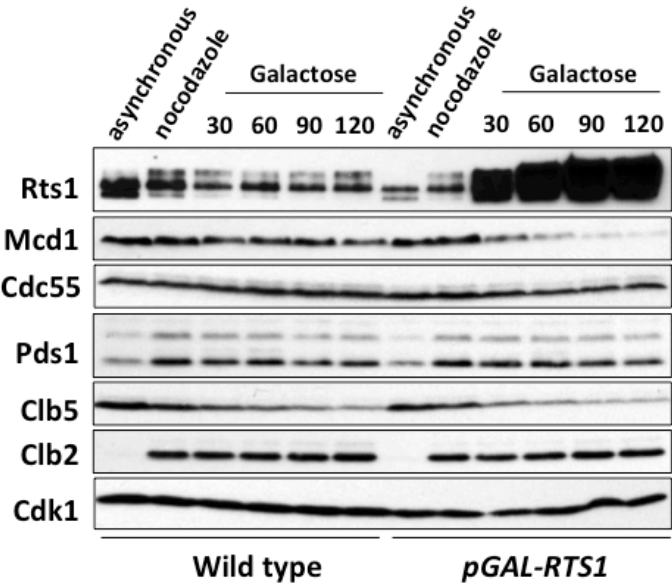


Figure A2.5. Over expression of Rts1 resulted in Mcd1 degradation required for cell cycle progression. Wild type (ADR4006) and Gal-Rts1 (ADR4006 pAR1126) expressed from a plasmid. Cells were arrested in mitosis with nocodazole and kept arrested for the indicated time, then harvested, lysed and blotted with the indicated antibodies. Note the decrease level of Mcd1 (a cohesion subunit, Scc1), indicating degradation of the cohesion complex.

A2.1.6. The role of Cak1 in Cdk1-Y19 phosphorylation

To investigate the role of Cdk1-T169 phosphorylation by Cak1 on the subsequent Cdk1-Y19, I used two temperature sensitive mutations of Cak1 (*cak1-23* and *cak1-95*) to look at growth defects and phosphorylation state of Cdk1. Phosphorylation of Cdk1-T169 is activating for Cdk1 activity (Kaldis et al., 1996). Cak1 phosphorylates Cdk1 at T169 which is in a large flexible loop that can block the Y19 phosphorylation site when unphosphorylated and stabilizes the cyclin-CDK interaction site (Kaldis et al., 1996; Russo et al., 1996; Thure et al., 1996). T169 can be dephosphorylated by Ptc2 and Ptc3, although the significance of this dephosphorylation is unknown as in wild type cells T169 phosphorylation does not vary over the cell cycle (Cheng et al., 1999; Ross et al., 2000). Interestingly, deletion of *MIH1* increased the temperature sensitive phenotype in both *cak1-23* and *cak1-95* (Figure A2.6), indicating a possible Mih1 regulation of the Cdk1-T169 phosphorylation.

To assess the phosphorylation of the Cdk1-T169 and Y19 sites, cells were grown over night in YEP raffinose for Gal inducing strains or YEP dextrose, arrested in nocodazole, induced with galactose for 1 hr if required, and samples taken for immunoblotting. Samples were run on standard acrylamide gels or acrylamide gels containing phos-tag reagent, transferred to nitrocellulose and blotted using total Cdk1 and Cdk1-Y169-P or Cdk1-Y19-P phosphorylation specific antibodies. Loss of Cak1 function results from exposure to non-permissive temperature, as observed by *cak1-23* and *cak1-95* having reduced T169 and Y19 phosphorylation (Figure A2.7.C). Consistent with spot assay results, the decreased viability of *cak1-23* at 37°C likely accounts for the absence of increased Y19 phosphorylation even at permissive temperature. The *cak1-95* mutation had consistent increase in Y19 phosphorylation with the loss of *MIH1* at permissive temperature,

Figure A2.6.

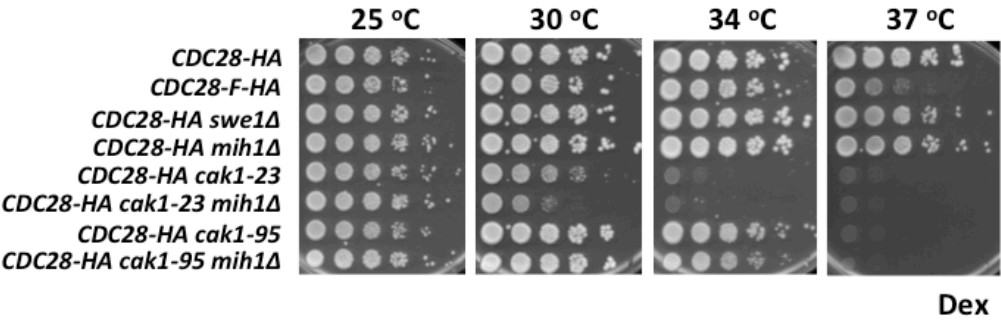
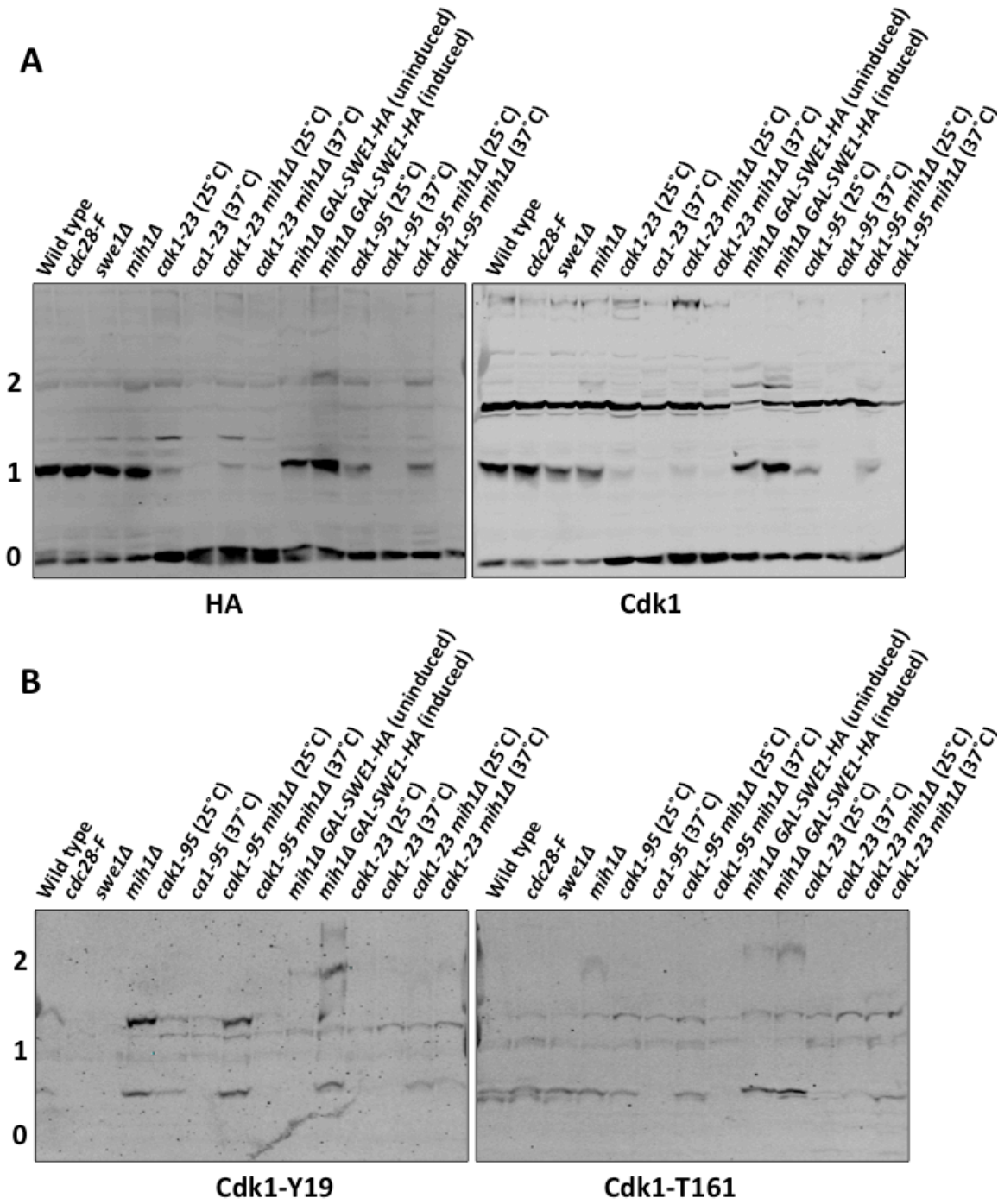


Figure A2.6. *cak1-23* temperature sensitivity was exacerbated by *mih1*Δ. All strains contain *CDC28-HA* for use in future analysis. Ten-fold serial dilutions of wild type (ADR477), *cdc28-F* (ADR510), *swe1*Δ (ADR1314), *mih1*Δ (ADR684) and *cak1-23* (ADR9946), *cak1-23 mih1*Δ (ADR9949), *cak1-95* (ADR9950), and *cak1-95 mih1*Δ (ADR9952) cells were spotted onto YPD plates and incubated at the indicated temperatures. *cak1-23* and *cak1-95* are temperature sensitive mutations of *CAK1* reducing phosphorylation on T169 of Cdk1 and subsequent Y19 phosphorylation.

Figure A2.7.



C

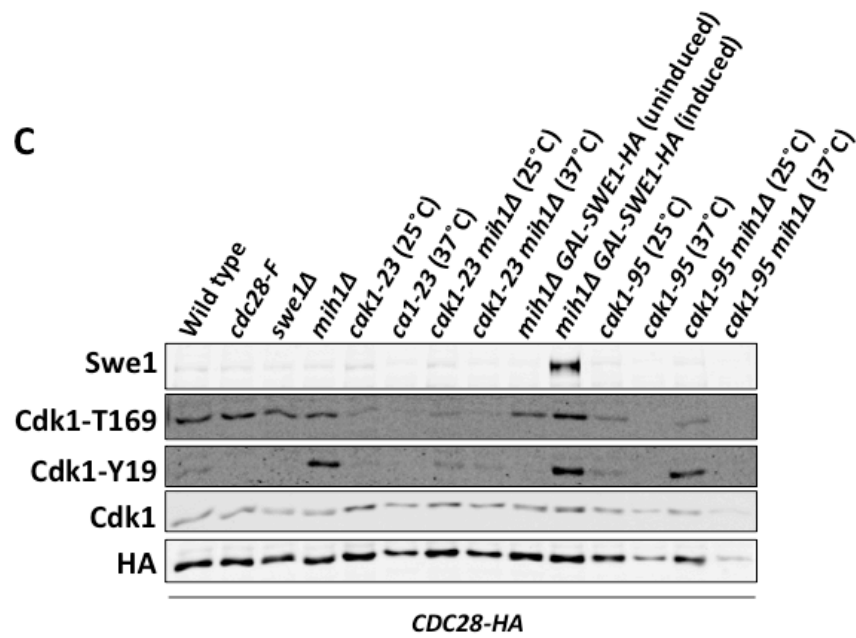


Figure A2.7. Mutating *CAK1* resulted in altered Cdk1-T169 and Y19 phosphorylation. All strains contain *CDC28-HA* for use in future analysis: wild type (ADR477), *cdc28-F* (ADR510), *swe1Δ* (ADR1314), *mih1Δ* (ADR684) and *cak1-23* (ADR9946), *cak1-23 mih1Δ* (ADR9949), *mih1Δ GAL-SWE1-HA* (ADR1374), *cak1-95* (ADR9950), and *cak1-95 mih1Δ* (ADR9952). Cells were grown in YPD or YEP + raffinose, arrested in mitosis with nocodazole and switched to YEP + galactose if necessary media to induce expression of Swe1. After 1 h, Samples were taken for immunoblotting, and run on a polyacrylamide gel containing Phos-tag reagent (**A, B**), or a standard polyacrylamide gel (**C**) and immunoblotted with the indicated antibodies. Note that running and transferring of Phos-tag polyacrylamide gels is inconsistent and cell cycle-dependent changes in protein abundance observed in these panels may not accurately reflect changes in protein abundance. Both *CAK1* temperature sensitive mutations resulted in loss of the Cak1 protein, although *cak1-95 mih1Δ* had increased Cdk1-Y19 phosphorylation at permissive temperature consistent with *mih1Δ* alone.

as observed in *mih1*Δ alone. Over expression of Cdk1-Y19 kinase Swe1 in an *mih1*Δ strain increased both Y19 and T169 phosphorylation.

Phos-tag reagent will slow the migration of phosphorylated proteins in proportion to the amount of phosphorylation present in the protein. The single phosphorylation is proposed to be the activating T169 phosphorylation, consistence with its absence in both *cak1-23* and *cak1-95* at non-permissive temperature as shown by probing with total Cdk1 (Figure A2.7.A), and both phospho antibodies to Y19 and T169 (Figure A2.7.B). Based on probing total Cdk1 with anti-HA or anti-Cdk1 antibodies (Figure A2.7.A), the majority of Cdk1 is singly phosphorylated, with mutations in *CAK1* resulting in the majority of Cdk1 being un-phosphorylated. When observing only the phosphorylated portion of Cdk1 (Figure A2.7.B), deletion of *MIH1* increases both the Y19 and T169 phosphorylation amount compared to wild type. Shifting the *cak1-95* mutant to the non-permissive temperature resulted in the loss of T169 phosphorylation but Y19/T169 double phosphorylation persisted, possibly sheltered from phosphatases; this is consistent with and without *mih1*Δ.

Table A2.1. Strains used in this analysis

Strains used in this study are derivatives of W303-1 (*ura3-1 leu2-3,112 trp1-1 his3-11 ase2-1 can1-100*).

Strain	MAT	Genotype
ADR477	a	<i>CDC28-HA-CaURA3</i>
ADR510	a	<i>CDC28-F-HA-CaURA3</i>
ADR684	a	<i>swe1Δ::TRP1 CDC28-HA-CaURA3</i>
ADR1314	a	<i>mih1Δ::LEU2 CDC28-HA-CaURA3</i>
ADR1374	a	<i>mih1Δ::LEU2 swe1::pGAL-SWE1-HA-LEU2 cdc28::CCD28-HA-URA3</i>
ADR3173	a	<i>swe1Δ::TRP1 bar1Δ</i>
ADR4006	a	wild type <i>bar1Δ</i>
ADR6385	a	<i>CDC55-AID-kanMX bar1Δ leu2::pGDP1-OsTIR1-LEU2</i>
ADR6876	a	<i>cdc55Δ::hphMX bar1Δ</i>
ADR6929	a	<i>rts1Δ::hphMX bar1+</i>
ADR7368	a	<i>CDC55-AID-kanMX bar1Δ mih1Δ::HYG^R, leu2::pGDP1-OsTIR1-LEU2</i>
ADR7445	a	<i>RTS1-AID-kanMX bar1Δ mih1Δ::hphMX, leu2::pGDP1-OsTIR1-LEU2</i>
ADR7471	a	<i>RTS1-AID-kanMX bar1Δ mih1Δ::hphMX, leu2::pGDP1-OsTIR1-LEU2</i>
ADR7519	a	<i>RTS1-AID-kanMX bar1Δ leu2::pGDP1-OsTIR1-LEU2</i>
ADR7550	a	<i>bar1Δ ppz1Δ::hphMX</i>

ADR7960	a	<i>bar1Δ ltp1Δ: kanMX</i>
ADR8279	a	<i>bar1Δ rrd1Δ::hphMX</i>
ADR8282	a	<i>bar1Δ rrd1Δ::hphMX</i>
ADR8300	a	<i>ptp3Δ::CaURA3</i>
ADR9946	a	<i>CDC28-HA-URA3 cak1-23</i>
ADR9949	α	<i>CDC28-HA-URA3 cak1-23 mih1Δ::LEU2</i>
ADR9950	a	<i>CDC28-HA-URA3 cak1-95</i>
ADR9952	a	<i>CDC28-HA-URA3 cak1-95 mih1Δ::LEU2</i>

A2.2. Additional methods

A2.2.1. PhosTag polyacrylamide gels

3.0×10^7 cells were harvested for immunoblotting and cell pellets were washed twice with 50mM HEPES pH 8.0. Cell extracts were prepared by bead beating frozen cell pellets in a Mini- Beadbeater (BioSpec Products) in 1X urea sample buffer (2% SDS, 65 mM Tris-Cl pH 6.8, 10% glycerol, 4M Urea, 0.02% bromophenol blue, 5% betamercaptoethanol, and 1mM PMSF) and an excess of acid washed glass beads (BioSpec Products) for 60 sec. Samples were run on 10% acrylamide gels with 50 μ M Phos-tag reagent (Wako), 200 μ M MnCl_2 and 0.1% SDS. Gels were run for 2 h at 200V and 25mA. Following electrophoresis, gels were washed 2 x 10 min in transfer buffer with 1 mM EDTA, 1 x 10 min in transfer buffer and transferred to nitrocellulose using standard wet-transfer protocol at 60 V and 500 mA for 2 hr at 4°C.

Erin Kennedy – Curriculum Vitae

Education

Doctorate in Biochemistry,

2013 - 2018

University of Ottawa, Ottawa, ON

Dr. A. Rudner and Dr. P. Chakraborty, Department of Biochemistry, Microbiology, and Immunology

- Using yeast to study the pathobiology of SIFD, a genetic rare disease
- Redundant Regulation of Cdk1 Tyrosine Dephosphorylation in *S. cerevisiae*
- Graduate level courses: Analytical Techniques in Proteomics, Lipidomics, Metabolomics, and Genomics; and RNA Molecular Biology

Master's in Molecular Biology and Genetics,

2005 - 2008

University of Alberta, Edmonton, AB

Dr. F. Nargang, Department of Biological Sciences, Molecular Biology and Genetics

- The Role of Tob37 and Tob38 in Mitochondrial b-Barrel Protein Assembly
- Graduate level courses: Genetic; and Ethics in Science

Bachelor of Science, Honours, First Class Distinction

2000 - 2005

Dalhousie University, Halifax, NS

Dr. R. Lee, Department of Biology,

- Unique Mitochondrial genome architecture in the *Oogamochlamys*-Clade
- Graduate level courses: Bio-statistical Analysis; Gene Expression; and Bioinformatics

Relevant Work Experience

Radiation Research Technologist,

2009 – 2011

Defence Research and Development Canada, Ottawa

Capabilities for Asymmetric & Radiological Defence and Simulation

- Government representative on multi-departmental product development project
- Developed a novel assay for assessing DNA damage, published in scientific literature
- Conducted diagnostic testing and statistical analyses for a regulatory need accordance with ISO 19238 and IAEA Tech Report 405

Laboratory Manager,

2008 - 2009

University of Ottawa, Ottawa, ON

Dr. A. Rudner, Department of Biochemistry, Microbiology, and Immunology

- Conducted independent and collaborative research in yeast
- Prepared and participated in a Canadian Nuclear Safety Commission (CNSC) audit
- Trained students; managed supplies, purchasing, and finance; developed and implemented new laboratory techniques, member of Fire Safety Committee, maintained laboratory safety requirements and records

**University Teaching Assistant,
Dalhousie University and University of Alberta**

2003 – 2008

- Introduction to Scientific Writing, University of Alberta, 2007 – 2008
 - Taught 100+ students an introduction to scientific writing, marked assignments
 - Marked final Cell Biology exam
- Cell Biology Laboratory Techniques, University of Alberta, 2005 – 2007
 - Guided students through weekly lab sessions
 - Marked lab reports and final Cell Biology exam
- Introduction to Microbiology, Dalhousie University, 2003 – 2005
 - Guided students through weekly lab sessions and marked assignments
 - Evaluated in lab performance and presentations, proctored lab exam

Honours, and Awards

- CIHR National Poster Competition 2017, Honorable Mention
- Ontario Graduate Scholarship 2013 - 2017, University of Ottawa
- Excellence Scholarship 2013 - 2017, University of Ottawa
- Susan Eberlein Graduate Scholarship in Genetics, 2007, University of Alberta
- Queen Elizabeth II Graduate Scholarship – Master's level, 2007, University of Alberta
- Teaching Assistant Letter of Commendation, 2007, University of Alberta
- Mary Louise Imrie Graduate Student Award, 2006, University of Alberta
- Graduate Scholarship, 2007, Alberta Advanced Education Achievement program
- Dalhousie University Dean's List, 2003 - 2005, Dalhousie University
- David Andrew Dougall Memorial Bursary, 2004, Dalhousie University
- Sarah Lawson Research Award in Botany, 2004, Dalhousie University
- Entrance Scholarship, 2000, Dalhousie University

Publications

- Lianga, N., Doré, C., **Kennedy, E.K.**, Yeh, E., Williams, E.C., Fortinez, C.M., Wang, A., Bloom, K.S., Rudner, A.D. 2018. Cdk1 phosphorylation of Separase functions with PP2A and Slk19 to regulate the localization of pericentric Cohesin and anaphase onset. *PLoS Genetics* 14(3): e1007029.
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- Chakraborty, P., Schmitz-Abe, K., **Kennedy, E.**, Mamady, H., Naas, T., Durie, D., et al. 2014. Mutations in TRNT1, encoding the CCA-adding enzyme, cause congenital sideroblastic anemia with B-cell immunodeficiency, periodic fevers, and developmental delay (SIFD). *Blood*. 124(18): 2867-2871.
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- J. G. Wideman, N. E. Go, A. Klein, **E. Redmond**, S. W. K. Lackey, T. Tao, H. Kalbacher, D. Rapaport, W. Neupert and F. E. Nargang. 2010. Roles of the Mdm10, Tom7, Mdm12, and Mmm1 Proteins in the Assembly of Mitochondrial Outer Membrane Proteins in *Neurospora crassa*. *Molecular Biology of the Cell*. 21: 1725-1736.
- T. Borza, **E. K. Redmond**, M. Laflamme, R. W. Lee. 2009. Mitochondrial DNA in the *Oogamochlamys*-Clade (Chlorophyceae): High GC content and unique genome architecture for green algae. *Journal of Phycology* 45, 1323-1334.

Presentations and Posters

- **E. Kennedy**, M. Holcik, P. Chakraborty, A. Rudner. Using yeast to determine the pathobiology of SIFD.
 - Poster: CIHR Research Poster Competition. Winnipeg, MB. 2017.
 - Poster: Canadian Society of Molecular Biosciences. Ottawa, ON. 2017.
 - Talk: Gordon Research Seminar, Translation Machinery in Human Health and Disease, Galveston, TX, USA. 2017.
 - Poster: Gordon Research Conference, Translation Machinery in Human Health and Disease, Galveston, TX, USA. 2017.
 - Talk: University of Ottawa Graduate Student Research Day, Ottawa, ON. 2017.
 - Poster: Canadian Human and Statistical Genetics Meeting, Halifax, NS. 2016.
 - Poster: University of Ottawa Graduate Student Research Day, Ottawa, ON. 2016. ; Awarded 2nd Place
- **E. K. Kennedy**, M. Dysart, N. Lianga, C. Dore, A. D. Rudner. Is tyrosine dephosphorylation of Cdk1 required for anaphase onset?
 - Poster: University of Ottawa Graduate Student Research, Ottawa, ON. 2015.
 - Talk: University of Ottawa Graduate Student Research, Ottawa, ON. 2013.
 - Poster: University of Ottawa Graduate Student Research, Ottawa, ON. 2013.
 - Poster: OISB Annual Meeting. Ottawa, ON. 2013
- **E. K. Kennedy**, L. Prud'homme Lalonde, R. Liu, B. C. Foster, D. Wilkinson. Botanical Extracts as Medical Countermeasures for Radiation Induced Damage
 - Poster: Health Physics Society Annual Conference. Florida, USA. 2011.
 - Poster: Radiation Research Annual Conference. Maui, USA. 2010.

- Poster: Defence Military Personnel Research Symposium. Ottawa, ON. 2010.
- Talk: Canadian Radiation Protection Association. Edmonton, AB. 2010.
- **E. K. Redmond**, N. E. Go, F. Nargang. Tob37 and Tob38 in Mitochondrial Beta-Barrel Assembly.
 - Poster: The Genetics Society of Canada Annual Conference, Montreal, QC. 2007.
 - Poster: University of Alberta, Graduate Student Research Day. Edmonton, 2007.
- **E. K. Redmond**, T. Borza, R. W. Lee. Conformation and partial sequence analysis of the *Chlamydomonas culleus* and *Chlamydomonas segnis* mitochondrial genomes.
 - Poster: Maritimes Protistology Conference. Halifax, NS. 2005.
 - Talk: Atlantic Universities Biology Conference, Antigonish, NS. 2005.

Current Professional Affiliations

- Member - Genetics Society of America
- Member - Canadian Society for Molecular Biosciences

Current Community Involvement

- Executive Board Member, Needham Preschool, Halifax, NS, 2016 - present
- Canadian Society for Molecular Biosciences Trainee Committee 2017 - 2018
- Co-Manager, Crichton Park Critter Soccer Club, Halifax, NS, 2016 - present