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Factors modulating tRNA biogenesis and function in *Saccharomyces cerevisiae*

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Abstract

Transfer RNA (tRNA) genes are transcribed by RNA polymerase III as precursors that undergo multiple processing steps to form mature tRNAs. These steps include processing of the 5' leader and 3' trailer sequences, addition of a 3' CCA tail, removal of introns, and formation of modified nucleosides. The mature tRNAs carry amino acids to the ribosome where proteins are synthesized. The aim of this thesis is to identify and characterize factors that influence tRNA biogenesis and function in *Saccharomyces cerevisiae*.

Nonsense suppressor tRNAs are encoded by mutated tRNA genes and able to read stop codons. The *SUP4* gene encodes such a suppressor tRNA that base-pairs with UAA stop codons. By screening for mutations that impair the nonsense suppression of the *SUP4*-encoded tRNA, we identified a loss-of-function mutation in the *YPK9* gene. Inactivation of Ypk9p causes a reduction in the readthrough of UAA stop codon. We found that phenotypes of *ypk9Δ* cells including decreased UAA readthrough and sensitivity to Mn^{2+} are counteracted by increasing the cellular levels of putrescine, one type of polyamine. Importantly, cells lacking Ypk9p show reduced levels of putrescine. Our results suggest that the *YPK9* gene product influences the cellular levels of putrescine, which plays a role in maintaining the fidelity of translation termination.

The Elongator complex, consisting of Elp1p-Elp6p six proteins, catalyzes the formation of U_{34} modifications in the anticodon region of 11 tRNA species. Elongator mutants display pleiotropic phenotypes that are caused by decreased tRNA functionality. We found that the genetic background, largely due to a polymorphism at the *SSD1* locus, influences the pleiotropic phenotypes of Elongator mutants.

In a genetic screen for factors that are essential for the survival of cells encoding a destabilized $tRNA^{Ser}_{CGA}$, several gene products were identified. We demonstrate that mutations in these genes result in reduced levels of the destabilized $tRNA^{Ser}_{CGA}$, suggesting a role for these gene products in $tRNA^{Ser}_{CGA}$ biosynthesis.

Abbreviations

tRNA	Transfer RNA
Pre-tRNA	Precursor transfer RNA
$tRNA_i^{Met}$	Initiator methionine acceptor tRNA
mRNA	Messenger RNA
UTR	Untranslated region
eIFs	Eukaryotic translation initiation factors
eEFs	Eukaryotic translation elongation factors
eRFs	Eukaryotic release factors
PIC	Preinitiation complex
mRBP	Messenger RNA-binding protein
ORF	Open reading frame
NMD	Nonsense-mediated mRNA decay
SEN	Splicing endonuclease
ODC	Ornithine decarboxylase
SAM	*S*-adenosylmethionine
dcSAM	Decarboxylated *S*-adenosylmethionine
U_{34}	Uridine at position 34 in tRNA
ψ	Pseudouridine
Um	2'-*O*-methyl-uridine
cm^5U	5-carboxymethyl-uridine
ncm^5U	5-carbamoylmethyl-uridine
ncm^5Um	5-carbamoylmethyl-2'-*O*-methyl-uridine
mcm^5U	5-methoxycarbonylmethyl-uridine
mcm^5s^2U	5-methoxycarbonylmethyl-2-thio-uridine
m^1A	1-methyl-adenosine
m^1G	1-methyl-guanosine

ac^4C	N^4-acetyl-cytidine
KAT	Lysine acetyltransferase
HPLC	High-performance liquid chromatography
CWI	Cell wall integrity
FD	Familial dysautonomia
ID	Intellectual Disability

Papers in this thesis

This thesis is based on the following papers that are referred to by their Roman numerals.

I. Ypk9p influences putrescine levels and the fidelity of translation termination.

Xu F, Huang B, Saiardi A, Johansson MJO, and Byström AS.

Manuscript

II. *SSD1* suppresses phenotypes induced by the lack of Elongator-dependent tRNA modifications.

Xu F, Byström AS, and Johansson MJO.

PLoS Genetics. 2019;15(8): e1008117

III. Identification of factors that promote biogenesis of $tRNA^{Ser}_{CGA}$.

Xu F, Zhou Y, Byström AS, and Johansson MJO.

RNA Biology. 2018;15(10): 1286-94

Introduction

1. Translation

1.1 Translation initiation and elongation

During translation, mRNAs are decoded by the ribosome to produce polypeptide chains (**Figure 1**). The translation process can be divided into four steps: initiation, elongation, termination, and ribosome recycling (Schuller and Green 2018).

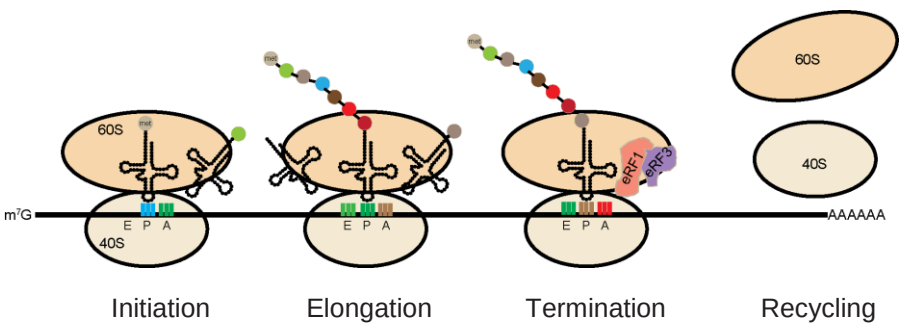

Figure 1. Overview of translation in eukaryotes.

Translation is predominantly regulated at the stage of translation initiation, which involves the following steps: formation of a 43S preinitiation complex (PIC), binding of the 43S PIC to the 5' end of an mRNA, ribosome scanning of the 5' untranslated region (5'-UTR), and assembly of an 80S ribosome complex at the start site of translation (Jackson et al. 2010). Translation initiation factor 2 (eIF2), met-tRNA$_i^{Met}$, and GTP form a ternary complex that attaches to the 40S small ribosome subunit together with eIF1, eIF1A, eIF3, and eIF5, to form a 43S PIC. This 43S complex binds close to the m^{7}G cap structure of an mRNA via a process assisted by the eIF4F complex. The eIF4F complex consists of

eIF4A (an ATP-dependent RNA helicase), eIF4E (a cap-binding protein), and eIF4G, which acts as an adaptor molecule between the mRNA and the 43S complex. The eIF4B (an RNA-binding protein) enhances the RNA helicase activity of eIF4A. Following the unwinding of secondary structures in the 5' UTR, the ribosome complex scans the mRNA in the 3' direction. Upon recognition of the first AUG codon by met-tRNA$_i^{Met}$, eIF5 and eIF5B displace the translation initiation factors and a 60S ribosome subunit joins resulting in an 80S initiation complex (Jackson et al. 2010). In addition to the canonical translation initiation model, translation initiation may also occur at internal ribosome entry sites (Haimov et al. 2015) and the initiation codon can be a non-AUG codon (Kearse and Wilusz 2017).

At the start of translation elongation, the 80S complex has an empty A-site and a met-tRNA$_i^{Met}$ in the P-site (Dever et al. 2018). In the next step, the translation elongation factor eEF1A, aminoacyl-tRNA, and GTP form a ternary complex and the tRNA anticodon undergoes base-pairing with the A-site codon **(Figure 1**). Following GTP hydrolysis by eEF1A, GDP and eEF1A are released. Thereafter, the amino acid attached to the A-site tRNA forms a peptide bond with the P-site peptidyl-tRNA, which is suggested to be promoted by the hypusine-modified eIF5A (Saini et al. 2009; Schuller et al. 2017). Upon peptide bond formation, the elongation factor eEF2 translocates the tRNA and mRNA relative to the ribosome and leaves a vacant A-site for incorporation of a new aminoacyl-tRNA ternary complex. During translocation, the P-site tRNA is moved to the E-site and released from the ribosome (Dever et al. 2018). In fungi, translation elongation requires a third factor, eEF3, which may promote dissociation of deacylated tRNA from the E-site after translocation (Triana-Alonso et al. 1995; Andersen et al. 2006; Kasari et al. 2019).

1.2 Translation termination and recycling

The termination phase of translation starts when a stop codon is present at the ribosomal A-site (**Figure 1**). Facilitated by eRF3 and GTP, release factor eRF1 interacts with the A-site stop codon and induces peptide release in generating a post-termination complex (Hellen 2018). The hypusine-modified eIF5A is likely able to stimulate the process of peptide release (Saini et al. 2009; Schuller et al. 2017). In the ribosome recycling phase, the ATP-binding protein Rli1p binds to eRF1 in the post-termination complex where eRF3 has been dissociated. Upon ATP binding and hydrolysis, the post-termination complex is dissociated generating a 60S subunit, an mRNA/tRNA-bound 40S subunit, eRF1, and Rli1p. Thereafter, the mRNA and deacylated tRNA are released from the 40S subunit, a step involving several factors including Tma20p, Tma22p, and Tma64p (Hellen 2018; Young et al. 2018).

1.3 Readthrough of stop codons

Translation usually stops when a ribosome encounters a stop codon in the A-site. When the ribosome encounters a premature termination codon, the factors Upf1p, Upf2p, and Upf3p of the nonsense-mediated mRNA decay pathway (NMD) are recruited and trigger ribosome dissociation and mRNA degradation (He and Jacobson 2015). However, several factors such as eRF3 in its [*PSI*$^{+}$] prion form, increased levels of Mg^{2+}, and suppressor tRNAs are known to cause readthrough of stop codons (Celis and Piper 1981; Liebman and Derkatch 1999; Johansson and Jacobson 2010). The prion form of eRF3 sequesters the release factors eRF3 and eRF1 from translation termination (Liebman and Derkatch 1999). Mg^{2+}, an essential co-factor for translation, is required for efficient translation and, at elevated levels, it can induce readthrough of

stop codons (Johansson and Jacobson 2010). Genes encoding tRNAs can be mutated to generate suppressor tRNAs that base-pair with stop codons (Beier and Grimm 2001). Several suppressor tRNAs decoding stop codons have been identified in *Saccharomyces cerevisiae* (Celis and Piper 1981). A well-studied example of such a suppressor tRNA is the *SUP4*-encoded suppressor tRNA (Gilmore et al. 1971; Goodman et al. 1977). The *SUP4* gene, which is a mutant form of the *tY(GUA)J2* gene, encodes a tRNA with a U instead of a G at position 34. The suppressor tRNA is able to read UAA and, to a lesser extent, UAG stop codons (Gilmore et al. 1971; Johansson et al. 2008).

1.4 Polyamines

Polyamines (putrescine, spermidine, and spermine) are positively charged molecules that are important for life (Hoyt and Davis 2004). The biosynthesis of polyamines begins with the decarboxylation of ornithine by the ornithine decarboxylase (ODC) Spe1p generating putrescine, which is the rate-limiting step in the biosynthesis pathway (**Figure 2**) (Fonzi and Sypherd 1987; Hoyt and Davis 2004). In the presence of decarboxylated *S*-adenosylmethionine (dcSAM), putrescine is converted to spermidine by Spe3p and sequentially to spermine by Spe4p. The biosynthesis of polyamines is downregulated by the antizyme Oaz1p, which binds and inhibits the activity of Spe1p (**Figure 2**). Expression of the *OAZ1* gene is autoregulated and requires a programmed +1 frameshifting, which is regulated by polyamines (Rom and Kahana 1994; Kurian et al. 2011). In addition to a role in +1 frameshifting, polyamines promote translational readthrough of premature stop codons in bacteria and human cells (Hryniewicz and Vonder Haar 1983; Yoshida et al. 2002; Petros et al. 2005). The hypusine modification of eIF5A (see section 1.1)

is synthesized from spermidine (Park 1989). Since polyamines are positively charged molecules, they can neutralize polyanions and stabilize membranes through binding to negatively charged molecules (Hoyt and Davis 2004; Lightfoot and Hall 2014). Polyamines are also required for the biosynthesis of pantothenic acid, a precursor of coenzyme A (White et al. 2001).

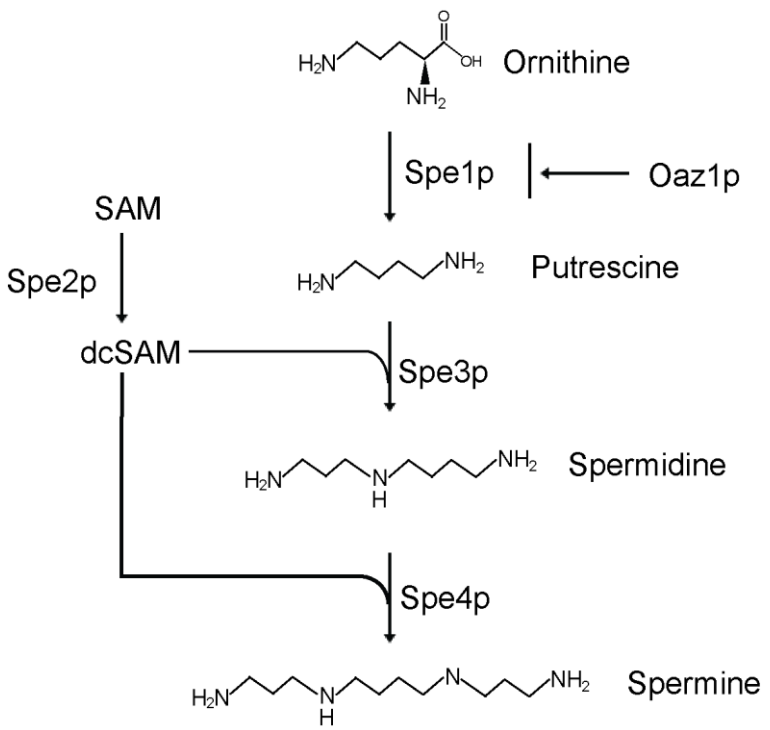

Figure 2. Pathway of polyamine (putrescine, spermidine, and spermine) biosynthesis in *S. cerevisiae*.

1.5 Messenger RNA-binding proteins

In eukaryotes, primary mRNA transcripts are produced in the nucleus and mature mRNAs are translated in the cytoplasm. In addition to regulation at the transcriptional level, gene expression is also extensively regulated posttranscriptionally by mRNA-binding proteins (mRBPs) (Glisovic et al. 2008). In yeast, more than one thousand mRBPs have been identified (Hentze et al. 2018). These proteins function in almost every aspect of RNA biology including biogenesis, modification, localization, translation, and turnover resulting in fine-tuning of gene expression (Hentze et al. 2018). The mRBP Ssd1p associates with more than 150 mRNAs (Uesono et al. 1997; Hogan et al. 2008; Jansen et al. 2009). Of the Ssd1p-associated mRNAs, a large fraction encodes proteins that are involved in cell wall biogenesis (Hogan et al. 2008; Jansen et al. 2009). In addition to a role in cell wall biogenesis, Ssd1p has been shown to influence many other cellular processes such as aging, stress response, and transcription of RNA polymerases I, II, and III (Stettler et al. 1993; Kaeberlein and Guarente 2002; Kaeberlein et al. 2004). Several effects of Ssd1p on its binding targets have been suggested, including repressing translation, sequestering mRNAs to P-bodies, and increasing mRNA turnover (Jansen et al. 2009; Li et al. 2009a; Kurischko et al. 2011; Hu et al. 2018). Therefore, it is believed that the effect of Ssd1p on cell physiology is influenced by its effect on gene expression.

2. Transfer RNA

2.1 Maturation of the 5’ leader and 3’ trailer of precursor tRNA

Nuclear-encoded tRNAs are transcribed by RNA polymerase III as precursors with a 5’ leader and a 3’ trailer sequence (**Figure 3**) (Hopper 2013). tRNA maturation begins with cleavage of the 5’ leader by a multisubunit RNase P and the 3’ trailer is trimmed by RNase Z and Rex1p (Hopper 2013). Subsequently, a CCA tail is attached to the 3’ terminus by the nucleotidyltransferase Cca1p (Aebi et al. 1990).

2.2 tRNA shuttling and splicing

Following maturation of the 5’ and 3’ ends, tRNAs are exported from the nucleus to the cytoplasm (**Figure 3**). tRNA transport is bidirectional, which means that tRNAs can shuttle between the nucleus and cytoplasm (Shaheen and Hopper 2005; Takano et al. 2005a). tRNA shuttling is of three types: primary nuclear export, retrograde nuclear import, and nuclear re-export (Hopper 2013). A majority of primary tRNA nuclear export is performed by Los1p (Hellmuth et al. 1998). Cells lacking Los1p accumulate intron-containing pre-tRNAs in the nucleus since in yeast the removal of introns occurs in the cytoplasm (**Figure 3**) (Hopper et al. 1980; Murthi et al. 2010). The proteins Msn5p, Crm1p, and the Mex67p·Mtr2p heterodimer have also been suggested to play a role in primary nuclear export (Takano et al. 2005a; Murthi et al. 2010; Wu et al. 2015).

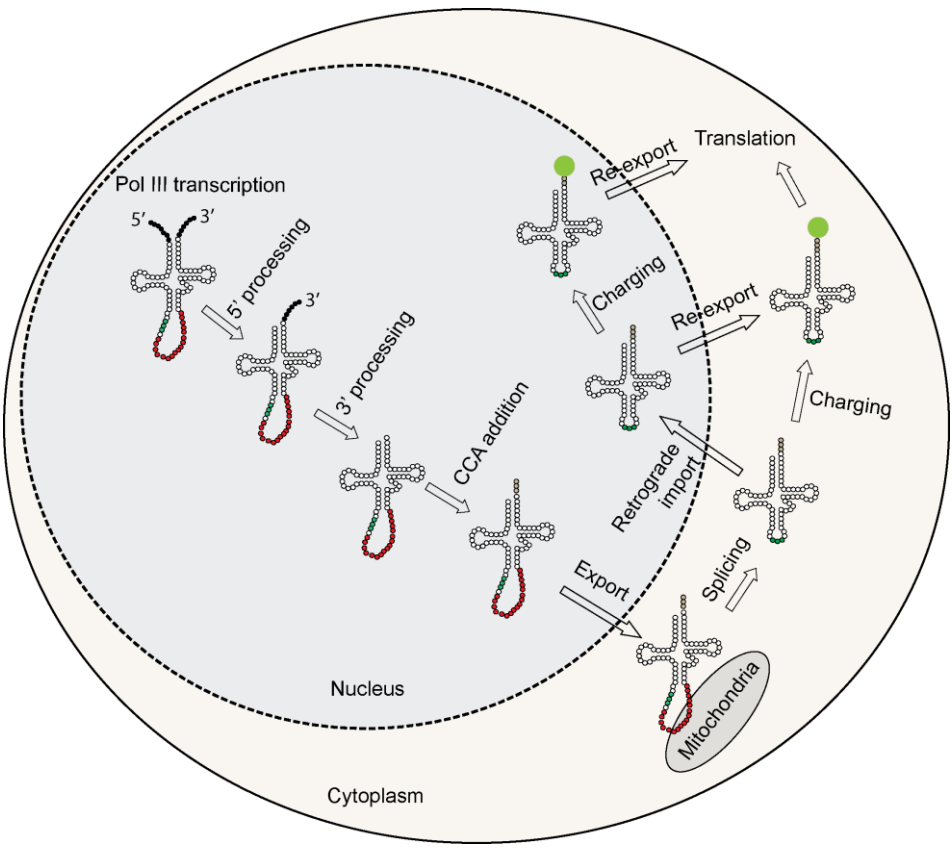

Figure 3. Processing of tRNAs encoded by intron-containing genes in *S. cerevisiae*.

Of the nuclear-encoded tRNAs in *S. cerevisiae*, 61 out of 275 (≈22%) genes contain an intron (Chan and Lowe 2016). The introns in pre-tRNAs are removed by a splicing endonuclease (SEN) complex, which is a heterotetramer consisting of four essential protein subunits, namely, Sen2p, Sen15p, Sen34p, and Sen54p (Trotta et al. 1997). The yeast SEN complex is located on the outer membrane of mitochondria (**Figure 3**) (Yoshihisa et al. 2003). Therefore, following maturation of the 5' and 3' ends in the nucleus, pre-tRNAs with an intron must be exported to the cytoplasm for intron removal. Spliced fragments are ligated by the tRNA ligase Trl1p, and the splicing is completed by the phosphotransferase Tpt1p (Phizicky and Hopper 2010).

Since the splicing of pre-tRNAs takes place on the mitochondrial outer membrane, the nuclear accumulation of spliced tRNAs transcribed from intron-containing tRNA genes indicates that certain species of tRNAs are able to move from the cytoplasm to the nucleus, a process called tRNA retrograde nuclear import (**Figure 3**) (Shaheen and Hopper 2005; Takano et al. 2005b). The proteins Mtr10p and Ssa1p are required for retrograde nuclear tRNA import (Shaheen and Hopper 2005; Takano et al. 2015). The nuclear re-export of tRNAs is the process in which retrograde-imported tRNAs are exported to the cytoplasm (**Figure 3**). Proteins involved in tRNA re-export include Msn5p (Wu et al. 2015) and Los1p (Huang and Hopper 2015). Moreover, the proteins eEF1A, the Mex67p·Mtr2p heterodimer, and aminoacyl-tRNA synthetases are also implicated in tRNA re-export (Chatterjee et al. 2018). tRNA retrograde import and re-export are important for the biosynthesis of wybutosine modification in $tRNA^{Phe}$. After splicing in the cytoplasm, intronless $tRNA^{Phe}$ is imported into the nucleus for the formation of m^1G_{37} and re-exported to the cytoplasm where several additional steps are required for the wybutosine formation (Ohira and Suzuki 2011). The two trafficking

processes are also suggested to regulate the cytosolic tRNA pools depending on nutrient availability (Murthi et al. 2010).

2.3 Modified nucleosides in tRNA

A common feature of tRNAs across all three domains of life is that they all contain posttranscriptionally modified nucleosides (Bjork and Hagervall 2014; Agris et al. 2017). In *S. cerevisiae*, 25 different types of modified nucleosides have been identified in cytosolic tRNAs (Phizicky and Hopper 2010). These modifications are important for tRNA stability, tRNA discrimination, and promotion of anticodon-codon interactions (Agris et al. 2017). For example, m^1A_{58}, Um_{44}, and ac^4C_{12} are required for stability of certain tRNA isoacceptors (Anderson et al. 1998; Johansson and Bystrom 2004; Kotelawala et al. 2008). The ribosylation of adenosine at position 64 (A_{64}) catalyzed by Rit1p functions as a discriminator for the initiator $tRNA_i^{Met}$. The initiator $tRNA_i^{Met}$ is prevented from interacting with the elongation factor eEF1A by the A_{64} modification and thereby functions only in the initiation but not elongation of translation (Astrom and Bystrom 1994; Shin et al. 2011). The modifications of U_{34} in the anticodon loop of tRNAs restrict and improve the anticodon-codon interactions (Bjork et al. 2007; Johansson et al. 2008; Kurata et al. 2008).

The catalytic reactions for the formation of modified nucleosides can take place in either the nucleus or the cytoplasm (Phizicky and Hopper 2010). The pseudouridine synthases Pus1p and Pus7p appear to be localized in the nucleus (Simos et al. 1996; Huh et al. 2003). Many tRNA-modifying enzymes, such as Trm3p, Trm7p, and Pus8p, are localized in the cytoplasm, indicating that the modifications catalyzed by these enzymes are formed in the cytoplasm (Huh et al. 2003; Hopper 2013).

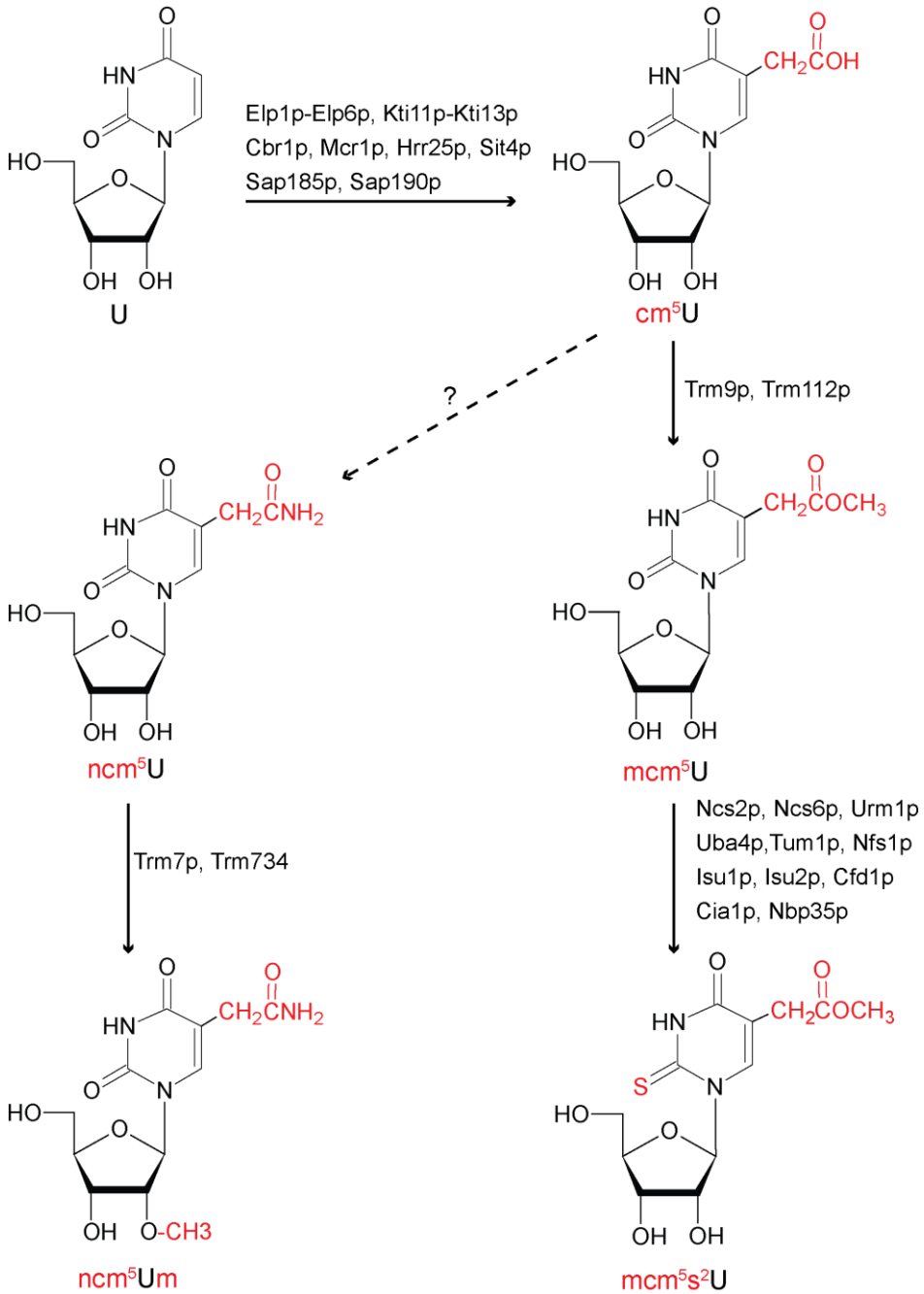

Figure 4. Biosynthesis of ncm^{5}U, ncm^{5}Um, mcm^{5}U, and mcm^{5}s^{2}U in *S. cerevisiae*.

2.4 Wobble uridine modifications

There are 42 species of cytosolic tRNAs in *S. cerevisiae* and 13 of them contain a uridine at the wobble position (U_{34}) (**Figure 4**) (Hani and Feldmann 1998). Except for $tRNA^{Leu}_{UAG}$ that contains an unmodified U_{34}, the U_{34} of the other 12 tRNA species is all modified (Randerath et al. 1979; Johansson et al. 2008). The U_{34} in $tRNA^{Ile}_{UAU}$ is converted to pseudouridine (ψ_{34}) by Pus1p (Szweykowska-Kulinska et al. 1994). Of the remaining 11 tRNA species, five ($tRNA^{Val}_{UAC}$, $tRNA^{Pro}_{UGG}$, $tRNA^{Ser}_{UGA}$, $tRNA^{Thr}_{UGU}$, and $tRNA^{Ala}_{UGC}$) contain a 5-carbamoylmethyl-uridine (ncm^5U_{34}), and two ($tRNA^{Arg}_{UCU}$ and $tRNA^{Gly}_{UCC}$) with a 5-methoxycarbonylmethyl-uridine (mcm^5U_{34}) (Kuntzel et al. 1975; Yamamoto et al. 1985; Keith et al. 1990; Johansson et al. 2008; Lu et al. 2008). The U_{34} in $tRNA^{Leu}_{UAA}$ is modified to ncm^5Um_{34}. $tRNA^{Lys}_{UUU}$, $tRNA^{Gln}_{UUG}$, and $tRNA^{Glu}_{UUC}$ contain a 5-methoxycarbonylmethyl-2-thio-uridine ($mcm^5s^2U_{34}$) (Smith et al. 1973; Kobayashi et al. 1974; Glasser et al. 1992; Lu et al. 2005).

Many gene products have been implicated in the formation of ncm^5U, ncm^5Um, mcm^5U, and mcm^5s^2U at the wobble position (**Figure 4**) (Johansson et al. 2018). At least 15 proteins are required for the first step for biosynthesis of the suggested intermediate 5-carboxymethyl-uridine (cm^5U), which is further modified to mcm^5U by the Trm9p·Trm112p complex or to ncm^5U by an uncharacterized mechanism (Kalhor and Clarke 2003; Huang et al. 2008; Mazauric et al. 2010; Songe-Moller et al. 2010; Chen et al. 2011a; Selvadurai et al. 2014; Lin et al. 2016). In $tRNA^{Lys}_{UUU}$, $tRNA^{Gln}_{UUG}$, and $tRNA^{Glu}_{UUC}$, the nucleoside mcm^5U is further modified to mcm^5s^2U, a step that involves at least 11 proteins (**Figure 4**) (Nakai et al. 2004; Bjork et al. 2007; Nakai et al. 2007; Dewez et al. 2008; Huang et al. 2008; Nakai et al. 2008; Schlieker et al. 2008; Leidel et al.

2009; Noma et al. 2009). Mutant cells lacking the mcm^5 modification show reduced levels of the s^2 group indicating that formation of the mcm^5 side chain is a prerequisite for s^2 group formation (Nakai et al. 2008; Leidel et al. 2009; Noma et al. 2009).

2.5 The Elongator complex

Of the proteins required for the formation of U_{34} modifications, six proteins Elp1p-Elp6p form the Elongator complex which contains two copies of each subunit (**Figure 4**) (Krogan and Greenblatt 2001; Winkler et al. 2001; Dauden et al. 2017). Two copies of Elp1p-Elp3p forms a symmetric subcomplex where Elp1p in a homodimer acts as a scaffold for Elp2p and Elp3p. Elp4p-Elp6p assemble into an asymmetric heterohexameric ring that binds to the Elp1p-Elp3p subcomplex (Glatt et al. 2012; Lin et al. 2012; Dauden et al. 2017; Setiaputra et al. 2017). All six components are essential for the activity of Elongator complex, because cells lacking any of the subunits show identical defects in the formation of U_{34} modifications (Butler et al. 1994; Yajima et al. 1997; Frohloff et al. 2001; Huang et al. 2005; Esberg et al. 2006).

The scaffold proteins Elp1p appears to play a regulatory role because phosphorylation of the S1209, S1198, and S1202 residue of Elp1p is important for the activity of Elongator holoenzyme (Abdel-Fattah et al. 2015). Elp3p of the Elongator complex is a 4Fe-4S cluster protein containing a radical *S*-adenosylmethionine (rSAM) and a lysine acetyltransferase (KAT) domain (Wittschieben et al. 1999; Paraskevopoulou et al. 2006). Of the six Elp proteins, Elp3p is the most conserved and it also exists in archaea and some bacteria strains (Selvadurai et al. 2014). Biochemical and structural studies have suggested that Elp3p harbors the catalytic activity, which initiates the

formation of wobble uridine modifications (Selvadurai et al. 2014; Lin et al. 2019). It has also been shown that the acetyl-CoA hydrolysis activity of archaeal Elp3p can be induced by tRNA binding (Lin et al. 2019). The archaeal Elp3p was also suggested to catalyze the formation of a cm^5 side chain on the U_{34} (Selvadurai et al. 2014). However, the enzymatic reaction could not be reproduced in an independent study (Glatt et al. 2016).

Elp4p, Elp5p, and Elp6p form a ring-like complex consisting of two copies of each protein (Glatt et al. 2012; Lin et al. 2012). The complex is able to bind tRNA in an ATP-dependent manner, implying a possible role of the complex in tRNA recruitment or dissociation during the tRNA modification processes (Dauden et al. 2018). Elp4p and Elp5p were shown to be phosphorylated in *sit4Δ* cells, and the phosphorylation of Elp4p was also observed in rapamycin-treated cells (Soulard et al. 2010; Abdel-Fattah et al. 2015). A recent study in *Schizosaccharomyces pombe* suggested that the phosphorylation of Elp4p may be involved in the sensing of nutrient availability (Candiracci et al. 2019).

2.6 Phenotypes of Elongator mutants

Genes encoding Elongator subunits were originally isolated in genetic screens for mutations generating resistance to zymocin, a toxin secreted by *Kluyveromyces lactis* (Butler et al. 1991; Butler et al. 1994; Yajima et al. 1997; Frohloff et al. 2001; Schaffrath and Meinhardt 2005). Subsequently, the Elongator complex was shown to be required for the formation of wobble uridine modifications in tRNAs (Huang et al. 2005). The γ-subunit of zymocin, which is an endonuclease, cleaves the anticodon region of tRNAs containing the $mcm^5s^2U_{34}$, which explains the resistance of Elongator mutants to zymocin (Lu et al. 2005). In addition to formation of the ncm^5 and mcm^5 side chains, the Elongator complex was thought to be associated with RNA polymerase II, and Elongator mutants show phenotypes of transcriptional defects such as a reduction in histone H3 acetylation and delay in transcriptional activation (Otero et al. 1999; Wittschieben et al. 1999). Moreover, inactivation of the Elongator complex causes many other phenotypes such as slow growth, delay in the G1 phase of cell cycle, sensitivity to many stress conditions, increased +1 frameshifting, mitochondrial dysfunction, and defects in exocytosis, filamentous growth, protein homeostasis, and telomeric gene silencing (Otero et al. 1999; Wittschieben et al. 1999; Frohloff et al. 2001; Rahl et al. 2005; Abdullah and Cullen 2009; Li et al. 2009b; Klassen et al. 2015; Nedialkova and Leidel 2015; Tigano et al. 2015; Tukenmez et al. 2015; Klassen et al. 2017). However, except for the defect in tRNA modification, the pleiotropic phenotypes of Elongator mutants can be suppressed by overexpression of $tRNA^{Lys}_{UUU}$, $tRNA^{Gln}_{UUG}$, and $tRNA^{Glu}_{UUC}$, which in wild-type cells contain the $mcm^5s^2U_{34}$ (Esberg et al. 2006; Chen et al. 2011b; Nedialkova and Leidel 2015; Klassen et al. 2017). Therefore, it is now believed that the pleiotropic phenotypes of Elongator mutants are caused by a translational defect induced by the hypomodified tRNAs.

2.7 Proteins influencing Elongator activity

The known proteins influencing the activity of Elongator complex include Kti11p, Kti12p, Kti13p, Cbr1p, Mcr1p, Hrr25p, Sit4p, Sap185p, and Sap190p (**Figure 4**). Among these proteins, Kti11p and Kti13p form a complex that physically interacts with the Elongator complex (Fichtner et al. 2003; Bar et al. 2008; Zabel et al. 2008; Glatt et al. 2015). Kti11p and its regulatory interactor Kti13p are suggested to provide electrons to the 4Fe-4S cluster-containing Elongator complex during the formation of U_{34} modifications (Bar et al. 2008; Glatt et al. 2015; Kolaj-Robin et al. 2015). A similar role of the Kti11p·Kti13p complex as an electron donor has also been described for the formation of diphthamide modification of elongation factor eEF2 (Zhang et al. 2010; Dong et al. 2014). Recently, Cbr1p and Mcr1p were shown to act as reductases that provide electrons to the Kti11p·Kti13p complex (Lin et al. 2016). The protein kinases Kti12p and Kti14p, the phosphatase Sit4p, and the Sit4p-associated proteins Sap185p and Sap190p modulate the activity of the Elongator complex by regulating the phosphorylation status of Elp1p (Luke et al. 1996; Jablonowski et al. 2001; Mehlgarten and Schaffrath 2003; Jablonowski et al. 2004; Mehlgarten et al. 2009; Abdel-Fattah et al. 2015).

2.8 Elongator complex and human disease

The role of Elongator complex in the formation of wobble uridine modifications is conserved in plants (Mehlgarten et al. 2010), mice (Lin et al. 2013), worms (Chen et al. 2009), and humans (Karlsborn et al. 2014; Yoshida et al. 2015). Familial dysautonomia (FD), a disorder of the autonomic nervous system, is caused by a mutation in the human *ELP1* gene (Anderson et al. 2001; Slaugenhaupt et al. 2001; Slaugenhaupt and Gusella 2002). Analysis of brain tissue and fibroblast cells derived from FD patients shows a reduction of the formation of wobble uridine modifications (Karlsborn et al. 2014; Yoshida et al. 2015). Mutations in the human *ELP1* and *ELP3* gene are linked to bronchial asthma in children and Intellectual Disability (ID), respectively (Takeoka et al. 2001; Najmabadi et al. 2011; Cohen et al. 2015). In addition, mutations in the human *ELP3* gene is also linked to motor neuron degeneration based on human population association studies (Simpson et al. 2009), which is also supported by studies using animal models (Bento-Abreu et al. 2018). Variants of the *ELP4* gene are associated with several neurological symptoms indicating a neurodevelopmental disorder (Addis et al. 2015) and with Rolandic Epilepsy (Strug et al. 2009). In addition to a role in neurodevelopment, a subset of the Elongator-encoding genes (*ELP1*, *ELP3*, *ELP5*, and *ELP6*) are also suggested to promote invasion and metastasis of tumor cells (Delaunay, 2016 #2311;Close, 2012 #2209). The *ELP1* and *ELP3* genes are also reported to be important for the survival and drug resistance of melanoma cells (Rapino et al. 2018).

Results and discussion

I. Ypk9p influences putrescine levels and the fidelity of translation termination

In the tRNA encoded by the *SUP4* gene, a mcm^5U is present at position 34 (Gilmore et al. 1971; Goodman et al. 1977; Huang et al. 2005). The *SUP4*-encoded tRNA can decode UAA and, to a lesser extent, UAG codons (Gilmore et al. 1971; Johansson et al. 2008). Strains in the W303 background, harboring the nonsense *ade2-1* and *can1-100* alleles with premature UAA stop codon, require adenine for growth and are resistant to a toxic arginine analog canavanine (Fiorentini et al. 1997). However, a W303 strain containing the *SUP4* gene does not require adenine and is sensitive to canavanine, due to translational readthrough of UAA stop codons in the *ade2-1* and *can1-100* mRNAs. In this study, we performed a genetic screen to identify novel factors that are required for nonsense suppression by the SUP4-encoded tRNA.

Mutants derived from *SUP4*-containing strains that show reduced nonsense suppression, *i.e.*, growth in the presence of canavanine and no growth on plates lacking adenine, were scored as *SUP4* anti-suppressors. Recessive anti-suppressors define twelve complementation groups. Ten groups contain mutations in genes (*ELP1-6*, *KTI11*, *KTI12*, *MOD5*, and *PUS7*) that encode known factors for formation of modified nucleosides in the *SUP4*-encoded tRNA (Laten 1984; Behm-Ansmant et al. 2003; Huang et al. 2005; Klassen and Schaffrath 2018). One of the two remaining groups contains a mutation in the *TYS1* gene, encoding the tyrosyl-tRNA synthetase (Chow and RajBhandary 1993). Since the *SUP4* gene is a mutant form of the *tY(GUA)J2* gene encoding a $tRNA^{Tyr}$, anti-suppression phenotypes of the *tys1* mutant is likely through a defect in aminoacylation of the *SUP4*-encoded tRNA.

The last complementation group consists of a *ypk9* mutant. The *YPK9* gene encodes a putative vacuolar membrane P-type ATPase (Schmidt et al. 2009). Consistent with the anti-suppression phenotype, cells with inactivated Ypk9p exhibit reduced translational readthrough of UAA stop codons. To address whether the anti-suppression of Ypk9p inactivation is due to a defect in biogenesis of the suppressor tRNA, we analyzed total tRNA for levels of modified nucleosides present in the suppressor tRNA. Levels of the suppressor tRNA were also determined. Results suggest that Ypk9p does not appear to influence modification or levels of the suppressor tRNA.

To determine the role of Ypk9p in translation readthrough, we conducted a genetic screen for high-copy suppressors that restored the nonsense suppression of *ypk9Δ SUP4* cells. We found that the *SPE1* gene, encoding an ornithine decarboxylase, in high dosage restores nonsense suppression of *ypk9Δ SUP4* cells. Spe1p catalyzes the formation of putrescine from ornithine, which is the first and rate-limiting step of polyamine biosynthesis (Hoyt and Davis 2004). Polyamines (putrescine, spermidine, and spermine) have been implicated in the regulation of translation (see section 1.4). Analysis of polyamines showed that the levels of putrescine were reduced in *ypk9Δ* cells, suggesting a role for Ypk9p in the maintenance of the cellular levels of putrescine. In addition to restoring the nonsense suppression, we showed that overexpression of the *SPE1* gene also overcame the sensitivity of *ypk9Δ* cells to Mn^{2+}. Collectively, these findings suggest that Ypk9p influences the cellular levels of putrescine, which is required for maintaining the fidelity of translation termination and the tolerance to Mn^{2+}.

II. *SSD1* modifies phenotypes of Elongator mutants

The Elongator complex (Elp1p-Elp6p) is required for the formation of mcm^5 and ncm^5 side chains on uridine at position 34 (U_{34}) in the anticodon region of 11 tRNA species (Johansson et al. 2008). Inactivation of the Elongator complex not only leads to a lack of U_{34} modifications but also many other phenotypes (see section 2.6) (Wittschieben et al. 1999; Frohloff et al. 2001; Abdullah and Cullen 2009; Li et al. 2009b; Klassen et al. 2015). Although the pleiotropic phenotypes are believed to be consequences of a translational defect induced by hypomodified tRNAs, the mechanisms for individual phenotypes are not well understood (Esberg et al. 2006; Chen et al. 2011b; Nedialkova and Leidel 2015). Moreover, phenotypes associated with Elongator inactivation are not the same between strains of two genetically similar backgrounds, which are W303 and S288C (Bjork et al. 2007; Klassen et al. 2015; Nedialkova and Leidel 2015). In this study, we show that the mRNA-binding protein Ssd1p influences the phenotypic penetrance of Elongator mutations.

The sensitivity of Elongator mutants to stress conditions, such as high temperature and compounds that induce cell wall stress, indicates a possible cell wall defect (Frohloff et al. 2001; Levin 2005). Accordingly, we observed that the sensitivity to stress conditions of *elp3Δ* cells in the W303 background is suppressed by activation of the cell wall integrity (CWI) pathway. However, activation of the CWI pathway could not counteract stress-induced growth defects of *elp3Δ* cells in the S288C background. This difference indicates that the strain background influences the suppression efficiency of the CWI activation. Strains in the W303 background often contain the nonsense *ssd1-d2* allele, whereas the S288C strains produce a full-length Ssd1 protein (Sutton et al. 1991; Jorgensen et al. 2002). The *SSD1* gene encodes an mRNA-binding protein that participates in many cellular processes including the CWI

pathway (Hogan et al. 2008; Jansen et al. 2009). Therefore, the influence of CWI pathway activation on phenotypes of *elp3Δ ssd1-d2* cells was compared with that of *elp3Δ SSD1* cells in both the W303 and S288C backgrounds. We found that increased activation of the CWI pathway counteracts the stress-related phenotypes of *elp3Δ* cells only when the mutant *ssd1-d2* allele is present in the background. Moreover, we showed that the variations in stress-related phenotypes of *elp3Δ* cells between the two genetic backgrounds are largely due to the polymorphism at the *SSD1* locus. Furthermore, we demonstrated that the defects in telomeric gene silencing and histone acetylation of *elp3Δ* cells are also dependent on the presence of the mutant *ssd1-d2* allele.

Since pleiotropic phenotypes of Elongator mutants are believed to be caused by the hypomodified tRNAs, the influence of Ssd1p on the modification and levels of tRNAs was investigated (Esberg et al. 2006; Chen et al. 2011b; Nedialkova and Leidel 2015; Klassen et al. 2017). HPLC and northern blot analysis indicated that Ssd1p does not appear to influence the modification or levels of tRNAs. We conclude that the mechanism by which Ssd1p modulates the phenotypes of Elongator mutants does not likely involve tRNA biogenesis. Ssd1p not only influences the translation and levels of its associated mRNAs, but also the abundance of mRNAs that are not bound by Ssd1p (Jansen et al. 2009; Li et al. 2009a). Therefore, the effect of Ssd1p on the phenotypes of *elp3Δ* mutants could be caused by direct or indirect influence on protein expression.

III. Identification of factors that promote biogenesis of $tRNA^{Ser}_{CGA}$

Precursor tRNA transcripts undergo multiple processing steps to form mature tRNAs. Many gene products are required for these tRNA maturation processing steps (Hopper 2013). The *tS*(*CGA*)*C* gene encodes the only serine tRNA able to decode UCG codons in *S. cerevisiae* (Etcheverry et al. 1982). Previous studies have shown that several altered forms of the $tRNA^{Ser}_{CGA}$ are sensitized to mutations in genes inducing tRNA modification defects (*TRM2*, *PUS4*, *TRM1*, and *TRM3*) and in the *LHP1* gene encoding a tRNA chaperone (Johansson and Bystrom 2002). A screen was performed to identify synthetic lethal mutations with a mutant *tS(CGA)C* allele (s*up61-T47:2C)*, which encodes a destabilized $tRNA^{Ser}_{CGA}$ (Johansson and Bystrom 2004).

The identified recessive mutants from the synthetic lethal screen defined twelve complementation groups. Mutations of one group were genetically linked to the *tS*(*CGA*) gene indicating a second-site mutation in the mutant *sup61-T47:2C* allele. Analysis of the remaining groups identified mutations in the *TAN1*, *BUD13*, *TRM1*, *DUS2*, and *MOD5* genes that impair the formation of modified nucleosides in the $tRNA^{Ser}_{CGA}$ (Dihanich et al. 1987; Johansson and Bystrom 2004; Xing et al. 2004; Zhou et al. 2013). In addition, mutations in the *LOS1* and the *SES1* genes were also identified. Los1p mediates the nuclear export of tRNAs (Hellmuth et al. 1998). We found that the levels of the altered $tRNA^{Ser}_{CGA}$ were reduced in the *los1* mutant. Ses1p aminoacylates $tRNA^{Ser}_{CGA}$ and stimulates the formation of m^3C_{32} in $tRNA^{Ser}_{CGA}$ (Weygand-Durasevic et al. 1987; Han et al. 2017). We found that the abundance but not modification of the altered $tRNA^{Ser}_{CGA}$ was affected by the mutation in the *SES1* gene.

In addition to the genes encoding known factors for tRNA biogenesis, the screen also identified strains with mutations in the *RPA49*, *RRN3,* *MOT1*, and *ALR1* genes. Northern blot analysis indicated that mutations in the *RPA49*, *RRN3,* and *MOT1* genes lead to a reduction in levels of the altered $tRNA^{Ser}_{CGA}$, which provides an explanation for the synthetic lethality. Since proteins encoded by the three genes are implicated only in the transcription of RNA polymerases I and II, their influence on the tRNA levels is likely to be indirect (Auble et al. 1994; Yamamoto et al. 1996; Gadal et al. 1997; Dasgupta et al. 2007). The *ALR1* gene encodes a Mg^{2+} transporter that is responsible for Mg^{2+} uptake (MacDiarmid and Gardner 1998). The nonviability of the double mutant *alr1-11 sup61-T47:2C* suggests that the function or stability of the destabilized $tRNA^{Ser}_{CGA}$ is sensitive to a reduction of the Mg^{2+} levels. In summary, the screen identified several novel factors that promote biogenesis of the destabilized $tRNA^{Ser}_{CGA}$.

Conclusions

1. The *YPK9* gene product influences the cellular levels of putrescine, which is required for maintaining the fidelity of translation termination and tolerance to Mn^{2+}.

2. The mRNA-binding protein Ssd1p modulates many phenotypes of Elongator mutants without influencing tRNA biogenesis.

3. The *sup61-T47:2C* mutant allele is a potent genetic tool for identification of novel tRNA biogenesis factors. Gene products of *RPA49*, *RRN3*, *MOT1*, and *ALR1* are implicated in tRNA biogenesis.

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