Functional aspects of wobble uridine modifications in yeast tRNA

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Abstract

Transfer RNAs (tRNA) function as adaptor molecules in the translation of mRNA into protein. These adaptor molecules require modifications of a subset of their nucleosides for optimal function. The most frequently modified nucleoside in tRNA is position 34 (wobble position), and especially uridines present at this position. Modified nucleosides at the wobble position are important in the decoding process of mRNA, i.e., restriction or improvement of codon-anticodon interactions. This thesis addresses the functional aspects of the wobble uridine modifications.

The *Saccharomyces cerevisiae* Elongator complex consisting of the six Elp1-Elp6 proteins has been proposed to participate in three distinct cellular processes; elongation of RNA polymerase II transcription, regulation of polarized exocytosis, and formation of modified wobble nucleosides in tRNA. In Paper I, we show that the phenotypes of Elongator deficient cells linking the complex to transcription and exocytosis are counteracted by increased level of $tRNA^{G\text{In}}_{\text{mcm}^5\text{s}^2\text{U}^G}$ and $tRNA^{5\text{m}^{\text{mcm}^5\text{s}^2\text{U}^G}}_{\text{U}^U\text{U}^U}$. These tRNAs requires the Elongator complex for formation of the 5-methoxycarbonylmethyl (mcm$^5$) group of their modified wobble nucleoside 5-methoxycarbonylmethyl-2-thiouridine (mcm$^5$s$^2$U). Our results therefore indicate that the relevant function of the Elongator complex is in formation of modified nucleosides in tRNAs and the defects observed in exocytosis and transcription are indirectly caused by inefficient translation of mRNAs encoding gene products important for these processes.

The lack of defined mutants in eukaryotes has led to limited understanding about the role of the wobble uridine modifications in this domain of life. In Paper II, we utilized recently characterized mutants lacking the 2-thio (s$^2$) or 5-carbamoylmethyl (ncm$^5$) and mcm$^5$ groups to address the *in vivo* function of eukaryotic wobble uridine modifications. We show that ncm$^5$ and mcm$^5$ side-chains promote reading of G-ending codons, and that presence of a mcm$^5$ and an s$^2$ group cooperatively improves reading of both A- and G-ending codons.

Previous studies revealed that a *S. cerevisiae* strain deleted for any of the six Elongator subunit genes shows resistance towards a toxin (zymocin) secreted by the dairy yeast *Kluyveromyces lactis*. In Paper III, we show that the cytotoxic $\gamma$ subunit of zymocin is a tRNA endonuclease that target the anticodon of mcm$^5$s$^2$U$_{34}$ containing tRNAs and that the wobble mcm$^5$ modification is required for efficient cleavage. This explains the $\gamma$-toxin resistant phenotype of Elongator mutants which are defective in the synthesis of the mcm$^5$ group.
Main References:

This thesis is based on the following papers, which are referred to in the text by their roman numerals.

    *Molecular Cell* 24, 139–148.

    *Manuscript.*

    *RNA* 11, 1648-1654.
1. Introduction

The central dogma

The central dogma of molecular biology describes the flow of genetic information from DNA to protein (Figure 1).

![Diagram of the central dogma](image)

Figure 1. The central dogma of molecular biology.

DNA is composed of four building blocks: the deoxyribonucleotides containing the bases Adenine (A), Guanine (G), Cytosine (C), or Thymine (T). The function of DNA is to provide long-term storage for vital genetic information, and to function as a blueprint for essential components like proteins and RNA molecules. The first process in protein synthesis, transcription, is where an RNA molecule, messenger RNA (mRNA), is synthesized (transcribed) from a selective DNA region (gene). In the second process, translation, the mRNA is decoded to generate a specific amino acid chain (protein) according to the rules defined by the genetic code. The mRNA nucleotides are decoded in a triplet manner (codons) where each three-letter code specifies a specific amino acid. However, the mRNA codons are not directly recognized by amino acids. Translation of mRNA codons into specific amino acids is dependent on adaptor molecules, which
are able to bind a defined amino acid and recognize a specific codon. These adaptor molecules are small RNA molecules called transfer RNA (tRNA) that contain a nucleotide-triplet (anticodon) designed to match and decode the different mRNA codons. The decoding occurs in the ribosome, which is a large ribonucleoprotein complex capable of binding the mRNA and guide the tRNA into the correct position on the mRNA. When matching occurs between codon and anticodon, the amino acid linked to the tRNA will be incorporated into the growing amino acid chain. This thesis will focus on the functional aspects of modified uridines present at the first position in the anticodon.

**Transfer RNA**

Transfer RNA was discovered by Hoagland et al. 1956 and later described as a molecule important in translating mRNA into proteins (Hoagland et al., 1956; For review, RajBhandary and Kohrer, 2006). These small RNA molecules are composed of four different nucleosides Uridine (U), Adenosine (A), Guanosine (G), and Cytidine (C). Transfer RNAs are between 75 and 95 nucleotides in length and usually visualized in the canonical cloverleaf structure in which the tRNA is divided into the variable arm, acceptor stem, D-, ΨC-, and anticodon-loop (Figure 2A). All tRNAs fold into a similar L-shaped three-dimensional structure (Figure 2B) promoted by base pairs between well-conserved positions within the tRNA (Dirheimer et al., 1995; Holley, 1965; Holley et al., 1965).
In eukaryotes, the RNA polymerase III (Pol III) transcription machinery transcribes the genes coding for tRNAs. The promoter present in tRNA genes has internal A- and B-boxes, which correspond to the D- and TΨC-stem loops of the transcribed tRNA. Transcription by Pol III involves a multi-step assembly of initiation factors into a pre-initiation complex that directs recruitment of Pol III. To activate transcription of tRNA genes, the TFIIIC binds to the A- and B-boxes and acts as an assembly factor to direct binding of the TFIIIB to an upstream position of the tRNA gene. Assembled TFIIIB recruits the Pol III enzyme and directs multiple-rounds of transcription (For review, Schramm and Hernandez, 2002).

Figure 2. Schematic structure of a tRNA. (A) Two-dimensional structure. (B) Tertiary-structure (Kim et al., 1974). Positions 34, 35, and 36 (anticodon), are shown in black.
All eukaryotic tRNA molecules are transcribed as precursors with extra sequences at both the 5’ and 3’ ends, which have to be processed to generate a mature tRNA. Removal of the 5’ leader is accomplished by a ribonucleoprotein, RNaseP, whereas the 3’ end is processed by the endoribonuclease RNaseZ. The primary tRNA transcript from eukaryotic cells does not contain the 3’ CCA sequence, which is essential for aminoacylation (Figure 2). The 3’ CCA sequence is added by the tRNA-nucleotidyltransferase. Ten *S. cerevisiae* tRNA species contain an intervening sequence in their primary transcript. The introns can vary in size between 14-60 nucleotides, and are always located one nucleotide 3’ of the anticodon. Splicing involves multiple-steps, first, the intron is removed by the tRNA-splicing endonuclease. Secondly, the RNA-ends are ligated together by the tRNA-ligase, and finally the 2´ phosphate group present after ligation is removed by a phosphotransferase. For the fidelity of translation, each tRNA must be charged with its cognate amino acid. The aminoacyl-tRNA synthetases (aaRS) link the correct amino acid to the 3’ CCA end of the cognate tRNA. In general, there is one aaRS for each of the 20 amino acids used in protein synthesis. Finally, to generate a fully functional adaptor molecule, specific tRNA modifications have to be introduced post-transcriptionally. The different modified nucleosides present in the tRNA are derivatives of the normally present nucleotides A, C, G, or U, and up till now twenty-five different tRNA modifications have been found in cytoplasmic *S. cerevisiae* tRNA (For reviews, Björk, 1995; Hopper and Phizicky, 2003; Ibba and Söll, 2000; Johansson and Byström, 2005).
The genetic code

The mRNA sequence is decoded in a triplet manner into a specific amino acid sequence by a three-nucleotide complementary region, the anticodon, in the tRNA (Figure 3).

![Diagram of codon-anticodon interaction](image)

Figure 3. A schematic drawing of codon-anticodon interaction. The mRNA codon is composed of three nucleotides $A_1$, $A_{II}$, and $A_{III}$. The anticodon of the tRNA is composed of three nucleotides at position 34, 35, and 36. $U_{36}$ will interact with the first position ($A_1$), $U_{35}$ with the second- ($A_{II}$), and $U_{34}$ (wobble base) with the third-position ($A_{III}$) of the mRNA codon.

The deciphering of the three-letter codons generated what we today call the “Genetic Code”. The genetic code is in principle universal, consisting of 64 triplet letters, where 61 codons code for amino acids and three codons terminate translation. The genetic code is degenerate, i.e., many amino acids are specified by more than one codon. Two observations provide an explanation for the degeneracy of the genetic code. First, the existence of
isoaccepting tRNA species charged with the same amino acid. Second, the “wobble hypothesis” proposed by Crick, which predicts that some tRNA species can decode more than one codon (Crick, 1966; Crick et al., 1961). The wobble hypothesis stated that the nucleoside at the first position of the anticodon (wobble position) could form either a canonical or a wobble base pair with the third nucleoside in the codon, provided that the nucleosides at position 35 and 36 in the tRNA form Watson-Crick base pairs with the first two nucleosides of the codon (Crick, 1966) (Figure 3).

The “Codon Table” summarizes all the 64 possible triplets and clarifies which codon specifies which amino acid (Figure 4). The two first letters of the three letter codon create 16 different combinations, constructing the 16 codon boxes in the codon table (Figure 4). There are two types of codon boxes, the family- and the split-codon boxes. In a family codon box, all four codons code for the same amino acid, whereas a split codon box contains codons for more than one amino acid.
Figure 4. The codon table. The genetic code is composed of four different letters U, C, A, and G, generating in total 64 different three-letter combinations. The codon table summarizes all 64 codons and clarifies which codon specifies which amino acid.

**Translation**

Protein translation is one of the most conserved processes in gene expression. Translating mRNA into protein requires three different RNA species, mRNA, tRNA, and rRNA. In addition, this process also requires a number of proteins important for assistance throughout the translation process. The eukaryotic ribosome is a large multi-subunit complex composed of 4 RNA molecules and ~76 proteins, which assemble into the small 40S and the large 60S subunit, which together form the mature 80S ribosome. The ribosome is
capable of binding tRNA in three separate binding sites: the A-site, P-site, and E-site. Each of these sites has a distinct function, the A-site binds the aminoacyl-tRNA, the P-site binds the peptidyl-tRNA, and the E-site (Exit-site) binds the deacyl-tRNA. Translation of mRNA into protein can be divided into three distinct steps, initiation, elongation, and termination.

Initiation of translation is a sequence of events important for identification of the correct start codon and determination of the correct reading frame. According to the scanning model of translation initiation, the pre-initiation complex (40S, initiation factors, and the initiator methionine tRNA$^{Met,\ CAU}$) binds to the 5´ end of the mRNA (Figure 5A). The pre-initiation complex scans the mRNA in the 5´ to 3´ direction searching for the start codon (Figure 5B). Once the start codon is identified, the initiation factors are released and the 60S is recruited, assembling the 80S ribosome (Figure 5C). There are three potential reading frames in an mRNA and the reading frame is fixed by the identification of the start codon. Normally a reading frame is maintained and the mRNA translated in one continuous motion from start to finish (Hershey and Merrick, 2000).

During translation elongation, the ribosome moves stepwise on the mRNA, codon by codon, guiding the tRNA into the correct position. The ribosome faithfully selects aminoacyl-tRNA based on how well it fits into the A-site and the strength of the codon-anticodon interaction. Each elongation cycle will add one amino acid to a growing amino acid chain. An elongation cycle starts when a ternary complex, consisting of the aminoacyl-tRNA, elongation factor 1A (eEF1A), and GTP, enters the
ribsomal A-site, (Figure 5D). If matching occurs between the
codon-anticodon, hydrolysis of the GTP promotes eEF1A-GDP release
(Figure 5E). The amino acid chain is transferred from the P-site tRNA to the
A-site tRNA by the enzymatic activity of the ribosome peptidyl transferase
center (Figure 5F). In this process, a new amino acid is added to the growing
amino acid chain. Following peptide transfer, the elongation factor 2 (eEF2)
promotes translocation of the peptidyl-tRNA to the P-site (Figure 5G). In
addition to eEF1A and eEF2, elongation factor 3 (eEF3) promotes release of
the E-site tRNA from the ribosome, and the elongation factor 1B (eEF1B)
recycles the eEF1A-GDP to eEF1A-GTP (Figure 5) (Merrick and Nyborg,
2000).

Of the 64 different codons, three are designated to terminate protein
translation (UAA, UAG, and UGA) (Figure 4). These codons are not
decoded by a tRNA into an amino acid, instead these codons are recognized
by the eukaryotic release factor 1 (eRF1) (Figure 5H). When the eRF1 binds
a stop codon, it will together with release factor 3 (eRF3) promote
translation termination. In this process, the amino acid chain dissociates
from the P-site tRNA, the mRNA is released, and ribosomal subunits
dissociate (Figure 5I) (Welch et al., 2000).
Figure 5. Simple view of eukaryotic protein translation. (A-B) Initiation of translation. (C-G) Translation elongation. (H-I) Translation termination.
**Translational fidelity**

A vital task in protein synthesis is to incorporate the correct amino acid and maintain the correct reading frame at the same time sustaining speed. On average, the translation machinery incorporates ~20 amino acids per second; however, the machinery is not perfect since an incorrect amino acid is occasionally incorporated. Cells have in the course of evolution, evolved numerous proofreading steps to improve translational accuracy.

To assure translational fidelity, each tRNA species has to be charged with its cognate amino acid. To increase the accuracy of tRNA aminoacylation, the aaRS accepts only the correct amino acid into the substrate-binding site. To further reduce misaminacylation of incorrect substrate tRNAs, a number of identity elements are present within the different tRNA species. In general, the identity element lies in the two distal parts of the tRNA, with the major determinants in the anticodon loop and the acceptor stem. Moreover, modified nucleosides, especially those located in the anticodon region of the tRNA, have been shown to be important for charging (For reviews, Giege et al., 1998; Ibba and Söll, 2000).

According to current models, two independent proofreading steps assure selection of a correct aminoacyl-tRNA into the A-site of the ribosome (Thompson, 1988). The first proofreading step involves the initial binding of the ternary complex to the ribosome. Here the primary strength of the codon-anticodon interaction determines if the tRNA should be accepted into the ribosome (Figure 5D). The second proofreading step is after GTP hydrolysis and release of the eEF1A-GDP, here the ribosome senses the
codon-anticodon strength and the A-site fitness, and together these features determine whether the aminoacyl-tRNA should be rejected or not (Figure 5E) (For review, Ogle and Ramakrishnan, 2005). In the selection of the cognate aminoacyl-tRNA into the A-site of the ribosome, modifications present at the wobble position have been shown to play a vital role, primarily by influencing the codon-anticodon interaction (Agris, 1991; Lim, 1994; Takai and Yokoyama, 2003; Yokoyama and Nishimura, 1995; Yokoyama et al., 1985). In addition to these proofreading steps, the E-site tRNA has also been shown to be important for the selection of correct A-site tRNA, but the mechanism for this proofreading step is not clear (Nierhaus, 2006).

**Translational errors**

Even though the translational machinery is capable of translating long mRNA faithfully, errors now and then occur. During the translation elongation process, three classes of translational errors can be distinguished.

(I) *Progressive errors*, the release of either a shorter or a longer form of the amino acid chain. At least three different events can cause this kind of translational error, (1) read through of stop codons, (2) incorrect translational termination, or (3) drop-off of peptidyl-tRNA.

(II) *Missense errors*, an incorrect amino acid incorporated at sense codons. This kind of error is usually caused by misreading of the third nucleotide of the mRNA codon by the wobble base of the tRNA. In addition, tRNA can also be misaminoacylated and thereby incorporate an incorrect amino acid even though it pairs with the matching codon.
Frameshifting errors, a shift in the reading frame. Hypothetically, there are four different ways to induce frameshifting: (1) Transcriptional errors expanding or contracting the codon size, (2) incoming aminoacyl-tRNA binds out of frame, (3) translocation error, or (4) slippage of the peptidyl-tRNA. After a frameshifting error, the original genetic message is completely altered and can produce either longer or shorter form of proteins.

It is broadly accepted that a single mRNA that encodes for a defined amino acid chain will give rise to identical protein products, assuming that these proteins go through the same post-translational modifications. This notion was recently challenged by the findings that altered translational rate could change protein folding and modify the protein function (Kimchi-Sarfaty et al., 2007). It was hypothesized that temporal separation of folding events throughout synthesis of a protein molecule might be vital to ensure correct protein function. This implies that cells have a high demand for a fully functionally translational machinery to maintain correct translational speed to assure accurate protein folding.

Wobble uridine modifications
Transfer RNA modifications are found in all organisms and all over the tRNA molecule. These modifications are introduced post-transcriptionally and are derivatives of the normal nucleotides A, C, U, and G (For reviews, Björk, 1995; Johansson and Byström, 2005). So far, 91 different tRNA modifications have been found in the three kingdoms of life. Some tRNA
modifications are present at the same position, in the same population of tRNA, and within all phylogenetic domains, suggesting a conserved function (Björk et al., 2001; RNAmods@lib.med.utah.edu).

The modifications present in tRNA have been shown to play a vital role in numerous processes e.g., folding and stability of tRNA (Anderson et al., 1998; Helm, 2006), recognition and anti-determinants for proteins interacting with tRNAs (Åström and Byström, 1994; For review, Giege et al., 1998), and intracellular localization of tRNA (Kaneko et al., 2003). The most frequently modified base in tRNA is positions 34, and especially tRNA carrying a uridine at the wobble position. Modifications at wobble position are primarily thought to influence the decoding properties of the tRNA.

In total, there are 42 cytoplasmic S. cerevisiae tRNA species, and out of these, thirteen contain a uridine at the wobble position in the primary transcript. Of the thirteen $U_{34}$ containing tRNA species, one carries an unmodified U (tRNA$^{Leu}_{UAG}$) and one has a pseudouridine ($\Psi$) (tRNA$^{Ile}_{UAG\Psi}$). The remaining eleven $U_{34}$ containing tRNAs have either a 5-carbamoylmethyl (ncm$^5$) or a 5-methoxycarbonylmethyl (mcm$^5$) group at the position 5 (Figure 6) (in many cases the ncm$^5$ and the mcm$^5$ modifications will be referred to as xm$^5$) (Huang et al., 2005; For review, Johansson and Byström, 2005) (Paper II). Three of the mcm$^5$ containing tRNAs are thiolated at position 2, generating the hyper modified 5-methoxycarbonylmethyl-2-thiouridine nucleoside (mcm$^5$s$^2$U) (Kobayashi et al., 1974; Smith et al., 1973) (Paper III) (Figure 6).
Figure 6. Examples of uridine or uridine derivatives present at position 34 (wobble position). From the left; uridine (U), 5-carbamoylmethyl-uridine (ncm$^5$U), 5-methoxycarbonylmethyl-uridine (mcm$^5$U), and 5-methoxycarbonylmethyl-2-thio-uridine (mcm$^5$s$^2$U).

**The role of wobble uridine modifications in decoding**

In the decoding process, an aminoacyl-tRNA can potentially direct decoding of three different types of codons. Cognate codon: the tRNA is able to form three Watson-Crick base pairs, Near-cognate codons: two Watson-Crick base pairs and one wobble base pair at the third position, and Non-cognate codon: one or less Watson-Crick base pair. In the translational process, a unmodified U$^{34}$ will according to Crick’s original wobble hypothesis preferentially decode cognate A- and near cognate G-ending codons, assuming that the nucleotides at position 35 and 36 in the tRNA form Watson-Crick base pairs with the two first nucleotides of the mRNA codon (Crick, 1966). At the time when Crick presented his hypothesis, it was not known that cytoplasmic tRNA almost never contains an unmodified U$^{34}$. Since then, Crick’s hypothesis has been revised and it was suggested that an
unmodified U₃⁴ recognizes all four nucleotides U, C, A, and G, whereas a modified uridine can alter the decoding capacity (Agris, 1991; Björk, 1995; Lim, 1994; Takai and Yokoyama, 2003; Yokoyama and Nishimura, 1995; Yokoyama et al., 1985). Based on results from *in vitro* studies, as well as structural considerations, it has been proposed that the presence of an xm⁵ group at a wobble uridine would prevent pairing with U- and C-ending codons (Lim, 1994; Yokoyama and Nishimura, 1995; Yokoyama et al., 1985). For the decoding of A- and G-ending codons, two models are proposed. The first and most accepted model states that presence of an xm⁵ side-chain at U₃⁴ improves reading of A- and causes inefficient decoding of G-ending codons (Yokoyama and Nishimura, 1995; Yokoyama et al., 1985). The second model suggests that an xm⁵U nucleoside efficiently reads both A- and G-ending codons (Kruger et al., 1998; Lim, 1994; Murphy et al., 2004; Takai and Yokoyama, 2003; Yarian et al., 2002).

Three mcm⁵U₃⁴ containing tRNAs are thiolated at position 2 of the base. Interestingly, the 2-thio group is only found in tRNAs decoding in split codon boxes. Similar to an xm⁵U₃⁴ nucleoside, presence of an mcm⁵s²U₃⁴ residue was suggested to prevent pairing with U- and C-ending codons, allow efficient reading of A-, and simultaneously reduce the ability to pair with G-ending codon (Lustig et al., 1981; Sekiya et al., 1969; Yokoyama et al., 1985). More recent data have indicated that a mcm⁵s²U₃⁴ residue would allow reading of both A- and G-ending codons (Murphy et al., 2004).
Proteins required for wobble uridine modifications

Today over 50 gene products are dedicated for synthesis of tRNA modifications in *S. cerevisiae* (Huang et al., 2005; For review, Johansson and Byström, 2005; Nakai et al., 2007) (Lu J. personal communication). Surprisingly, at least twenty-four of these are required for the synthesis of wobble uridine modifications.

At this moment, fourteen gene products have been identified that are required for synthesis of the \( \text{xm}^5 \) modification at \( \text{U}_{34} \). The Elongator complex, consisting of six proteins Elp1-Elp6, is required for the formation of \( \text{ncm}^5 \) and \( \text{mcm}^5 \) side-chains at wobble uridines (Figure 6) (Huang et al., 2005). In addition to a role in tRNA modification, the Elongator complex was proposed to participate in two other distinct cellular processes. Originally the Elongator complex was identified based on its association with the hyper-phosphorylated elongating form of RNA polymerase II (Pol II) (Otero et al., 1999). Purified Elongator complex from yeast cells is able to transfer acetyl groups from acetyl-CoA to histones H3 and H4 *in vitro* (Hawkes et al., 2002; Kim et al., 2002; Winkler et al., 2002; Wittschieben et al., 1999). Moreover, an *elp3Δ* strain shows a decreased acetylation level of primarily histone H3 *in vivo* (Winkler et al., 2002). Together these data suggested that the Elongator complex mediates Pol II transcription elongation through chromatin structure. Another distinct function described for the Elongator complex is in polarized transport of secretory vesicles to the bud tip (Rahl et al., 2005). This transport event requires activation of the vesicle-associated GTPase Sec4p by its guanine nucleotide exchange factor.
Sec2p (Salminen and Novick, 1987; Walch-Solimena et al., 1997). It was found that Sec2p associated with Elp1p and that polarized localization of Sec2p was dependent on the presence of Elp1p, suggesting that the Elongator complex regulates exocytosis by influencing the localization of Sec2p (Rahl et al., 2005). In this thesis, we address whether the Elongator complex has three distinct functions or whether it regulates one key process that sequentially leads to downstream effects in the two other processes (Paper I). We also utilized an elp3 null mutant to address the function of the ncm5 and mcm5 side-chains at wobble uridines (Paper II). In addition to the elp1–elp6 mutants, the formation of ncm5 and mcm5 modified wobble uridines is abolished in the kti11, kti12, sit4, kti14, and the double sap185 sap190 mutants, whereas a kti13 mutant show significantly reduced levels (Huang et al., 2005) (Lu J. personal communication).

The esterified methyl constituent of the mcm5 group is synthesized by the tRNA carboxyl methyltransferase, Trm9 protein (Kalhor and Clarke, 2003). The TRM9 gene was identified in a search for S. cerevisiae proteins that contained putative AdoMet binding motifs, and later it was shown that Trm9p used AdoMet as the donor for the tRNA methylation reaction (Kalhor and Clarke, 2003; Niewmierzycka and Clarke, 1999). A strain deleted for the TRM9 gene did not contain mcm5U or mcm5s2U in total tRNA (Figure 6), and lacked methyl-esterified nucleosides in purified tRNAArg and tRNAGlu. Extracts from wild-type but not the trm9 mutant catalyzed incorporation of methyl-esters into a substrate tRNA. This suggested that the Trm9 protein catalyzed formation of methyl-esters in both mcm5U and mcm5s2U in
tRNA (Kalhor and Clarke, 2003). In this thesis, we used a trm9 null mutant to address the role of the esterified methyl group in decoding (Paper II).

Three tRNAs, tRNA\textsubscript{Gln\text{mcm}5\text{s}2UUG}, tRNA\textsubscript{Lys\text{mcm}5\text{s}2UUU}, and tRNA\textsubscript{Glu\text{mcm}5\text{s}2UUC} carry the hyper-modified nucleoside mcm\textsuperscript{5} s\textsuperscript{2}U at the wobble position (Figure 6). At the moment, there are ten gene products known to be required for the synthesis of the 2-thio group at U\textsubscript{34}. The Tuc1 protein was identified by sequence homology to the \textit{Escherichia coli} TtcA protein, which is required for the synthesis of s\textsuperscript{2}C in position 32 of a subset of tRNAs (Björk G. personal communication). However, no s\textsuperscript{2}C modification has been identified in \textit{S. cerevisiae} tRNA, so it was speculated if this protein could be required for the formation of the 2-thio group present in the mcm\textsuperscript{5} s\textsuperscript{2}U\textsubscript{34} nucleoside. Accordingly, total tRNA isolated from a \textit{tuc1} null strain lack the s\textsuperscript{2} group of the mcm\textsuperscript{5}s\textsuperscript{2}U wobble nucleoside (Björk G. personal communication). The \textit{Kluvyveromyces lactis} zymocin is a heterotrimeric toxin consisting of a α, β, and γ subunit (Schaffrath and Meinhardt, 2005). The γ-toxin subunit is a tRNA endonuclease cleaving tRNA\textsubscript{Gln\text{mcm}5\text{s}2UUG}, tRNA\textsubscript{Lys\text{mcm}5\text{s}2UUU}, and tRNA\textsubscript{Glu\text{mcm}5\text{s}2UUC} at the 3’ side of the wobble nucleoside mcm\textsuperscript{5}s\textsuperscript{2}U (Paper III). Presence of the mcm\textsuperscript{5}s\textsuperscript{2}U\textsubscript{34} nucleoside is important for efficient cleavage of substrate tRNA and mutants defective in formation of either mcm\textsuperscript{5} or s\textsuperscript{2} side-chains are resistant to zymocin (Paper III) (Lu J. personal communication). In a screen for zymocin resistant mutants, we identified five mutants defective in formation of the s\textsuperscript{2} group of the mcm\textsuperscript{5}s\textsuperscript{2}U\textsubscript{34}, among these the \textit{tuc1} and the \textit{tuc2} mutants (Lu J. personal communication). In addition to Tuc1p and Tuc2p, the Nfs1p, Yor251Cp, Nbp35p, Cia1p,
Urm1p, Uba4p, Isu1p, and Isu2p are also required for the formation of \( s^2U \) at wobble position (Nakai et al., 2007) (Lu J. personal communication). In this thesis, we use the \textit{tuc1} and \textit{tuc2} mutant (also called \textit{NCS6} and \textit{NCS2}, respectively) to address the function of the 2-thio group in decoding (Paper II).
2. Results and discussion

**Paper I: Elevated levels of two tRNA species bypass the requirement for the Elongator complex in transcription and exocytosis.**

The *S. cerevisiae* Elongator complex, consisting of the Elp1-Elp6 proteins, has been proposed to participate in three distinct cellular processes: transcriptional elongation, exocytosis, and formation of modified wobble uridines in tRNA. It was important to clarify whether the Elongator complex has three distinct functions or whether it regulates one key process that sequentially leads to downstream effects. A strain lacking Elongator function displays a multitude of phenotypes, including temperature sensitive (Ts) growth at 38°C (Frohloff et al., 2001; Jablonowski et al., 2001a; Krogan and Greenblatt, 2001).

We investigated if there were genes in high dosage that could bypass the requirement for an *elp3* null mutant. Except for plasmids carrying the *ELP3* gene, we identified five plasmids that partially suppressed the Ts phenotype. All these plasmids contained inserts carrying a tK(UUU) gene, which codes for tRNA_{Lys}^{mcms^2U}U. In a strain lacking Elongator function, this tRNA species contains s^2U rather than mcms^5s^2U at the wobble position. Since the *ELP1-ELP6* genes are required for formation of wobble mcms^5 and mcms^5 side-chains in 11 tRNA species (Huang et al., 2005) (Paper II) (Paper III), we investigated whether elevated levels of any of the remaining 10 tRNAs
species would suppress the Ts phenotype of the *elp3Δ* strain. Weak suppression of the Ts phenotype of the *elp3Δ* strain was obtained by increased dosage of a tQ(UUG) gene which codes for mcm\(^5\)s\(^2\)U containing tRNA\(^{\text{Gln}}_{\text{mcm}^5\text{s}^2\text{UG}}\). Simultaneously increased expression of the tK(UUU) and tQ(UUG) genes in an *elp3Δ* strain resulted in a cooperative growth improvement at both 30°C and 38°C. The tRNA\(^{\text{Gln}}_{\text{mcm}^5\text{s}^2\text{UG}}\) species contain the same wobble nucleoside (mcm\(^5\)s\(^2\)U) as tRNA\(^{\text{Lys}}_{\text{mcm}^5\text{s}^2\text{UUU}}\) and contain s\(^2\)U in Elongator deficient cells (Paper III). Because strains with a mutation in any of the *ELP1-ELP6* genes display similar phenotypes, we tested if the suppression generated by increased dosage of the tK(UUU) and tQ(UUG) genes is also true in other Elongator mutants. Increased dosage of the tK(UUU) and tQ(UUG) genes suppressed the Ts phenotype of all the *elp1-elp6* mutants. These results indicate with respect to growth defects, elevated levels of the hypomodified tRNA\(^{\text{Gln}}_{\text{mcm}^5\text{s}^2\text{UG}}\) and tRNA\(^{\text{Lys}}_{\text{mcm}^5\text{s}^2\text{UUU}}\) species bypass the requirement for the Elongator complex.

The second function proposed for the Elongator complex was acetylation of histones, and the histone acetyl transferase (HAT) activity was primarily associated with the Elp3 protein. HATs transfer acetyl groups from acetyl-CoA to the amino group of certain lysine residues within histone N-terminal tails (Sterner and Berger, 2000). Lysine 14 in histone H3 has been shown to be the major target for the HAT activity of Elp3p (Kuo et al., 1996; Winkler et al., 2002). Histones isolated from *elp3Δ* cells with or without elevated levels of tRNA\(^{\text{Lys}}_{\text{mcm}^5\text{s}^2\text{UUU}}\) and tRNA\(^{\text{Gln}}_{\text{mcm}^5\text{s}^2\text{UG}}\) were analyzed with anti-histone H3 and anti-acetyl-lys14-histone H3 antiserum. Increased levels
of the hypomodified tRNA$_{\text{Lys}}^{\text{Lys}}$ and tRNA$_{\text{Gln}}^{\text{Gln}}$ restored the Lysine 14 acetylation levels of histone H3 in elp3Δ cells. Thus, the low acetylation level of Lysine 14 in histone H3 isolated from elp3Δ cells is most likely caused by a reduced function of the hypomodified tRNA$_{\text{Lys}}^{\text{Lys}}$ and tRNA$_{\text{Gln}}^{\text{Gln}}$, strongly questioning a role of Elp3p as a bona fide HAT.

The third distinct function proposed for the Elongator complex was the delivery of transport vesicles to the bud-site (Rahl et al., 2005). Sec2p is a guanine nucleotide exchange factor that triggers GDP to GTP exchange of Sec4p (Walch-Solimena et al., 1997). Activated GTP-bound Sec4p participates in delivery of vesicles to a specific region of the plasma membrane (Salminen and Novick, 1987) (Walch-Solimena et al., 1997). A sec2-59 mutant displays a Ts phenotype (Nair et al., 1990) and introduction of an elp1 null allele into the sec2-59 mutant strain suppress the Ts phenotype (Rahl et al., 2005). Increased expression of the hypomodified tRNA$_{\text{Lys}}^{\text{Lys}}$ and tRNA$_{\text{Gln}}^{\text{Gln}}$ in the elp1Δ sec2-59 double mutant generated a Ts phenotype, as observed in the sec2-59 single mutant. Thus, increased levels of these tRNAs mimic the presence of Elp1p. Sec2p is a cytosolic protein that normally concentrates in a distinct polarized manner to the site of exocytosis (Elkind et al., 2000). In an elp1Δ strain, Sec2p is mislocalized and it was suggested that a physical interaction between Elp1p and Sec2p was important for the polarized localization of Sec2p (Rahl et al., 2005). Elevated levels of the hypomodified tRNA$_{\text{Lys}}^{\text{Lys}}$ and tRNA$_{\text{Gln}}^{\text{Gln}}$ in the elp1Δ strain restored polarized localization of Sec2p. This shows that an interaction to Elp1p is not a requirement for a polarized localization of...
Sec2p, rather it reflects the importance of the mcm\(^5\) side-chain in tRNA\(^{\text{Lys}}_{\text{mcm}^5\text{s}^2\text{UUU}}\) and tRNA\(^{\text{Gln}}_{\text{mcm}^5\text{s}^2\text{UG}}\) for post-transcriptional expression of gene products crucial in the exocytosis process.

The mcm\(^5\) or s\(^2\) groups of the wobble mcm\(^5\)s\(^2\)U residue are both believed to affect decoding in a similar manner (see above). Phenotypes caused by removal of the s\(^2\) or the mcm\(^5\) group in tRNA\(^{\text{Lys}}_{\text{mcm}^5\text{s}^2\text{UUU}}\) and tRNA\(^{\text{Gln}}_{\text{mcm}^5\text{s}^2\text{UG}}\) are expected to be a consequence of less efficient decoding Lys and Gln codons. We recently found that a strain with a \(\text{tuc2}\) (also called \(\text{ncs2}\)) null allele lacks the s\(^2\) group in mcm\(^5\)s\(^2\)U containing tRNA species (see above). A \(\text{tuc2}\) strain displays similar phenotypes as \(\text{elp1}\) and \(\text{elp3}\) mutant strains e.g., delayed transcriptional activation, chromatin remodeling-, and exocytosis-defects. Increased levels of the mcm\(^5\) containing tRNA\(^{\text{Lys}}_{\text{mcm}^5\text{UUU}}\) and tRNA\(^{\text{Gln}}_{\text{mcm}^5\text{UG}}\) suppressed the phenotypes of a \(\text{tuc2}\) null strain, suggesting that the defects were caused by lack of the 2-thio group at wobble position. We conclude that phenotypes proposed to be caused by a direct effect of Elongator on exocytosis or transcription are observed in a \(\text{tuc2}\) mutant where the functionality of tRNA\(^{\text{Lys}}_{\text{mcm}^5\text{s}^2\text{UUU}}\) and tRNA\(^{\text{Gln}}_{\text{mcm}^5\text{s}^2\text{UG}}\) has been reduced by an Elongator-independent mechanism.

**Summary:**

In Paper I, we show that increased levels of the hypomodified tRNA\(^{\text{Lys}}_{\text{s}^2\text{UUU}}\) and tRNA\(^{\text{Gln}}_{\text{s}^2\text{UG}}\) could bypass the requirement for the Elongator complex in transcription and exocytosis. As the Elongator complex is required for the formation of the mcm\(^5\) group at position 34 in these tRNAs, we suggest that
the relevant function of the Elongator complex is to modify wobble uridine in tRNA, and that transcription and exocytosis defects in Elongator mutant are indirect effects resulting from a primary defect in translation (Figure 7).

Figure 7. Model: The Elongator complex is required to modify uridines at the wobble position. The defects observed in exocytosis and transcription are indirectly caused by inefficient translation of mRNAs encoding gene products important for these processes.
Modified nucleosides in the anticodon region of tRNA are important in the decoding process. Since defined mutants defective in formation of the $s^2$ or $ncm^5$ and $mcm^5$ groups at wobble uridines have not been available, the \textit{in vivo} roles of these modifications have not been investigated. The Elongator complex was recently shown to be required for formation of $ncm^5$ and $mcm^5$ side-chains at position 34 (Huang et al., 2005). In addition, the last methyl group in the $mcm^5$ side-chain is synthesized by the Trm9p (Kalhor and Clarke, 2003). Furthermore, the Tuc2p is required for the formation of the 2-thio group in $mcm^5s^2U_{34}$ containing tRNA species (Paper I) (Björk G. personal communication). Strains with mutations in any of the \textit{ELP1-ELP6}, \textit{TRM9}, or \textit{TUC2} genes are viable, making it possible to investigate the \textit{in vivo} role of the wobble $ncm^5$, $mcm^5$, and $s^2$ groups.

According to current models based on mainly physicochemical studies, a tRNA with an unmodified $U_{34}$ may decode codons ending with any of the four nucleotides (Lim, 1994; Yokoyama and Nishimura, 1995). To investigate if an unmodified wobble uridine is able to read codons ending with any of the nucleotides \textit{in vivo}, we utilized the distribution of tRNA species in the Leucine and Proline family codon boxes. In the Leucine box, there are only two tRNA species present, the $tRNA_{U_{34}}^{Leu}$ containing an unmodified $U_{34}$ (Randerath et al., 1979), and the $tRNA_{G_{34}}^{Leu}$ harboring a $G_{34}$. Interestingly, a strain lacking the $G_{34}$ containing $tRNA_{G_{34}}^{Leu}$ species is viable,
suggesting that an unmodified wobble uridine can read all four nucleotides.

In the Proline box, there are two tRNA species present, one contains ncm\(^5\)U\(_{34}\) \(\text{tRNA}^{\text{Pro}}_{\text{ncm}^5\text{UGG}}\) and the other one contains an A at the wobble position (most likely converted to I\(_{34}\)). A strain lacking the A\(_{34}\) containing \(\text{tRNA}^{\text{Pro}}_{\text{AGG}}\) species is viable with no apparent growth defect. Introduction of an \(\text{elp3}\Delta\) allele into this strain did not generate a synthetic growth defect, suggesting that the unmodified U\(_{34}\) in \(\text{tRNA}^{\text{Pro}}_{\text{UGG}}\) is also able to decode codons ending with any of the four nucleotides. The ability of \(\text{tRNA}^{\text{Pro}}_{\text{ncm}^5\text{UGG}}\) to decode U- and C-ending codons is independent of the ncm\(^5\) group, which could imply that reading of these codons involves a two out of three interaction (Lagerkvist, 1978).

To investigate if the ncm\(^5\) group in \(\text{tRNA}^{\text{Val}}_{\text{ncm}^5\text{UAC}}\) is important for decoding A-ending codons, we reduced the copy number of the genes coding for ncm\(^5\) containing \(\text{tRNA}^{\text{Val}}_{\text{ncm}^5\text{UAC}}\) in yeast cells lacking the \(\text{elp3}\) allele. In a strain deleted for one of the two genes coding for \(\text{tRNA}^{\text{Val}}_{\text{ncm}^5\text{UAC}}\), introduction of the \(\text{elp3}\Delta\) allele did not generate a synthetic growth defect. Since a tRNA species with an I\(_{34}\) residue is present in the Valine family codon box \(\text{tRNA}^{\text{Val}}_{\text{IAC}}\), it is possible that lack of a synergistic phenotype is due to the ability of the I\(_{34}\) containing tRNA to decode GUA codons. However, a strain lacking the ncm\(^5\) containing \(\text{tRNA}^{\text{Val}}_{\text{ncm}^5\text{UAC}}\) species is inviable, showing that the I\(_{34}\) containing \(\text{tRNA}^{\text{Val}}_{\text{IAC}}\) cannot decode the GUA codons. We conclude that the absence of an ncm\(^5\) side-chain at U\(_{34}\) does not reduce the ability of the \(\text{tRNA}^{\text{Val}}_{\text{ncm}^5\text{UAC}}\) to decode the GUA codons.

To investigate if the ncm\(^5\) group in \(\text{tRNA}^{\text{Val}}_{\text{ncm}^5\text{UAC}}\) is important for
decoding G-ending codons, we deleted the two genes coding for the C$_{34}$ containing tRNA$_{_{\text{Val}}}^{\text{C\text{AC}}}$ species. As this strain was viable, the ncm$^5$U$_{34}$ containing tRNA$_{_{\text{Val}}}^{\text{ncm}^5\text{UAC}}$ must be able to decode GUG codons. However, introduction of the *elp3* allele into a strain lacking the C$_{34}$ containing tRNA$_{_{\text{Val}}}^{\text{C\text{AC}}}$ generated lethality, showing that the ncm$^5$ group in tRNA$_{_{\text{Val}}}^{\text{ncm}^5\text{UAC}}$ is required for reading the GUG codons. We obtained similar results using comparable genetic strategies for the Threonine and the Serine codon boxes. Together these data supports a model where the ncm$^5$U$_{34}$ nucleoside does not enhance the ability to decode A-, but improves reading of G-ending codons.

To test if the mcm$^5$ group at U$_{34}$ is important for decoding A-ending codons, we deleted two of the three genes coding for the mcm$^5$U$_{34}$ containing tRNA$_{_{\text{Gly}}}^{\text{mcm}^5\text{UCC}}$. Introduction of the *elp3*Δ allele into this strain did not generate a synthetic growth defect, suggesting that the mcm$^5$ group does not notably affect the ability of tRNA$_{_{\text{Gly}}}^{\text{mcm}^5\text{UCC}}$ to decode GGA codons. To address the role of the mcm$^5$ group in decoding G-ending codons, we introduced an *elp3*Δ allele into a strain lacking the C$_{34}$ containing tRNA$_{_{\text{Gly}}}^{\text{CCC}}$ species. A synthetic growth defect was observed, suggesting that the presence of a mcm$^5$ side-chain improves decoding of G-ending codons. We obtained similar results using a comparable genetic approach in the Arginine split codon box (AGN) to address the role of the mcm$^5$ group in decoding G-ending codons. Taken together, this suggests that the mcm$^5$ residue improves reading of G-, but has no significant influence on decoding A-ending codons.
To investigate the role of the mcm\(^5\)s\(^2\)U\(_{34}\) residue in the decoding of G-ending codons, a strain with a deletion of the single copy C\(_{34}\) containing tRNA\(_{\text{CUG}}^{\text{GlnG}}\) gene was constructed. This strain is inviable, suggesting that tRNA\(_{\text{CUG}}^{\text{GlnG}}\) is unable to read the G-ending codons. The presence of either a mcm\(^5\) or an s\(^2\) group could potentially prevent reading of G-ending codons. To test this hypothesis we deleted the single copy C\(_{34}\) containing tRNA\(_{\text{CUG}}^{\text{GlnG}}\) gene in either an elp3\(\Delta\) or a tuc1\(\Delta\) null strain. Lack of neither the mcm\(^5\) nor the s\(^2\) group suppressed the inviability of a strain lacking the C\(_{34}\) containing tRNA\(_{\text{CUG}}^{\text{GlnG}}\) species. Thus, normal levels of the tRNA\(_{\text{CUG}}^{\text{GlnG}}\) carrying either the mcm\(^5\)s\(^2\)U\(_{34}\), mcm\(^5\)U\(_{34}\) or the s\(^2\)U\(_{34}\) at wobble position are not able to decode G-ending codons. Interestingly, elevated levels of the tRNA\(_{\text{CUG}}^{\text{GlnG}}\) suppressed the need for C\(_{34}\) containing tRNA\(_{\text{CUG}}^{\text{GlnG}}\) in a wild-type background. This suppression was not observed in an elp3\(\Delta\) or tuc1\(\Delta\) background, suggesting that the mcm\(^5\) and the s\(^2\) groups cooperatively improve decoding of G-ending codons.

Summary
According to current models, an unmodified U\(_{34}\) should be able to decode all four nucleotides U, C, A, and G (Lim, 1994; Yokoyama and Nishimura, 1995). This is true for two of the tRNA species we have investigated. We show that the tRNA\(_{\text{UGU}}^{\text{Leu}}\) and the hypomodified tRNA\(_{\text{UGG}}^{\text{Pro}}\), both containing an unmodified U\(_{34}\), are able to decode codons ending with any of the four nucleosides. However, the decoding ability of U\(_{34}\) is highly specific as an unmodified U\(_{34}\) in tRNA\(_{\text{UGU}}^{\text{Thr}}\) or tRNA\(_{\text{UGA}}^{\text{Ser}}\), is incapable of reading their
respective G-ending codons. Therefore, we suggest that it is rather an exception than a role for an unmodified U_{34} to be able to decode all four nucleotides and we do not exclude the possibility that reading of these codons could involve a two out of three interaction (Lagerkvist, 1978).

According to current models, presence of an x{m}^5 side-chain is predicted to either restrict reading to A- or allow efficient decoding of both A- and G-ending codons (Kruger et al., 1998; Lim, 1994; Murphy et al., 2004; Takai and Yokoyama, 2003; Yarian et al., 2002; Yokoyama and Nishimura, 1995; Yokoyama et al., 1985). We found no evidence for a role of the x{m}^5 side-chain in tRNA_{Val}^{ncm5UAC}, tRNA_{Ser}^{ncm5UGA} and tRNA_{Gly}^{mcm5UCC} in the decoding of their respective A-ending codons. However, we could show that presence of an x{m}^5 side-chain in tRNA_{Val}^{ncm5UAC}, tRNA_{Ser}^{ncm5UGA}, tRNA_{Arg}^{mcm5UCU}, and tRNA_{Gly}^{mcm5UCC} improves reading of G-ending codons.

The presence of a mcm^5 s^2 U_{34} residue was originally proposed to allow the tRNA to efficiently read the cognate A-ending codon and simultaneously reduce the ability to decode G-ending codons (Lustig et al., 1981; Sekiya et al., 1969; Yokoyama et al., 1985). Together the data presented in Paper I and Paper II, suggests that the presence of the mcm^5 and the s^2 group cooperatively improves decoding of both A- and G-ending codons.
Figure 8. Summary of the result presented in Paper II. Circles connected with a line indicate one tRNA species. Transfer RNA species addressed in Paper II are labeled with black or gray circles. Black circles connected with a line shows that a tRNA species efficiently reads the indicated codons. Gray circles linked with a dashed line indicate that a tRNA species reads the specified codons only when over-expressed. The nucleoside at the wobble position is given for the U₃₄ containing tRNA species. The number of genes coding for a tRNA species is indicated next to the circle for the cognate codon, which also means that the primary anticodon sequence can be deduced from the figure. ¹ The gene(s) encoding the tRNA is nonessential. ² The gene(s) encoding the tRNA is essential.
Paper III: The *Kluyveromyces lactis* γ-toxin targets tRNA anticodons.

To gain competitive growth advantage, various microorganisms produce and secrete toxins. One example is the dairy yeast *K. lactis*, which secretes a heterotrimeric toxin (zymocin), that causes growth arrest of sensitive yeast cells (Gunge and Sakaguchi, 1981; For review, Schaffrath and Meinhardt, 2005; Sugisaki et al., 1983; White et al., 1989). Zymocin is composed of a α, β, and γ subunit. The α and β subunits assist transfer of the cytotoxic γ subunit into cells (Butler et al., 1991a; Gunge et al., 1981; For reviews, Schaffrath and Meinhardt, 2005; Stark et al., 1990; Tokunaga et al., 1989).

Two classes of *S. cerevisiae* mutants resistant to zymocin have been isolated (Butler et al., 1991a; Butler et al., 1994; For review, Schaffrath and Meinhardt, 2005; Sugisaki et al., 1983; White et al., 1989). Class I mutants are defective in binding and/or uptake of zymocin, as these mutants are resistant to extra genius zymocin, but sensitive to intracellular γ-toxin. Class II mutants are resistant to both extra genius zymocin and intracellular γ-toxin, and therefore considered target mutants. Interestingly, strains deleted for any of the *ELP1-ELP6* or *KTI11–KTI13* genes have been shown to be class II mutants (Butler et al., 1994; Fichtner and Schaffrath, 2002; Frohloff et al., 2001; Jablonowski et al., 2001b). As the Elongator complex was primarily thought to function in elongation of Pol II transcription, it was assumed that the γ-toxin was targeted to the Pol II transcription machinery in an Elongator-dependent manner, and that the growth arrest was caused by transcriptional inactivation (For review, Schaffrath and Meinhardt, 2005).
Previously, it was shown that elevated levels of the mcms\(^5\)s\(^2\)U\(_{34}\) containing tRNA\(^{Glu}_{mcm^5s^2UUC}\) suppressed the zymocin sensitivity of a wild-type S. cerevisiae strain (Butler et al., 1994). As all the class II mutants (elp1-elp6 and kti11-kti13) affect the synthesis of the mcms\(^5\) group present in tRNA\(^{Glu}_{mcm^5s^2UUC}\), we hypothesized that the killer toxin-resistant phenotype induced by these mutations could be a consequence of the inability to modify wobble uridine in tRNA\(^{Glu}_{mcm^5s^2UUC}\).

To test this hypothesis, we investigated the levels of tRNA\(^{Glu}_{mcm^5s^2UUC}\) in wild-type, elp3\(\Delta\), or trm9\(\Delta\) cells after induction of intracellular \(\gamma\)-toxin. The amount of tRNA\(^{Glu}_{mcm^5s^2UUC}\) was reduced with increased induction time in wild-type cells, whereas no reduction could be detected in elp3\(\Delta\) or trm9\(\Delta\) cells. This supported the idea that the entire mcms\(^5\) side-chain in tRNA\(^{Glu}_{mcm^5s^2UUC}\) is required for the cytotoxicity of the \(\gamma\)-toxin. In addition to the tRNA\(^{Glu}_{mcm^5s^2UUC}\), the mcms\(^5\) side-chain is present in tRNA\(^{Gly}_{mcm^5s^2UUC}\), tRNA\(^{Lys}_{mcm^5s^2UUC}\), tRNA\(^{Arg}_{mcm^5UCU}\), and tRNA\(^{Gln}_{mcm^5UCU}\), but no reduction of these tRNAs could be observed in wild-type cells after intracellular \(\gamma\)-toxin expression, suggesting that these tRNAs are not substrates \textit{in vivo}.

To investigate if \(\gamma\)-toxin acts directly on tRNA, we incubated total tRNA isolated from wild-type, elp3, or trm9 cells with purified \(\gamma\)-toxin. Cleavage of tRNA\(^{Glu}_{mcm^5s^2UUC}\), tRNA\(^{Lys}_{mcm^5s^2UUC}\), and tRNA\(^{Gln}_{mcm^5s^2UUG}\) could be detected of tRNA isolated from wild-type cells however, tRNA\(^{Lys}_{mcm^5s^2UUU}\) and tRNA\(^{Gln}_{mcm^5s^2UUG}\) were cleaved with lower efficiency. The mcms\(^5\) group is important for efficient cleavage \textit{in vitro}, as reduced \(\gamma\)-toxin cleavage efficiency was observed for tRNA isolated from elp3 or trm9 null mutants.
Analysis of the cleavage products revealed that the γ−toxin cleaves tRNA_{Glu}^{mcm5\hat{s}2UUC} , tRNA_{Lys}^{mcm5\hat{s}2UUU} , and tRNA_{Gln}^{mcm5\hat{s}2UG} between position 34 and 35, generating a 2´, 3´ cyclic-phosphate and a 5´ hydroxyl-group. This confirms that the γ-toxin acts directly on tRNA and suggests that tRNA_{Glu}^{mcm5\hat{s}2UUC} , tRNA_{Lys}^{mcm5\hat{s}2UUU} and tRNA_{Gln}^{mcm5\hat{s}2UG} might be γ-toxin targets in vivo.

Consistent with the γ-toxin reactivity of the substrate tRNA in vivo and in vitro, we showed that elevated levels of tRNA_{Glu}^{mcm5\hat{s}2UUC} , but not tRNA_{Lys}^{mcm5\hat{s}2UUU} or tRNA_{Gln}^{mcm5\hat{s}2UG} , made a wild-type strain resistant to exogenous zymocin. However, over-expression of tRNA_{Glu}^{mcm5\hat{s}2UUC} in combination with tRNA_{Lys}^{mcm5\hat{s}2UUU} or tRNA_{Gln}^{mcm5\hat{s}2UG} or all three mcm5\hat{s}2U34 containing tRNA species synergistically increased zymocin resistance. These data suggests that all three mcm5\hat{s}2U34 containing tRNAs, tRNA_{Glu}^{mcm5\hat{s}2UUC} , tRNA_{Lys}^{mcm5\hat{s}2UUU} , and tRNA_{Gln}^{mcm5\hat{s}2UG} , are γ-toxin substrates in vivo.

Inhibition of protein synthesis arrests yeast cells in the G1 phase of the cell cycle (Hohmann and Thevelein, 1992; Johnston et al., 1977; Pyronnet and Sonenberg, 2001; Unger and Hartwell, 1976; Wrobel et al., 1999). Interestingly, zymocin also arrests sensitive yeast cells in the G1 phase of the cell cycle (Butler et al., 1991b). Therefore, we propose that the zymocin induced G1 arrest is most likely a consequence of a translational defect caused by depletion or reduction of tRNA_{Glu}^{mcm5\hat{s}2UUC} , tRNA_{Lys}^{mcm5\hat{s}2UUU} , and tRNA_{Gln}^{mcm5\hat{s}2UG} .
Summary:
In Paper III, we characterize the first eukaryotic toxin shown to target tRNAs. We show that all three mcm\(^5\)s\(^2\)U\(_{34}\) containing tRNA species, tRNA\(^{\text{Glu}}\)\(_{\text{mcm5s2UUC}}\), tRNA\(^{\text{Lys}}\)\(_{\text{mcm5s2UUU}}\), and tRNA\(^{\text{Gin}}\)\(_{\text{mcm5s2UUU}}\) are cleaved by the \(\gamma\)-toxin between position 34 and 35. The \(\gamma\)-toxin resistance of elp1-elp6 and kti11-kti13 mutants can be explained by the fact that these mutants are defective in formation of the mcm\(^5 group present at position 34 which is required for efficient cleavage (Figure 9).

Figure 9. Model: *K. lactis* \(\gamma\)-toxin cleaves tRNA\(^{\text{Glu}}\)\(_{\text{mcm5s2UUC}}\), tRNA\(^{\text{Lys}}\)\(_{\text{mcm5s2UUU}}\), and tRNA\(^{\text{Gin}}\)\(_{\text{mcm5s2UUU}}\) between positions 34 and 35; the mcm\(^5\) group at position 34 is important for efficient cleavage.
3. Conclusions

Elevated levels of two tRNA species bypass the requirement for the Elongator complex in transcription and exocytosis.

Increased expression of tRNA\textsubscript{Gln\textsubscript{mcm\textsubscript{5}\textsubscript{s}\textsubscript{2}UUG}} and tRNA\textsubscript{Lys\textsubscript{mcm\textsubscript{5}\textsubscript{s}\textsubscript{2}UUU}} bypass the requirement for the Elongator complex in transcription and exocytosis. This observation suggests that the relevant function for the Elongator complex is in tRNA modification (Paper I) (Figure 7).

Eukaryotic wobble uridine modifications promote a functionally redundant decoding system.

The \textsubscript{mcm\textsuperscript{5}} side-chain present at the wobble uridine improves reading of G-ending codons. The \textsubscript{mcm\textsuperscript{5}} and \textsubscript{s\textsuperscript{2}} groups cooperatively improve reading of A- and G-ending codons (Paper II) (Figure 8).

The \textit{Kluyveromyces lactis} \(\gamma\)-toxin targets tRNA anticodons.

The \(\gamma\)-toxin cleaves the three \textsubscript{mcm\textsuperscript{5}\textsubscript{s\textsuperscript{2}U\textsubscript{34}}} containing tRNA species, tRNA\textsubscript{Gln\textsubscript{mcm\textsuperscript{5}\textsubscript{s\textsuperscript{2}UUG}}}, tRNA\textsubscript{Glu\textsuperscript{mcm\textsuperscript{5}\textsubscript{s\textsuperscript{2}UUCC}}}, and tRNA\textsubscript{Lys\textsubscript{mcm\textsuperscript{5}\textsubscript{s\textsuperscript{2}UUU}}} between position 34 and 35. The \(\gamma\)-toxin resistance of mutants defective in formation the \textsubscript{mcm\textsuperscript{5}} group can be explained by the fact that this group is important for efficient cleavage (Paper III) (Figure 9).
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