Physiological consequences of Elongator complex inactivation in Eukaryotes

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“Once you have a PhD, every meeting you go to becomes a doctor's appointment”

-Nick Offerman

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Appended papers

This thesis is based on the following appended papers, referred to by the corresponding Roman numerals in the text.

I. Loss of ncm^5 and mcm^5 wobble uridine side chains results in an altered metabolic profile.
Karlsborn T., Mahmud A.K.M.F.[†], Tükenmez H.[†] and Byström A.S.
(Submitted Manuscript)
[†] These authors contributed equally

II. Familial dysautonomia (FD) patients have reduced levels of the modified wobble nucleoside mcm^5s^2U in tRNA.
Karlsborn T., Tükenmez H., Chen C. and Byström A.S.
Biochemical and Biophysical Research Communications, 21 November (2014)
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III. Elongator complex enhances Rnr1p levels in response to DNA damage by influencing Ixr1p expression.
Tükenmez H., **Karlsborn T.**, Mahmud A.K.M.F., Chen C., Xu F. and Byström A.S.
(Manuscript)

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Abstract

Mutations found in genes encoding human Elongator complex subunits have been linked to neurodevelopmental disorders such as familial dysautonomia (FD), rolandic epilepsy and amyotrophic lateral sclerosis. In addition, loss-of-function mutations in genes encoding Elongator complex subunits cause defects in neurodevelopment and reduced neuronal function in both mice and nematodes. The Elongator complex is a conserved protein complex comprising six subunits (Elp1p-Elp6p) found in eukaryotes. The primary function of this complex in yeast is formation of the 5-methoxycarbonylmethyl (mcm^5) and 5-carbamoylmethyl (ncm^5) side chains found on wobble uridines (U_{34}) in tRNAs. The aim of this thesis is to investigate the physiological consequences of Elongator complex inactivation in humans and in the yeast *Saccharomyces cerevisiae*.

Inactivation of the Elongator complex causes widespread defects in a multitude of different cellular processes in *S. cerevisiae*. Thus, we investigated metabolic alterations resulting from Elongator complex inactivation. We show that deletion of the *S. cerevisiae ELP3* gene leads to widespread metabolic alterations. Moreover, all global metabolic alterations observed in the *elp3Δ* strain are not restored in the presence of elevated levels of hypomodified tRNAs that normally have the modified nucleoside mcm^5s^2U. Collectively, we show that modified wobble nucleosides in tRNAs are required for metabolic homeostasis.

Elongator mutants display sensitivity to DNA damage agents, but the underlying mechanism explaining this sensitivity remains elusive. We demonstrate that deletion of the *S. cerevisiae ELP3* gene results in post-transcriptional reduction of Ixr1p levels. Further, we show that the reduced Ixr1p levels prevent adequate Rnr1p levels upon treatment with DNA damage agents. These findings suggest that reduced Ixr1p levels could in part explain why Elongator mutants are sensitive to DNA damage agents.

Depletion of Elongator complex subunits results in loss of wobble uridine modifications in plants, nematodes, mice and yeast. Therefore, we investigated whether patients with the neurodegenerative disease familial dysautonomia (FD), who have lower levels of the ELP1 protein, display reduced amounts of modified wobble uridine nucleosides. We show that tRNA isolated from brain tissue and fibroblast cell lines derived from FD patients have 64–71% of the mcm^5s^2U nucleoside levels observed in total tRNA from non-FD brain tissue and non-FD fibroblasts. Overall, these results suggest that the cause for the neurodegenerative nature of FD could be translation impairment caused by reduced levels of modified wobble uridine nucleosides in tRNAs. Thus, our results give new insight on the importance of modified wobble uridine nucleosides for neurodevelopment.

Sammanfattning på svenska

Mutationer funna i gener som kodar för Elongator komplexets subenheter har associerats med de neurodegenerativa sjukdomarna Familjär dysautonomi (FD), Rolandisk epilepsi och amyotrofisk lateralskleros (ALS). Reducerade nivåer av Elongator komplex subenheter i möss och nematoder leder tillika till defekter i neurologisk funktion och utveckling. Elongator komplexet är ett konserverat protein komplex bestående av sex subenheter (Elp1p-Elp6p) i eukaryoter. Primära funktionen av komplexet är bildning av de kemiska sidokedjorna 5-methoxycarbonylmethyl (mcm^5) och 5-carbamoylmethyl (ncm^5) på uridiner i wobble position (U_{34}) av tRNA. Målet med denna avhandling är att undersöka de fysiologiska konsekvenserna vid inaktivering av Elongator komplexet i jästen *Saccharomyces cerevisiae* och människor.

I *S.cerevisiae* så leder förlust av ett funktionellt Elongator komplex till många defekter i flertalet cellulära processer. De många defekterna som påvisats vid förlust av Elongator komplexet föranledde oss att undersöka om det sker stora förändringar i metabolismen hos *S.cerevisiae* vid avsaknad av komplexet. Vi kan visa att inaktivering av *ELP3* genen ger upphov till avsevärda metabola förändringar. Överuttryck av hypomodifierade tRNA som normalt har den modifierade nukleosiden mcm^5s^2U leder inte till en global suppression av de metabola förändringarna som observerades vid avsaknad av *ELP3* genen. Överlag så visar våra data att modifierade wobble nukleosider i tRNA är viktiga för att vidhålla metabolisk homeostas.

Mutanter som har ett icke-funktionellt Elongator komplex påvisar känslighet mot kemikalier som orsakar DNA skada. De molekylära mekanismerna som leder till denna känslighet har hittills inte förklarats. Vi visar att inaktivering av *ELP3* genen leder till post-transkriptionell reduktion av Ixr1 protein-nivåer. Vi visar dessutom att reducerade nivåer av Ixr1 proteinet leder till lägre nivåer av Rnr1 proteinet som krävs vid DNA skada. Dessa resultat föreslår att lägre nivåer av Ixr1 proteinet kan vara orsaken till känsligheten mot DNA skadande kemikalier i celler med ett icke-funktionellt Elongator komplex.

Förlust av Elongator komplexet leder till avsaknad av modifierade wobble uridine nukleosider i växter, nematoder, möss och jäst. Av denna anledning undersökte vi om FD patienter, som påvisar lägre nivåer av Elp1 proteinet, har reducerade mängder av modifierade wobble uridine nukleosider i tRNA. Vi kan visa att hjärnvävnad och fibroblast-celler från FD patienter har 64-71% av mcm^5s^2U-nukleosidnivåerna som uppmättes i hjärnvävnad och fibroblast-celler från friska individer. Detta resultat föreslår att orsaken till de neurodegenerativa defekterna som ses hos FD patienter kan ha sitt ursprung i reducerad translations-effektivitet åsamkat av lägre nivåer av tRNA som har modifierade wobble uridiner. Dessa resultat ger oss en djupare inblick i betydelsen modifierade wobble uridine nukleosider har vid neurologisk utveckling.

Abbreviations

(eIFs) eukaryotic initiation factors

($tRNA_i^{Met}$) initiator methionyl-tRNA

($tRNA_e^{Met}$) elongation methionyl-tRNA

(PIC) pre-initiation-complex

(PABP) polyadenylate-binding protein or poly(A)-binding protein

(uORFs) upstream open reading frames

(IRES) internal ribosome entry sites

(PTC) peptidyl transferase center

(I) Inosine

(Gm) 2'-O-methylguanosine

(Cm) 2'-O-methylcytidine

(m^5C) 5-methylcytidine

(Ψ) Pseudouridine

(mcm^5U) 5-methoxycarbonylmethyluridine

(mcm^5s^2U) 5-methoxycarbonylmethyl-2-thiouridine

(ncm^5U) 5-carbamoylmethyluridine

(ncm^5Um) 5-carbamoylmethyl-2'-O-methyluridine

(m^1I) 1-methylinosine

(i^6A) N6-isopentenyladenosine

(m^1G) 1-methylguanosine

(yW) wybutosine

(t^6A) N6-threonylcarbamoyladenosine

(RTD) rapid tRNA decay

(FD) Familial Dysautonomia

(PCA) Principal component analysis

(PLS) Partial least squares projection to latent structures

Introduction

1 An overview of the translation process

Translation is a tightly regulated process during which information from the genetic code is transferred from mRNA to produce proteins. Translation also regulates gene expression, as translation of specific mRNAs varies in response to changes in environmental conditions (Wek et al., 2006). The translation process is divided into four phases: initiation, elongation, termination and ribosome recycling. During each phase, specific translation factors cooperate with the ribosome to ensure efficient translation (Rodnina and Wintermeyer, 2009).

1.1 Translation initiation and its regulation

The initiation phase of the translation process is heavily regulated, with at least 12 specific eukaryotic initiation factors (eIFs). These factors aid in assembly of the large ribosomal subunit (60S) with the small ribosomal subunit (40S) and $tRNA_i^{Met}$. The assembly results in a mature 80S ribosome that is ready for the next phase of the translation process (**Figure 1**) (Hinnebusch, 2011; Jackson et al., 2010; Sonenberg and Hinnebusch, 2009).

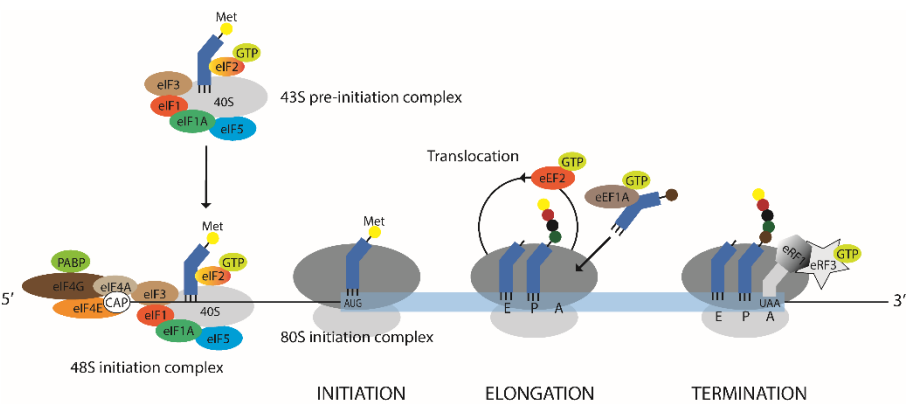

Figure 1. A simplified model illustrating translation.

Initiation begins with assembly of the 43S pre-initiation-complex (PIC) which comprises the small ribosomal subunit, eIFs 1, 1A, 2, 3, and 5, and the $tRNA_i^{Met}$. The eIF2 protein bound to GTP and $tRNA_i^{Met}$ is also called the ternary complex. After assembly of the ternary complex with eIFs 1, 1A, 3, and 5 and the small ribosomal subunit, the resulting 43S PIC is recruited to the m7G capped 5' end of mRNA. This recruitment depends on the cap-binding factors: eIF4E, eIF4G, and eIF4A, which together form the eIF4F complex.

Association of the cap-binding factors with the mRNA starts the mRNA on a downstream scan to find the codon complementary to $tRNA^{iMet}$ to form the 48S initiation complex (Sonenberg and Hinnebusch, 2009).

Formation of the eIF4F complex is inhibited when a set of proteins binds to the eIF4E subunit and blocks its interaction with the eIF4G scaffold. This blockade prevents formation of the eIF4F complex and, consequently, recruitment of the 43S PIC complex (Jackson et al., 2010). Furthermore, some yeast mRNAs rich in 5' untranslated region (UTR) secondary structures are believed to require the helicase Ded1p, in addition to eIF4A, for efficient mRNA scanning to locate the AUG start codon (Berthelot et al., 2004; Chuang et al., 1997; Sen et al., 2015). Moreover, eIF4G interacts with the polyadenylate-binding protein (PABP) located at the 3' poly-A end of the mRNA to form a closed loop (Costello et al., 2015; Hinton et al., 2007; Kahvejian et al., 2005). The PABP-eIF4G interaction is thought to stimulate translation by either: promoting ribosome recycling; stimulating 60S ribosome joining; or enhancing 40S ribosome recruitment by increasing interaction between eIF4F and the 5'-cap (Bi and Goss, 2000; Le et al., 1997; Otero et al., 1999b; Sachs and Davis, 1989; Searfoss et al., 2001; Tarun and Sachs, 1995).

Formation of the 48S initiation complex, and $tRNA_i^{Met}$ recognition of the first AUG start codon in a Kozak sequence context, trigger eIF5 to cause hydrolysis of the GTP bound by the eIF2 subunit. The eIF2 subunit thereby dissociates from the 48S initiation complex (Kozak, 1986). Activity of eIF2 is controlled by phosphorylation of the eIF2α subunit. Phosphorylation of the eIF2α subunit at Serine 51 prevents eIF2B-mediated recycling of eIF2 by GDP-GTP exchange, thus inhibiting formation of the ternary complex (Rowlands et al., 1988). Several pathways control eIF2α phosphorylation in response to physiological changes in the cell. For example, amino acid starvation causes Gcn2p activation and, subsequently, eIF2α phosphorylation (Harding et al., 2000; Wek et al., 1995).

Recruitment of the large ribosomal subunit to form a mature 80S ribosome for the elongation phase of translation requires another ribosome-activated hydrolysis step by the eIF1A-associated eIF5B (Lee et al., 2002; Pestova et al., 2000). Additionally, eIF5B interaction with 25S rRNA in the large ribosomal subunit is considered necessary for facilitating stringent AUG codon selection (Hiraishi et al., 2013). Notably, however, the start codon selected is not always the first AUG codon. This alternative to initiation at the first AUG codon is referred to as "leaky scanning" and occurs in mRNAs from several yeast genes (Slusher et al., 1991; Werner et al., 1987; Wolfe et al., 1994). In some mRNAs, initiation occurs both at AUG and at non-AUG codons, upstream of the coding

sequence. Initiation at these non-AUG codons generates upstream open reading frames (uORFs) with a potential regulatory function. Investigations of the presence of uORFs using ribosome occupancy studies show that ribosomes translate from upstream non-AUG codons during amino acid starvation (Ingolia et al., 2009).

1.2 Elongation during translation, and its regulation

The elongation phase involves transient binding of aminoacyl-tRNAs onto the mRNA in the 80S ribosome, resulting in formation of a polypeptide. This process is repeated with different species of aminoacyl-tRNA binding to the corresponding codons found on the mRNA as it passes through the ribosome. Elongation proceeds until the ribosome reaches a stop codon, triggering release of the polypeptide (**Figure 1**).

Elongation begins with recruitment of a complex consisting of the GTP-bound eEF1A and aminoacyl-tRNA to the 80S ribosome. GTP hydrolysis occurs when the aminoacyl-tRNA is accommodated in the A-site codon of the ribosome, causing dissociation of eEF1A-GDP from the tRNA and ribosome. The eEF1A-GDP sub-complex is then recycled by eEF1B to form eEF1A-GTP. Studies show that eEF1A is phosphorylated by several different kinases. These phosphorylations may alter eEF1A processivity in response to changes in cell physiology, and may upregulate or downregulate translation (Chang and Traugh, 1998; Eckhardt et al., 2007; Fan et al., 2010; Janssen et al., 1991; Lamberti et al., 2007; Lin et al., 2010; Mulner-Lorillon et al., 1994; Peters et al., 1995; Sheu and Traugh, 1999; Sivan et al., 2011; Venema et al., 1991).

Once the aminoacylated-tRNA is in the A-site, peptide bond formation occurs at the peptidyl transferase center (PTC) with the aminoacylated-tRNA in the P-site. The PTC consists mainly of ribosomal RNA located in the large subunit and is highly conserved between prokaryotes and eukaryotes (Rodnina and Wintermeyer, 2009). Immediately after peptide bond formation, the tRNAs in the P- and A-sites enter a hybrid P/E and A/P state in which the acceptor stem of the P-site tRNA shifts into the E-site and the A-site tRNA shifts into the P-site. The codon-anticodon base-pairing at the P- and A-sites remains unchanged (Rodnina and Wintermeyer, 2009). A complete shift of the tRNAs inside the ribosome requires the elongation factor eEF2 in a process called translocation.

Hydrolysis of GTP by eEF2 “unlocks” the ribosome from the mRNA and tRNAs, which shifts the tRNAs in the hybrid P/E and A/P states into the E- and P-sites and releases eEF2 from the ribosome (Rodnina and Wintermeyer, 2009). Like eEF1A, eEF2 is also phosphorylated with changes in cell physiology. In mammals, inhibitory phosphorylation of eEF2 occurs when the eEF2 kinase (eEF2K) is activated. Activation of eEF2K requires interaction of

eEF2K with the Ca^{2+}-ion-binding Calmodulin protein (Browne and Proud, 2002; Ryazanov et al., 1988). Phosphorylation of eEF2 kinase also decreases its activity. These phosphorylations are achieved by a plethora of kinases such as mTORC1, PKA and MAP kinases (Knebel et al., 2001; Redpath and Proud, 1993; Smith and Proud, 2008; Wang et al., 2001).

After translocation, a deacylated tRNA resides in the ribosome E-site and the A-site is ready to bind a new aminoacyl-tRNA-eEF1A complex. Researchers presume that entry of a new aminoacyl-tRNA-eEF1A complex into the A-site allows release of the deacylated tRNA from the E-site. However, this process is not fully understood. Some studies argue that binding of an aminoacyl-tRNA to the A-site is not coupled to release of the deacylated tRNA from the E-site (Dever and Green, 2012).

Yeast have an extra elongation factor, in addition to eEF1A and eEF2, called eEF3. Yeast eEF1A and eEF2 are sufficient for maintaining elongation in mammals, but mammalian eEF1A and eEF2 are unable to perform elongation in yeast without eEF3 (Skogerson and Engelhardt, 1977). Yeast eEF3 is an ATPase that presumably aids the release of the deacylated tRNA from the ribosomal E-site, but this function of eEF3 is not certain (Andersen et al., 2006; Kurata et al., 2013; Triana-Alonso et al., 1995).

1.3 Translation termination and ribosome recycling

When any stop codon (UAA, UGA, or UAG) enters the ribosomal A-site, it is recognized by eRF1 which is bound, in a complex, to eRF3 and GTP (**Figure 1**). Hydrolysis of GTP by eRF3 triggers the middle domain of eRF1 to terminate translation by protruding into the PTC and consequently releasing the polypeptide chain (Dever and Green, 2012; Jackson et al., 2012). At this stage the ribosome has two fates: (1) it dissociates from both the mRNA and the deacylated tRNA in the P-site, or (2) its 40S ribosomal subunit undergoes reinitiation.

The process of reinitiation could allow the ribosome to continue translation on an mRNA with two ORFs or it could allow re-translation of the mRNA by scanning of the 3' UTR and subsequently repositioning of the 40S subunit onto the 5' UTR for another cycle of translation (Hinnebusch and Lorsch, 2012; Kozak, 1984). Reinitiation may be aided by the interaction of eIF4G and PABP in the closed loop complex (Dever and Green, 2012).

Ribosome recycling is mediated by the ATP-dependent ATP-Binding Cassette, Sub-Family E Member 1 (ABCE1) protein (Rli1p in yeast). The ABCE1 protein facilitates release of eRF1, the 60S large ribosomal subunit, the 40S small ribosomal subunit bound to mRNA, and the deacylated tRNA (Barthelme et

al., 2011; Pisarev et al., 2010). Separation of the 40S subunit from the mRNA and the deacylated tRNA is presumed to be enhanced by the translation initiation factors eIF2D, eIF1, eIF1A, and the j-subunit of eIF3 (Dmitriev et al., 2010; Pisarev et al., 2007; Skabkin et al., 2010).

2 Transfer RNA

Transfer RNAs (tRNAs) are small RNA molecules under 100 nucleotides in length. They are often depicted two-dimensionally (**Figure 2A**) in the cloverleaf form rather than three dimensionally (**Figure 2B**). Base-pairing within the tRNA results in four stems: the cytosine-cytosine-adenosine tail (CCA-tail) and the three non-base-pairing loops: the D-loop, the Anticodon-loop and the TΨC-loop.

During translation, tRNAs decode the genetic information in mRNA to generate polypeptides. In total, 64 codons exist, 61 of which are sense codons that code for an amino acid, and 3 of which are stop codons. Eukaryotes always have less tRNA species than sense codons. For example, the yeast *S. cerevisiae* has 42 different tRNA species whereas chimpanzees have 55 (Goodenbour and Pan, 2006). The base in the wobble position (position 34) of the tRNA anti-codon varies, and therefore can pair with more than one unique base at the third position of the codon in mRNA (Crick, 1966). This wobble position enables 42 tRNAs to decode 61 codons.

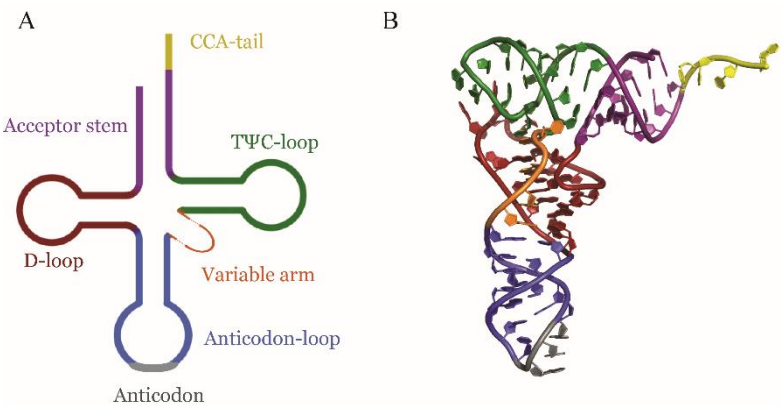

Figure 2. Transfer RNA (tRNA) depicted two-dimensionally (A) and three-dimensionally from crystal structure data on tRNA -phenylalanine (Shi and Moore, 2000) (B). The colours in Figure (A) match the colours in Figure (B). The colours of the descriptive text match the depicted structures in Figures (A) and (B).

RNA polymerase III (PolIII) transcribes tRNA genes. In yeast growing exponentially under optimal conditions, PolIII transcribes ~2-4 transcripts per tRNA gene each second, which ultimately amounts to transcription of ~3-

6 million tRNAs per cell cycle. This transcription rate is comparable to that of 35S rRNA which is transcribed by RNA polymerase I (PolI) at a rate of ~0.5 transcripts per gene each second (Cabart et al., 2008; Warner, 1999). The tRNAs also undergo heavy post-transcriptional processing and nucleoside modifications before they participate in translation (Phizicky and Hopper, 2010).

2.1 *Saccharomyces cerevisiae* tRNA modifications and tRNA modification enzymes

Posttranscriptional modifications always occur on tRNAs (**Figure 3**), but the extent and type of modification depends on the tRNA species and the host organism. Moreover, across all three domains of life, there are 92 different tRNA modifications and the average tRNA has at least 8 modified positions (Phizicky and Alfonzo, 2010; Phizicky and Hopper, 2010). Eukaryotes demonstrate a total of 50 different modifications, 25 of which are exhibited in *Saccharomyces cerevisiae*.

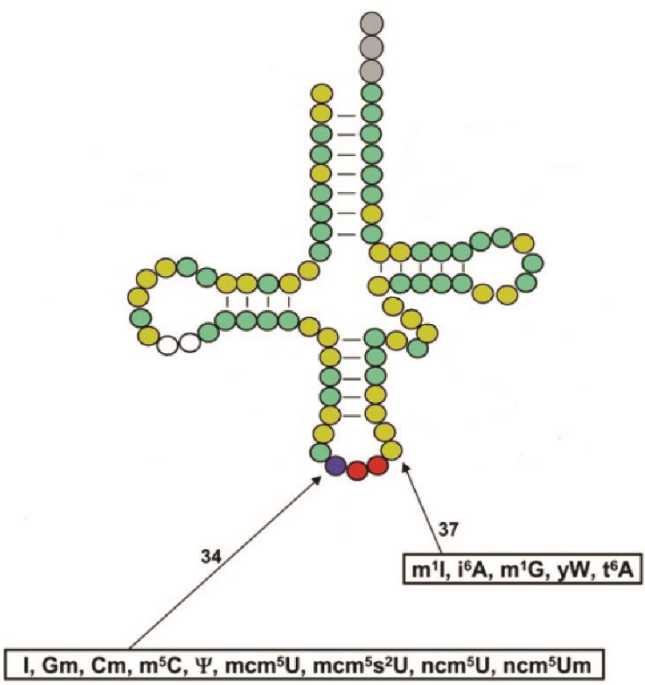

Figure 3. Modified tRNA residues in yeast. Green circles represent unmodified residues. Yellow circles represent residues that are modified in some or all tRNA species. White circles represent residues that are present and modified in some tRNA species. Red and blue circles represent the anticodon, with the wobble position in blue. Grey circles represent the CCA-tail. Adapted from (Phizicky and Hopper, 2010).

Scientists believe that posttranscriptional tRNA modifications evolved either to support translational efficiency, to increase tRNA stability, or as a means of distinguishing tRNA identity. The positions around the anticodon loop, such as positions 34 (the wobble position) or 37 are frequently modified. Loss of these modifications often leads to inviability or severe growth defects in yeast. For example, loss-of-function mutations in the *TAD2* or *TAD3* genes result in the absence of I_{34} which is lethal (Gerber and Keller, 1999). Furthermore, deletion of any of the *TRM5*, *SUA5*, *DEG1*, *TRM7*, *MOD5* or *ELP1-6* genes encoding gene products required for formation of m^1G_{37}, t^6A, $\Psi_{38,\,39}$, Nm_{34}, i^6A_{37} and ncm^5U, ncm^5Um, mcm^5U, mcm^5s^2U (see list of abbreviations), respectively, results in growth defects due to reduced translational efficiency or fidelity (Bjork et al., 2001; Dihanich et al., 1987; El Yacoubi et al., 2011; Huang et al., 2005; Johansson et al., 2008; Laten et al., 1978; Lecointe et al., 1998; Pintard et al., 2002).

The function of tRNA base modifications outside of the anticodon loop differs from the observed function of modifications of bases found within the anticodon region. The former tRNA base modifications are generally important for tRNA stability, and their absence is not as detrimental as the absence of modifications to bases within the anticodon region (Phizicky and Alfonzo, 2010; Phizicky and Hopper, 2010). Studies suggest that the presence or absence of these tRNA body modifications determines tRNA longevity. For example, deletion of the *TRM8* and *TRM4* genes abolishes the m^7G_{46} and m^5C_{49} modifications which leads to degradation of $tRNA^{Val(AAC)}$ through the rapid tRNA decay (RTD) pathway (Alexandrov et al., 2006; Chernyakov et al., 2008). Moreover, specific tRNA modifications or edits act as tRNA identifiers, e.g. G_{-1}, which makes $tRNA^{His}$ identifiable to the Histidyl-tRNA synthetase, and the loss of $Ar(p)_{64}$ due to *RIT1* gene deletion, which differentiates $tRNA_i^{Met}$ from $tRNA_e^{Met}$ during eIF2 recognition (Astrom and Bystrom, 1994; Astrom et al., 1993; Forster et al., 1993; Nameki et al., 1995).

2.2 Formation of the ncm^5U, mcm^5U and mcm^5s^2U tRNA wobble nucleosides in *Saccharomyces cerevisiae*

Saccharomyces cerevisiae has 42 cytoplasmic tRNA species, 13 of which have a uridine at the wobble position (U_{34}) (Percudani et al., 1997). One of the 13 wobble uridines has no modification, another is Pseudouridine (Szweykowska-Kulinska et al., 1994), and the remaining 11 tRNA species have either the ncm^5U, ncm^5Um, mcm^5U or mcm^5s^2U modified wobble uridine nucleoside (**Figure 4**) (Glasser et al., 1992; Johansson et al., 2008; Keith et al., 1990; Kobayashi et al., 1974; Kuntzel et al., 1975; Lu et al., 2005; Smith et al., 1973; Yamamoto et al., 1985).

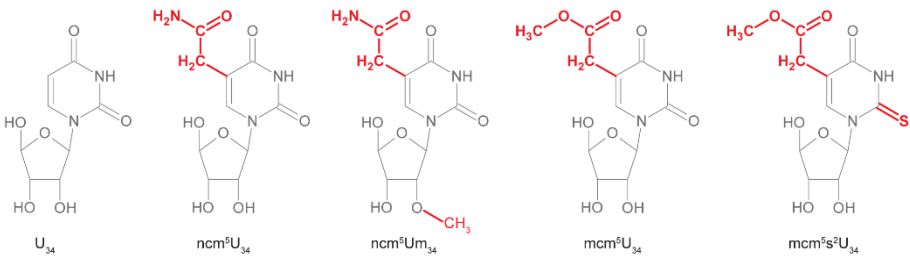

Figure 4. Wobble uridine modifications found in 11 out of 42 cytoplasmic tRNA species in *Saccharomyces cerevisiae*. Side-group modifications on uridine are labelled in red. Adapted from (Karlsborn et al., 2014b).

Formation of the ncm^{5}U and mcm^{5}U wobble uridine modifications requires at least 15 gene products. This number of gene products is unusual, as tRNA methylation modifications generally require only one or two gene products for formation (Johansson, 2005). A recent study reported that formation of t^6A_{37} in tRNA requires the KEOPS protein complex. The KEOPS complex consists of five protein subunits: Pcc1p, Pcc2p, Kae1, Bud32 and Cgi121 which, together with the Sua5p protein, bicarbonate, ATP and threonine, form t^6A_{37} (El Yacoubi et al., 2011; El Yacoubi et al., 2009; Perrochia et al., 2013; Srinivasan et al., 2011). Similarly, wybutosine (yW_{37}) formation on tRNA^{Phe} requires the genes *TRM5*, *TYW1*, *TYW2*, *TYW3*, and *TYW4* (Noma et al., 2006). Thus, formation of the ncm$^5U_{34}$, mcm$^5U_{34}$, t^6A_{37} and yW_{37} are quite unique in respect to the number of gene products required for their formation.

The first step in the formation of ncm^{5}U, ncm^{5}Um, mcm^{5}U and mcm^{5}s^{2}U, is most likely cm^{5}U generation (**Figure 5**). This step requires Elp1p-6p (the Elongator complex), Kti11p, Kti12p, Hrr25p, Sit4p, Sap185p, Sap190p and Kti13p. However, a *sap185Δ-sap190Δ* double mutant is required for total loss of ncm^{5}U, ncm^{5}Um, mcm^{5}U and mcm^{5}s^{2}U, and a *kti13Δ* strain only showed reduced levels of these modifications (Huang et al., 2005; Huang et al., 2008).

The next step, methyl-ester conversion of cm^{5}U to form mcm^{5}U, requires two additional gene products: Trm9p and Trm112p (Kalhor and Clarke, 2003; Mazauric et al., 2010). An enzyme responsible for ncm^{5}U formation (**Figure 5**) has yet to be discovered. However, methylation of the ncm^{5}U ribose to form ncm^{5}Um requires the enzyme Trm7p (**Figure 5**) (Pintard et al., 2002). Formation of the mcm^{5}s^{2}U nucleoside, which has a 2-thio-group on the uridine (s^2) in addition to the mcm-side group (mcm^5), requires 11 additional gene products (**Figure 5**) (Bjork et al., 2007; Dewez et al., 2008; Esberg et

al., 2006; Huang et al., 2005; Huang et al., 2008; Leidel et al., 2009; Nakai et al., 2008; Nakai et al., 2007; Nakai et al., 2004; Noma et al., 2009; Schlieker et al., 2008).

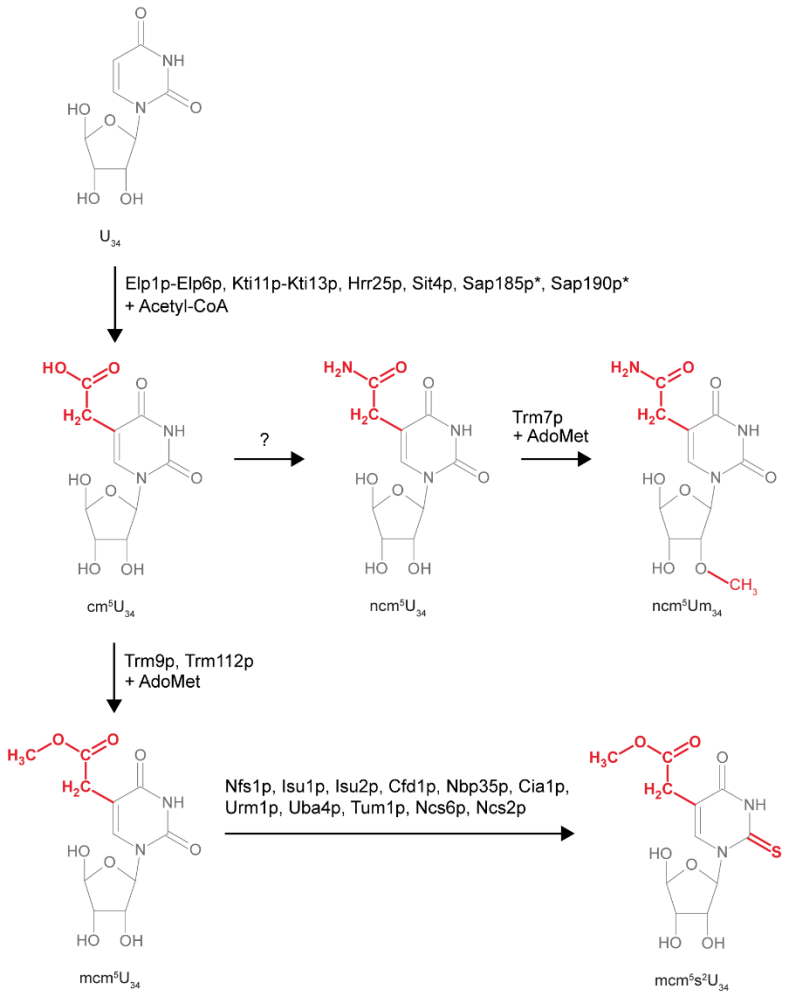

Figure 5. Gene products required for formation of the ncm^{5}U, ncm^{5}Um, mcm^{5}U and mcm^{5}s^{2}U wobble uridine nucleosides in tRNA. Formation of cm^{5}U requires Acetyl-CoA, and the methylation reactions by Trm7p, Trm9p and Trm112p require S-Adenosyl-methionine (AdoMet). (*) Ablation of both Sap185p and Sap190p is required for loss of wobble uridine modifications. (?) No enzyme has been discovered that convert cm^{5}U into ncm^{5}U.

Many of the genes involved in formation of the 2-thio group on tRNA wobble uridines are part of a sulfur relay system which provides sulfur for iron-sulfur cluster proteins that carry out radical reactions (Nakai et al., 2007; Nakai et al., 2004) The last step leading to 2-thio group formation on wobble uridines is catalyzed by Ncs2p and Ncs6p, with Urm1p as the sulfur carrier (Leidel et al., 2009). Thus, the mcm^5s^2U modified wobble nucleoside requires a total of at least 26 gene products for its formation, demonstrating the complexity of mcm^5s^2U wobble nucleoside formation.

2.2.1 Function of the tRNA wobble uridine ncm^5-, mcm^5- and s^2-side chains during mRNA decoding

S.cerevisiae has 13 tRNA species with a uridine in the wobble position. Of these 13 tRNA species, 11 are modified with ncm^5U, ncm^5Um, mcm^5U or mcm^5s^2U at the wobble position (**Figure 4**) (Glasser et al., 1992; Johansson et al., 2008; Keith et al., 1990; Kobayashi et al., 1974; Kuntzel et al., 1975; Lu et al., 2005; Smith et al., 1973; Yamamoto et al., 1985).

The contribution of modified wobble nucleosides to mRNA decoding was investigated *in vivo* by observing formation of the prokaryotic counterparts to the mcm^5 and s^2-side chains, mnm^5 and s^2, in mutants. The presence of these prokaryotic side chains on the wobble uridine in tRNAs facilitate decoding of both A- and G-ending codons (Kruger et al., 1998). An *in vitro* study investigating decoding by tRNAs having the mnm^5- and s^2-side chains on wobble uridine aligns with the *in vivo* study described above. According to the *in vitro* study, a crystal structure of $tRNA^{Lys}_{mnm^5s^2UUU}$ bound to the 30S small ribosomal subunit suggests that the modified wobble nucleoside facilitates decoding of both A- and G-ending codons (Murphy et al., 2004). Moreover, the presence of the eukaryotic mcm^5s^2U modification affects decoding by $tRNA^{Lys}$ in a manner similar to the presence of the prokaryotic mnm^5s^2U modification (Sundaram et al., 2000). The $tRNA^{Lys}_{mcm^5s^2UUU}$ is unique as poor stacking results in a more flexible anticodon loop than observed in other tRNAs.

Therefore, scientists have suggested that the presence of modifications in the anticodon loop result in stabilization by sterically hindering conformational changes (Durant et al., 2005). A study of *S. cerevisiae* investigated the decoding capacity of tRNAs with wobble uridine modifications. This *in vivo* study monitored growth of strains with specific tRNA gene deletions combined with loss-of-function mutations in genes required for formation of the mcm^5- ncm^5- or s^2-side chain groups on wobble uridines. The results showed that the mcm^5 and ncm^5 side chains promote decoding of G-ending codons. Moreover, the study found that the occurrence of both the mcm^5- and

s^2-side chains promote decoding of both A- and G-ending codons (Johansson et al., 2008).

2.2.2 The contribution of the ncm^5U, mcm^5U and mcm^5s^2U tRNA wobble nucleosides in maintaining translation efficiency

Efficient, error-proof decoding of the genetic code is crucial for maintaining appropriate gene expression. Section 1 above discusses global cellular approaches to altering mRNA translation. The current section focuses instead on alterations in translation efficiency due to loss of the ncm^5U, mcm^5U and mcm^5s^2U modifications at the wobble position in tRNA.

Strains with mutations that cause loss of the modified ncm^5U, mcm^5U and mcm^5s^2U wobble nucleosides in tRNA (such as *elp3Δ* strains) show pleiotropic phenotypes and grow more slowly than wild-type strains. As previously described, formation of the ncm^5U and mcm^5U modifications requires 15 gene products, and formation of mcm^5s^2U requires at least 26 gene products. The first step in formation of these nucleosides is most likely cm^5U, which requires Elp1p-6p (the Elongator complex), Kti11p, Kti12p, Hrr25p, Sit4p, Sap185p, Sap190p and Kti13p (Bjork et al., 2007; Dewez et al., 2008; Esberg et al., 2006; Huang et al., 2005; Huang et al., 2008; Kalhor and Clarke, 2003; Leidel et al., 2009; Mazauric et al., 2010; Nakai et al., 2008; Nakai et al., 2007; Nakai et al., 2004; Noma et al., 2009; Schlieker et al., 2008).

In *S. cerevisiae*, *elp3Δ* strains were initially used to investigate the link between altered translation efficiency and pleiotropic phenotypes resulting from loss of the modified ncm^5U, mcm^5U and mcm^5s^2U wobble nucleosides in tRNA. The investigation revealed that overexpression of hypomodified $tRNA^{Lys}_{s^2UUU}$ and $tRNA^{Gln}_{s^2UUG}$, which have the mcm^5s^2U wobble modification in wild-type strains, partially suppressed all phenotypes tested except for the defect in formation of the ncm^5U, mcm^5U and mcm^5s^2U wobble nucleosides in tRNA (Esberg et al., 2006). This finding suggests that the pleiotropic phenotypes observed in this strain arise from an inability of the hypomodified tRNAs to function properly during decoding of lysine-AAA and glutamine-CAA codons.

Since this initial study, others demonstrated that elevated levels of various combinations of hypomodified $tRNA^{Lys}_{s^2UUU}$, $tRNA^{Gln}_{s^2UUG}$ and $tRNA^{Glu}_{s^2UUC}$ suppress additional phenotypes observed in *S. cerevisiae* strains deleted for genes encoding Elongator complex subunits (Chen et al., 2011; Nedialkova and Leidel, 2015; Tigano et al., 2015; Zinshteyn and Gilbert, 2013). In addition, a proteomics approach has investigated proteins that are reduced when the mcm^5- and s^2-side chains are lost on wobble uridines in tRNA. This

study found that mRNAs enriched in primarily AAA (Bauer et al., 2012) and CAA (Rezgui et al., 2013) codons result in reduced protein expression when mcm^5- and s^2-side chains are absent at wobble uridine. Further, the findings showed that the presence of the mcm^5s^2U modified wobble nucleoside in tRNA enhances codon-anticodon interaction of $tRNA^{Lys}_{mcm^5s^2UUU}$ in the ribosome A-site (Rezgui et al., 2013).

The advent of ribosomal profiling (Ingolia et al., 2009) allowed scientists to investigate the impact, on global translation, of the presence of mcm^5- and s^2-side chains at the wobble uridine position in tRNA. Ribosomal profiling studies found that the absence of the mcm^5s^2U wobble modification in tRNA resulted in more ribosomes pausing when the AAA, CAA or GAA codon is in the ribosomal A-site (Nedialkova and Leidel, 2015; Zinshteyn and Gilbert, 2013). A recent investigation of +1 ribosomal frameshifting was performed in various mutants characterized by the loss of the mcm^5- or s^2-side chains. The study revealed that an *elp3Δ* strain has increased +1 frameshifting at the: Lys-tRNA AAA and AAG codons; Glu-tRNA GAA and GAG codons; Arg-tRNA AGA and AGG; Gly-tRNA GGA and GGG codons; Val-tRNA GUG codons; and Ser-tRNA UCA codons. Moreover, the results showed that the presence of the mcm^5s^2U wobble modification in Lys-tRNA enhances entry to the ribosomal A-site and thereby prevents +1 frameshifting (Tukenmez et al., 2015). These results implicate that mRNA enrichment with AAA, CAA and GAA codons in particular would negatively alter translation efficiency and, therefore, protein expression in strains lacking wobble uridine modifications. For this reason, in Paper III, my colleagues and I investigated the effects of CAA-codon enrichment in the *IXR1* gene using *elp3Δ* strains.

3 The Elongator complex

The Elongator complex is a conserved protein complex comprising six subunits. All eukaryotes investigated to date possess Elongator complex orthologues, and a six-subunit complex has been purified from yeast, plants and humans (Chen et al., 2009a; Close et al., 2012; Cohen et al., 2015; Gkampeta et al., 2014; Hawkes et al., 2002; Mei et al., 2014; Nelissen et al., 2010; Nelissen et al., 2005; Okada et al., 2010; Simpson et al., 2009; Winkler et al., 2001; Xu et al., 2015b; Zhu et al., 2015). As in yeast, loss-of-function mutations in genes encoding Elongator subunits in mice, plants and humans result in reduced levels of the modified ncm^5U, mcm^5U and mcm^5s^2U wobble nucleosides in tRNA (Paper II)(Karlsborn et al., 2014a; Lin et al., 2013; Mehlgarten et al., 2010; Okada et al., 2010; Yoshida et al., 2015).

Structural studies using the *S. cerevisiae* Elongator complex showed that the Elp4p-Elp6p sub-complex forms a hexameric ring that binds two copies of the

Elp1p-Elp3p core complex (**Figure 6**) (Glatt et al., 2012; Lin et al., 2012). The Elongator complex subunits have several different motifs. Elp3p contains a histone acetyltransferase domain (HAT) and an iron-sulfur cluster (Paraskevopoulou et al., 2006; Selvadurai et al., 2014; Wittschieben et al., 2000; Wittschieben et al., 1999), Elp1p is a phosphoprotein (Jablonowski et al., 2004; Mehlgarten et al., 2009) and Elp4p-Elp6p have a RecA-like ATPase domain (Glatt et al., 2012; Lin et al., 2012).

The Elongator complex activity has been suggested to be regulated by proteolysis of the Elp1p subunit resulting in an N-terminal truncation (Fichtner et al., 2003). However, this has been shown to be a preparation artefact during cell-lysis (Xu et al., 2015a). Some archaeal organisms require only the Elp3p subunit (and no other subunits of the Elongator complex) to form cm^5U in tRNA. Eukaryotes, however, require 13 gene products for efficient cm^5U formation (Huang et al., 2005; Huang et al., 2008; Paraskevopoulou et al., 2006; Selvadurai et al., 2014).

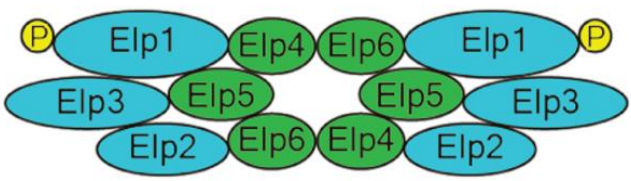

Figure 6. The Elongator complex with two copies of the core complex Elp1-3p (Turquoise) bound to the hexameric ring-shaped sub-complex consisting of Elp4-Elp6p (Green), according to crystal structure data (Glatt et al., 2012; Lin et al., 2012). Phosphorylation on Elp1p is indicated by (P) (Yellow).

3.1 The Elongator complex in *Saccharomyces cerevisiae*

Initially, scientists thought that the *S. cerevisiae* Elongator complex participated in RNA polymerase II (Pol II) transcription, since the Elongator core complex (Elp1p-Elp3p) subunits co-fractionated with the hyperphosphorylated C-terminal domain of Pol II (Otero et al., 1999a). Following this discovery a subcomplex consisting of Elp4p-6p was identified which interacts with the Elongator core complex (Elp1p-Elp3p) (Krogan and Greenblatt, 2001; Li et al., 2001; Winkler et al., 2001). The Elp3p subunit also demonstrates histone acetyltransferase activity that could, *in vitro*, transfer the acetyl-group from acetyl-CoA to histones (Wittschieben et al., 2000; Wittschieben et al., 1999). Consistent with the *in vitro* study, deletion of the *ELP3* gene resulted in reduced histone H3 and H4 acetylation (Winkler et al.,

2002). These studies suggested that the Elongator complex play an important role in Pol II elongation during transcription through histone acetylation.

However, a later study questioned the role of the Elongator complex in transcription elongation. Chromatin immunoprecipitation (ChIP) experiments provided no evidence of Elongator complex association with transcribing ORFs, and localization experiments found that the Elp1 and Elp3 proteins are localized in the cytosol (Pokholok et al., 2002). Another study proposed that Elp1p was involved in regulation of exocytosis in the cytosol through its interaction with Sec2p (Rahl et al., 2005). Sec2p is a guanine nucleotide exchange factor required for secretory vesicle transport that activates Sec4p (Salminen and Novick, 1987; Walch-Solimena et al., 1997).

Consistent with a cytosolic role for the Elongator complex, one study revealed deleting any gene encoding an Elongator complex subunit results in total loss of the modified ncm^{5}U, mcm^{5}U and mcm^{5}s^{2}U wobble nucleosides in tRNA (Huang et al., 2005). Another study further challenged the role of the Elongator complex in transcription and exocytosis. In that study, all phenotypes linked to loss of Elongator complex function were suppressed by elevated levels of hypomodified $tRNA^{Lys}_{s^2UUU}$ and $tRNA^{Gln}_{s^2UUG}$, except for the defect in formation of the ncm^{5}U, mcm^{5}U and mcm^{5}s^{2}U wobble nucleosides in tRNA (Esberg et al., 2006). Thus, the primary defect in Elongator mutants seem to be a translation defect that causes downstream effects in several cellular processes.

A more recent study investigated whether the only function of the Elongator complex was formation of tRNA wobble uridine modifications. The Elongator complex also interacted with proliferating cell nuclear antigen (PCNA), a protein associated with DNA replication and repair. The group hypothesized that such an interaction would explain the sensitivity to DNA damage agents and defects in telomeric gene silencing observed in Elongator mutants (Li et al., 2009). However, a later study demonstrated that the telomeric gene silencing defect and sensitivity to DNA damage agents in Elongator mutants were suppressed by elevated levels of hypomodified $tRNA^{Lys}_{s^2UUU}$, $tRNA^{Gln}_{s^2UUG}$ and $tRNA^{Glu}_{s^2UUC}$ (Chen et al., 2011). Moreover, in Paper III we show that inefficient translation of the CAA-codon enriched *IXR1* mRNA could in part explain the sensitivity of Elongator mutants to DNA damage agents.

Overall, these results indicate that the primary, if not the only, role of the Elongator complex in *S. cerevisiae* is formation of the ncm^{5}U, mcm^{5}U and mcm^{5}s^{2}U wobble nucleosides in tRNA. In addition, recent phenotypes discovered in *S. cerevisiae* Elongator mutants, such as mitochondrial defects at elevated temperature and defects in TOR signalling, can also be suppressed by elevated levels of various combinations of $tRNA^{Lys}_{s^2UUU}$, $tRNA^{Gln}_{s^2UUG}$ and

$tRNA^{Glu}_{s^2UUC}$ (Scheidt, 2014; Tigano et al., 2015). Given the multitude of phenotypes observed in Elongator mutants, my colleagues and I, in Paper I, investigated the extent of metabolic alterations observed in Elongator mutants and whether these metabolic alterations could be suppressed by elevated levels of hypomodified $tRNA^{Lys}_{s^2UUU}$, $tRNA^{Gln}_{s^2UUG}$ and $tRNA^{Glu}_{s^2UUC}$.

3.2 The Elongator complex associated proteins and their regulatory roles in *Saccharomyces cerevisiae*

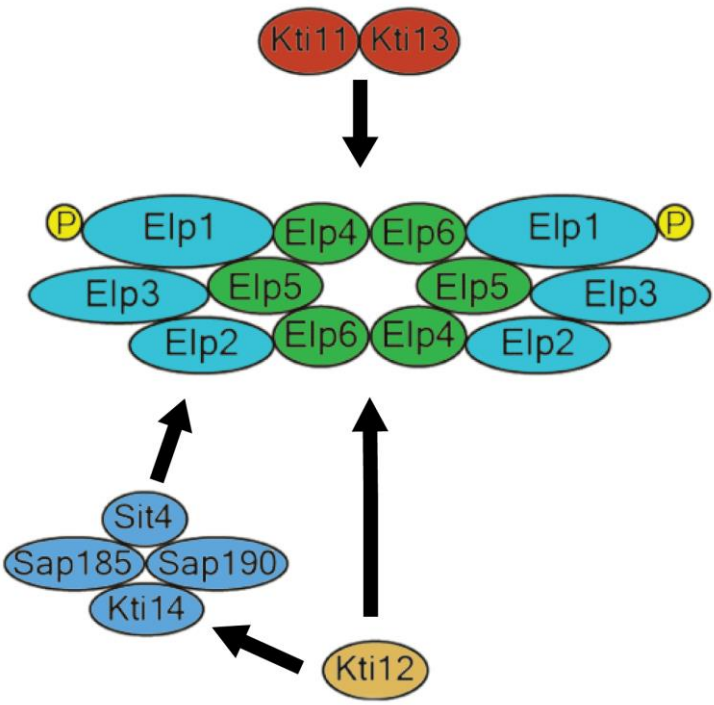

Figure 7. The Elongator complex with the Elp1p-3p core complex (Turquoise), and the Elp4-6p subcomplex (Green). Phosphorylation on Elp1p is indicated by the "P" (Yellow). Elongator complex associated proteins are arranged based on interaction studies (Gavin et al., 2002; Ho et al., 2002) and include: Sit4p, Sap185p, Sap190p, Kti14p (Hrr25p) portrayed in Blue. Kti12p porttrayed in Orange/Brown and Kti11p, Kti13p portrayed in Red.

3.2.1 The Sit4, Sap185, Sap190 and Hrr25 (Kti14) proteins in *Saccharomyces cerevisiae*

Sit4p belongs to a group of phosphatases called type 2A-related phosphatases. Sit4p was originally described to be involved in cell cycle progression from G1 phase to S phase (Sutton et al., 1991a; Sutton et al., 1991b). Later studies found that a group of proteins termed the SAPs associated with Sit4p, and these

SAPs were found to be crucial for Sit4p function (Luke et al., 1996). Another study discovered that Sit4p associated with the essential Tap42 protein which is involved in TOR-mediated signalling upon altered nutrient availability (Jiang and Broach, 1999).

A role for the Sit4p, Sap185p and Sap190p in mcm^5-side chain modifications of wobble uridines in tRNA was suggested by the discovery that loss of Sit4p or simultaneous loss of Sap185p and Sap190p gave *S.cerevisiae* resistance to the *Kluyveromyces lactis* Zymocin (Jablonowski et al., 2001). At this time it was not known that the γ-subunit of the Zymocin toxin targets tRNA and requires the mcm^5-side chain modification for proper targeting and cleavage of the tRNAs (Lu et al., 2005). With a mechanistic understanding of Zymocin function, another study could confirm that absence of the Sit4p resulted in loss of mcm^5U and mcm^5s^2U wobble modifications in tRNA (Huang et al., 2008).

Similarly to what was observed for loss of Sit4p function, loss of the Kti14p (Hrr25p) also resulted in resistance to Zymocin (Mehlgarten and Schaffrath, 2003). Hrr25p was initially described to be associated with DNA repair, but since its discovery this protein kinase has been suggested to be involved in additional cellular processes such as translation and secretory vesicle formation (DeMaggio et al., 1992; Hoekstra et al., 1991; Murakami et al., 1999). In addition, Hrr25p was later discovered to be crucial for formation of the mcm^5-side chain modifications on wobble uridines in tRNA (Huang et al., 2008). A regulatory role for Sit4p and its associated proteins Sap185 and Sap190 in formation of mcm^5-side chain modifications on wobble uridines was proposed upon discovery that resistance to Zymocin coincide with an altered phosphorylation pattern of the Elongator complex subunit Elp1p (Jablonowski et al., 2004).

Researchers observed that the kinase Hrr25p and the phosphatase Sit4p regulate Elongator activity in an antagonistic manner (Mehlgarten et al., 2009). In addition, a recent study has been able to identify at least two phosphorylation sites on Elp1p which are modified by Hrr25p (Abdel-Fattah et al., 2015). However, the question of why this antagonistic regulation exerted by Sit4p and Hrr25p takes place remains to be explained. No condition that would require altered Elongator complex activity to modulate formation of ncm^5- and mcm^5-side chain modifications on wobble uridines in tRNA has been identified to date.

3.2.2 The Kti12 protein in *Saccharomyces cerevisiae*

Like the *SIT4* and *HRR25* genes, the *KTI12* gene was discover by observing Zymocin resistance in *S. cerevisiae* strains disrupted for *KTI12* (Butler et al., 1994; Yajima et al., 1997). Interestingly, overexpression of the *KTI12* gene also

resulted in resistance to Zymocin (Butler et al., 1994). Furthermore, Kti12p interacts with the Elongator complex and this interaction is not essential for Elongator complex formation or stability (Fichtner et al., 2002; Petrakis et al., 2005). A role for Kti12p in formation of mcm^5-side chain modifications on wobble uridines in tRNA was established when it was observed that ablation of Kti12p results in loss of the mcm^5U and mcm^5s^2U modifications (Huang et al., 2008).

Little is known about the function of Kti12p in formation of modified wobble uridine nucleosides, but the presence of a P-loop motif suggests it has the ability to bind ATP or GTP (Fichtner et al., 2002). Yet, the effects of this P-loop on regulation of Elongator complex activity is unclear. Furthermore, Hrr25p binding to the Elongator complex depends on Kti12p (**Figure 6**) (Mehlgarten et al., 2009). Interestingly, the archaeal PSTK protein shares homology with yeast Kti12p at a region of the PSTK protein that is crucial for recognition of Ser-$tRNA^{Sec}$ in formation of Sep-$tRNA^{Sec}$ (Sherrer et al., 2008). So far no evidence has been presented implicating the Kti12p to interact with tRNA, but it has been suggested to interact with chromatin (Petrakis et al., 2005).

3.2.3 The Kti11 and Kti13 proteins in *Saccharomyces cerevisiae*

The *KTI11* (*DPH3*) and *KTI13* genes were initially identified by observing Zymocin resistance after deletion of these genes (Fichtner, 2002). In addition, loss of Kti11p results in total ablation of the ncm^5U, mcm^5U and mcm^5s^2U wobble modifications in tRNA, whereas loss of Kti13p results in severely reduced levels of these modified nucleosides (Huang et al., 2005; Huang et al., 2008). Kti11p has proven crucial for formation of the diphthamide modification on the translation factor eEF2 (Liu and Leppla, 2003).

The mechanistic role of Kti11p in both wobble uridine modification and diphthamide modification of eEF2 has been unknown until recently when it was suggested that it acts as an electron donor for the iron-sulfur clusters in Dph1p, Dph2p as well as Elp3p. This electron donor capacity of Kti11p resides in its ability to ligand an iron atom in its reduced state, which when oxidized leads to electron transfer (Dong et al., 2014; Glatt et al., 2015; Kolaj-Robin et al., 2015). One study suggests that the ability of Kti11p to undergo electron transfer is restricted by its interaction with Kti13p. Therefore, the Kti11p-Kti13p interaction may regulate the Kti11p electron transfer capability (Kolaj-Robin et al., 2015).

4 The Elongator complex in multicellular organisms

4.1 The Elongator complex in humans

The Elongator complex has several suggested roles in higher eukaryotes. The hELP1 protein (also named IKAP) in mammalian 293 cells is a proposed scaffolding protein in the IκB pathway. One study suggested that the role of hELP1 in the IκB pathway was to support assembly of the NIK-IKK kinase complex (Cohen et al., 1998). This complex is involved in pro-inflammatory cytokine signalling, thereby implying that hELP1 is required for cytokine signalling in mammalian cells (Cohen et al., 1998). The role of hELP1 in the IκB pathway was questioned when another study discovered that hELP1 is not associated with IKKs and has no role in cytokine-induced NF-Kappa B activation (Krappmann et al., 2000).

Another proposed role for hELP1 in mammalian cells eventually emerged. A two-hybrid screen demonstrated that hELP1 interacts with the c-Jun N-terminal kinase (JNK) (Holmberg et al., 2002). This interaction suggested that hELP1 is involved in regulating mammalian stress response by enhancing JNK activation (Holmberg et al., 2002). The hELP1 in mammalian cells also has a proposed role in histone acetylation. One study suggested that impaired transcription caused by inefficient histone acetylation due to loss of hELP1 resulted in reduced amounts of gene products involved in cell migration such as gelsolin, caveolin-1 and paxillin (Close et al., 2006). However, a later study could not repeat the aforementioned results (Johansen et al., 2008). Instead, this later study suggested that cell-migration defects in hELP1-depleted cells occurred due to reduced filamin A levels in membrane ruffles, thereby disturbing actin cytoskeleton organization (Johansen et al., 2008).

In addition to this contradictory study, another investigation suggested that microtubule destabilization by upregulation of the protein SCG10 (which destabilizes microtubules) in hELP1-depleted cells could also cause defective cytoskeleton organization in mammalian cells (Cheishvili et al., 2011). Yet another study suggested that hELP1 association with the JNK-pathway results in altered microtubule stabilization, implying that this association is required for functional cytoskeleton organization in mammalian cells (Abashidze et al., 2014).

Loss of the *hELP5* or *hELP6* gene reduces migration of melanoma cells (Close et al., 2012). Moreover, we showed that decreased levels of hELP1 in fibroblasts and brain tissue result in reduced levels of the modified mcm^5s^2U wobble nucleoside in tRNA (paper II,(Karlsborn et al., 2014a)). Thus, it is possible that in mammalian cells, as in yeast, loss of Elongator-complex-dependent wobble uridine modifications in tRNAs result in translation defects and consequently pleiotropic phenotypes.

4.2 The Elongator complex in the mouse *M. musculus*, the fruit fly *D. melanogaster*, the nematode *C. elegans* and the plant *A. thaliana*

The effects of depletion of Elongator complex subunits in multicellular organisms have been investigated in several model systems. Consistent with humans and yeast, loss-of-function mutations in genes encoding Elongator complex subunits in the mouse *M. musculus*, the nematode *C. elegans* and the plant *A. thaliana* result in loss of wobble uridine modifications (Chen et al., 2009a; Lin et al., 2013; Mehlgarten et al., 2010). In the plant *A. thaliana*, Elongator mutants display cell proliferation defects and altered abiotic stress responses (Nelissen et al., 2005; Zhou et al., 2009).

Homozygous *ikbkap*$^{-/-}$ (mouse *ELP1*) knockout mice die during embryonic development (Chen et al., 2009b). Conditional inactivation of *ikbkap* in mouse testes causes arrest of gamete formation by disrupting meiotic progression (Lin et al., 2013). Mouse zygotes depleted for the Elp1p, Elp3p or Elp4p homologues show a deficiency in paternal genome demethylation during embryogenesis (Okada et al., 2010). Furthermore, the Elp3p homologues in both mice and nematodes have been suggested to acetylate α-tubulin (Creppe et al., 2009; Solinger et al., 2010). However, such a function for the Elp3p homologue in nematodes was later dismissed as another study did not observe reduced α-tubulin acetylation upon loss of the Elp3p (Chen et al., 2009a). It was found that the MEC-17(ATAT1) and ATAT-2 proteins were the sole α-tubulin acetylases in *C.elegans* (Akella et al., 2010; Shida et al., 2010). Similarly, in mice Atat1 has been shown to be the major α-tubulin acetylase (Kim et al., 2013).

Furthermore, *C.elegans* Elongator mutants show translation defects and reduced levels of neuropeptides and acetylcholine in the synaptic cleft. These defects in Elongator mutants consequently cause reduced neuronal function as shown by a deficit in salt chemotaxis learning (Chen et al., 2009a). In the fruit fly *D.melanogaster,* ablation of the Elp3 protein leads to defects in larval- and neuro-development (Singh et al., 2010; Walker et al., 2011). It has been suggested that the *D.melanogaster* Elp3 protein acetylates Bruchpilot, a protein involved in neuronal differentiation (Miskiewicz et al., 2011). Thus, reduced Bruchpilot acetylation could possibly cause the defects observed upon ablation of Elp3 in *D.melanogaster*. Furthermore, loss of wobble uridine modifications in both nematodes and mice result in increased ribosomal pausing at codons requiring tRNAs with modified wobble uridines for decoding (Laguesse et al., 2015; Nedialkova and Leidel, 2015). Ribosomal pausing in mice may cause a defect in intermediate progenitor generation in the cerebral cortex, consequently resulting in microcephaly (Laguesse et al.,

2015). Thus, loss of modified wobble nucleosides is linked to neurological defects in both nematodes, mice and humans.

4.3 Human diseases caused by perturbations in Elongator complex function

Mutations in genes encoding Elongator complex subunits link the complex to several neurodegenerative diseases. Familial dysautonomia (FD) is caused by a recessive mutation in the *hELP1* (*IKBKAP*) that causes missplicing of the *hELP1* mRNA (Anderson et al., 2001; Slaugenhaupt et al., 2001). This missplicing causes skipping of exon 20 in the *hELP1* mRNA which results in reduced levels of the full length hELP1 protein (Dong et al., 2002). Moreover, the splicing defect appears tissue-dependent, with the most prominent missplicing and, consequently, reduction of hELP1 protein levels occurring in brain tissue (Cuajungco et al., 2003).

Reduced hELP1 protein levels result in a wide range of clinical features associated with defects in sensory and autonomic neurons (Axelrod, 2004; Norcliffe-Kaufmann et al., 2013). As mentioned previously, reduced hELP1 protein levels in fibroblasts and brain tissue from FD patients cause reduced levels of the mcm^{5}s^{2}U wobble modification in tRNA (paper II,(Karlsborn et al., 2014a)). However, no study has investigated whether reduced levels of modified wobble uridine modifications in tRNAs of FD patients are associated with ribosomal pausing at codons decoded by wobble uridine modified tRNAs. As increased ribosomal pausing occurs at these codons in yeast, nematodes and mice with depleted Elongator complex subunits, it is highly likely that cells from FD patients also display altered translation.

Altered expression or functionality of Elongator subunits other than hELP1 have also been implicated in several diseases. A missense variant of the *hELP2* gene was associated with intellectual disability (Cohen et al., 2015). Variants of the *hELP3* gene were associated with the progressive motor neuron disease amyotrophic lateral sclerosis (Simpson et al., 2009). The *hELP4* gene has been linked to the neurodevelopmental disorder rolandic epilepsy (Strug et al., 2009), but the role of hELP4 in this disorder is controversial (Reinthaler et al., 2014). Interestingly, studies performed using nematodes, mice or humans all point to an important role for the Elongator complex in maintaining normal neuronal function. Therefore, it is tempting to speculate that the neuronal defects observed are due to impaired translation caused by the absence of modified wobble uridines in tRNA.

5 "Omics" approaches to understanding defects in Elongator mutants

The advent of technologies that allow collection of data that describe global transcription, translation or metabolism makes it possible to get a "snapshot" of the state of these cellular activities. Such data could explain the extent and type of defects that occur within the cell with deletion of specific genes of interest.

A proteomics approach to investigating alterations in protein expression due to *ELP3* gene deletion in yeast revealed that genes enriched with either lysine AAA codons or glutamine CAA codons altered protein expression (Bauer et al., 2012; Rezgui et al., 2013). These results agree with ribosomal profiling studies which showed that ribosomal pausing occurs predominantly at the AAA and CAA codons in the absence of modified wobble uridine nucleosides (Nedialkova and Leidel, 2015; Zinshteyn and Gilbert, 2013). In *S. cerevisiae*, this increase in ribosomal pausing at AAA and CAA codons in *ncs2Δ* or *elp6Δ* strains was suppressed by overexpression of hypomodified $tRNA^{Lys}_{mcm^5/s^2UUU}$, $tRNA^{Gln}_{mcm^5/s^2UUG}$ and $tRNA^{Glu}_{mcm^5/s^2UUC}$. This finding offered a potential explanation for the suppressive effect of elevated levels of these tRNAs on all phenotypes tested in Elongator mutants (Nedialkova and Leidel, 2015).

One ribosomal profiling study discovered that Elongator mutants display accumulation of non-soluble protein aggregates. Surprisingly, after identifying the protein aggregates, there was no correlation between enrichment of specific codons and propensity for protein aggregation (Nedialkova and Leidel, 2015). Therefore, protein aggregation might be a secondary effect of impaired translation of certain proteins required for quality control processes for protein generation. Although ribosomal profiling studies provide evidence of altered translation in Elongator mutants, they also reveal how complex it is to determine which phenotypes arise directly from altered translation of specific mRNAs.

We performed metabolic profiling of a *S. cerevisiae elp3Δ* strain to explore the Elongator mutant metabolome. We observed alterations in levels of 36% or 46% of detected metabolites when strains were grown at 30°C and 34°C, respectively (Paper I). These results indicate major metabolic changes due to loss of wobble uridine modifications in tRNAs.

6 Metabolomics

Metabolism is the transformation of biologically-active organic molecules called metabolites. The rate of transformation of these metabolites provides a

readout of cell biochemical activity. Observing metabolism following deletion of a gene of interest provides valuable data on changes in biochemical activity associated with loss of the gene. These changes in biochemical activity might give significant insight into the function of the gene, or explain the phenotypes resulting from deletion of the gene.

Metabolomics uses a detection method to observe metabolism. This detection method largely depends on the range of metabolites of interest. Detection methods include various techniques, from mass spectrometry to nuclear magnetic resonance. The amount of biomass needed for metabolite detection using these devices is usually small, which therefore broadens the variety of samples that can be used in a metabolomics experiment. The ability to detect as many metabolites as possible is desirable in many studies. This approach, called untargeted metabolomics, provides an overview of cellular metabolism in the organisms being examined.
Untargeted metabolomics could create situations in which certain metabolites are detectable, and fragmentation spectra can be obtained from mass spectrometry. However, the metabolite represented by the fragmentation cannot be identified. This might be inconsequential for certain applications, such as diagnostic applications. Yet for other applications, such as explorative studies performed to describe gene function, the inability to identify metabolites is a major drawback. The following sub-sections focus on the use of untargeted metabolomics and statistical tools used to analyse the data generated.

6.1 Multivariate analysis methods in metabolomics

The fundamental aim of a metabolomics study is to distinguish different sample types by analysing all detectable metabolites, an approach called metabolic fingerprinting (Tyagi, 2010). The data sets generated in metabolomics studies are typically large and complex, and therefore require robust analysis methods to understand which specific metabolites discriminate the different sample types. Multivariate analysis methods have proven useful for metabolomics analysis and are now widely used in metabolic fingerprinting/profiling (Dettmer et al., 2007; Ellis and Goodacre, 2006). The two most popular multivariate analysis methods in metabolomics are principal component analysis (PCA) (Hotelling, 1933; Pearson, 1901) and partial least squares projection to latent structures (PLS) (Wold, 2001). Both methods aim to differentiate classes in highly complex data sets even if intra-class variability is present.

PCA is a multivariate projection method which is designed to extract and display the systematic variation in a data matrix. PCA is particularly valuable in studies where the number of observations (N) is smaller than the number

of variables (K). The data making up the variables can be made binary by converting “yes” or “no” into a numeric value. The data could also be more complex as in studies where the variable describes one metabolite and the concentration of this metabolite in a series of observations. This latter type of data sets is problematic when using traditional linear regression methods, as the data matrix used is not invertible (Johnstone and Titterington, 2009).

Here, I use a hypothetical dataset to display the features of PCA in metabolomics. This data set is much simpler than the data sets normally obtained from an untargeted metabolomics experiment. Normally, data pre-processing such as log2 (or binary) transformation, unit variance treatment, or mean-centering is required (Bijlsma et al., 2006; Katajamaa and Oresic, 2007). Also, data is often resampled for training and test datasets to test the reproducibility of the data analysis. Resampling is not shown in this hypothetical dataset. A thorough explanation of different parameters in the modelling is out of the scope of this thesis. Here the focus is mainly on presenting the procedures necessary to analyse metabolomics data, and different validation methods used to test the predictability of the models.

Differences in individual metabolite levels between the species are elucidated when the metabolite levels in the hypothetical dataset are compared (**Table 1**) However, defining whether the overall metabolism of compounds A-P in these species differ is cumbersome. Moreover, estimating how different the metabolism is between two groups of species is even more difficult. In order to answer these questions, we require a method that can compare all the variables within all the observations (triplicate samples from the species) with each other. Furthermore, we need an analysis output that can define the distance of separation between the observations for visual comparison.

Table 1. Hypothetical data set representing metabolite levels of a set of metabolites (A-P) in seven different species (sp_a - sp_g) sampled in triplicates.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
sp_a1	3	3	1	2	5	2	1	4	0	5	3	5	1	0	5	0
sp_a2	1	2	1	1	6	1	2	3	1	4	3	5	0	0	6	1
sp_a3	3	3	2	2	5	2	1	4	0	5	4	6	1	1	5	2
sp_b1	4	1	1	2	3	5	1	3	1	5	2	4	3	0	5	1
sp_b2	3	1	2	2	3	3	2	3	0	5	3	3	4	1	4	1
sp_b3	4	2	1	1	4	5	1	2	0	4	3	4	5	1	3	2
sp_c1	4	1	3	1	1	6	1	2	0	6	1	3	5	1	2	3
sp_c2	5	2	4	2	0	4	2	1	2	5	2	4	5	0	1	4
sp_c3	4	1	3	1	1	6	1	2	1	4	2	6	6	1	1	2
sp_d1	1	1	2	1	5	2	5	4	3	2	3	6	3	4	2	3
sp_d2	0	2	2	3	6	4	3	2	4	4	1	5	4	5	4	3
sp_d3	2	3	3	1	4	2	4	2	5	3	1	4	2	6	2	2
sp_e1	1	4	1	3	2	3	2	3	5	2	1	3	1	6	2	1
sp_e2	1	3	1	3	2	3	2	3	6	2	1	2	1	6	2	1
sp_e3	2	3	0	4	3	4	1	4	3	1	2	4	0	5	1	3
sp_f1	1	2	2	3	3	4	3	3	2	5	1	3	7	2	2	2
sp_f2	2	1	3	3	2	5	2	3	2	5	3	3	5	3	3	2
sp_f3	1	2	2	2	3	4	3	4	3	4	1	5	6	4	2	1
sp_g1	5	1	0	5	2	1	1	4	6	1	1	3	1	6	1	6
sp_g2	3	0	1	3	1	2	0	3	7	0	0	2	2	5	0	5
sp_g3	4	1	0	4	2	1	1	5	5	2	1	4	0	8	1	4

Data rows representing a specific species have been colour-coded for easier review of the data (some text is in red for better contrast against the background colour). Note that the colour-coding here aligns with the colours used in **Figure 8**.

A PCA model of the hypothetical dataset provides a visual output that represents an unbiased classification of the different species based on their metabolism of A-P. Notably, certain species seem to differ from each other in metabolism more than others, as displayed by the distance between sp_c, sp_g and sp_a (**Figure 8**). However, PCA is suboptimal at revealing any information regarding individual metabolite contributions to differences observed between the species. The PLS analysis method is required for extracting this information. PLS stands for projection to latent structures by means of partial least squares. It is a method for relating data matrix to each other by a linear multivariate model (Wold, 2001).

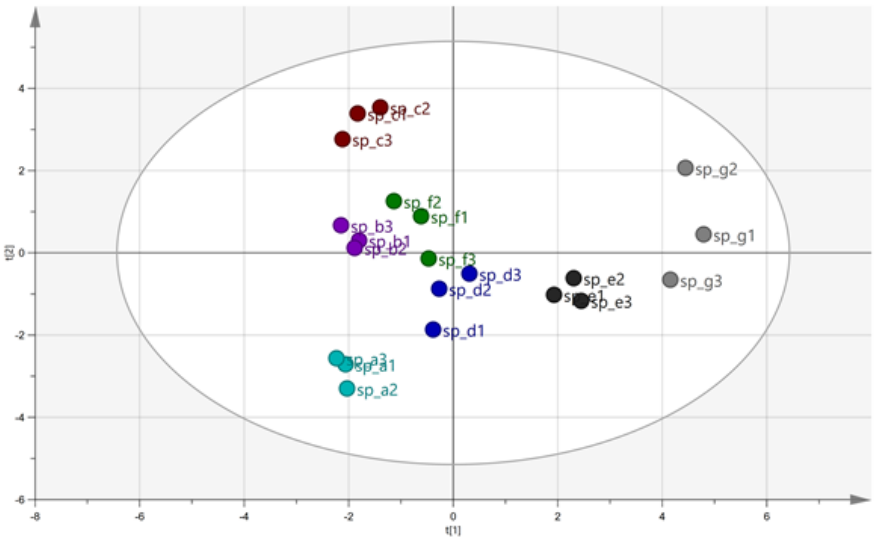

Figure 8. PCA-X performed using the data in **Table 1**. Component 1 t[1] explains most of the variation in metabolism between the species.

A PLS-Discriminant Analysis (PLS-DA) using only the sp_f and sp_d data allows us to investigate whether these two species differ in metabolism and, subsequently, identify the metabolites that differentiate them. The score scatter plot displays a good separation between these two species (**Figure 9**), indicating that they have distinguishable differences in metabolism. Several other validation methods should be used to further evaluate these differences. The loadings in this model need to be investigated to identify the metabolites differentiating these potentially different species (**Figure 10**).

The discriminatory power of a particular metabolite in the loading plot is determined by the distance of the metabolite X-variable to the projection (Y) (Wold, 2001). Observe that metabolites K and C have no discriminatory power in the model, whereas metabolites M and E have the highest discriminatory power (**Figure 10**). At this point in the analysis, investigating whether multicollinearity exists within the dataset would be beneficial. Multicollinearity is common in metabolomics, as a given pathway includes several chemical transformation steps that depend on each other, resulting in correlation between changes in levels of metabolites that share the same pathway (Bryan et al., 2008).

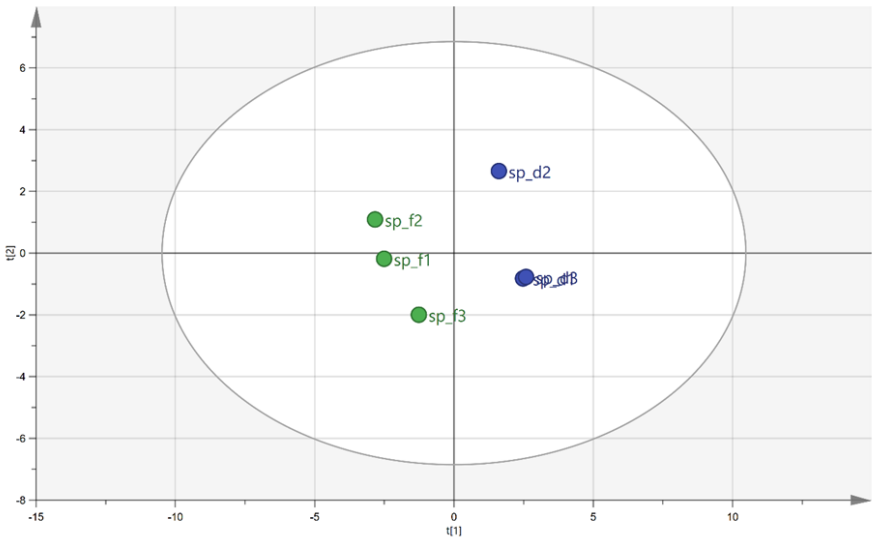

Figure 9. PLS-DA performed using the sp_f and sp_d data in **Table 1**. Component 1 t[1] explains most of the variation in metabolism between the two species.

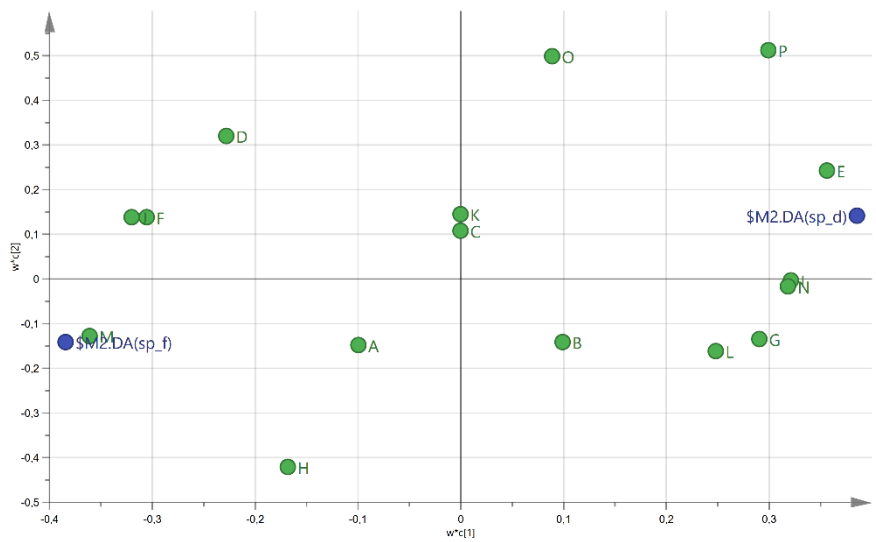

Figure 10. PLS-DA loading scatter plot. The sp_f and sp_d projections (Y) are labelled blue and the metabolites are labelled green.

The results from the PLS-DA loading scatter plot can also be portrayed on a plot showing the X-variation of specific metabolites relative to class identity. Observe that metabolite K, which is non-discriminatory, does not differ between the sp_f and sp_d samples (**Figure 11**). In addition, we can observe that metabolite M explains sp_f and metabolite E explains sp_d (**Figure 11**). Interestingly, these aforementioned metabolites are not the only metabolites that discriminate sp_f and sp_d.

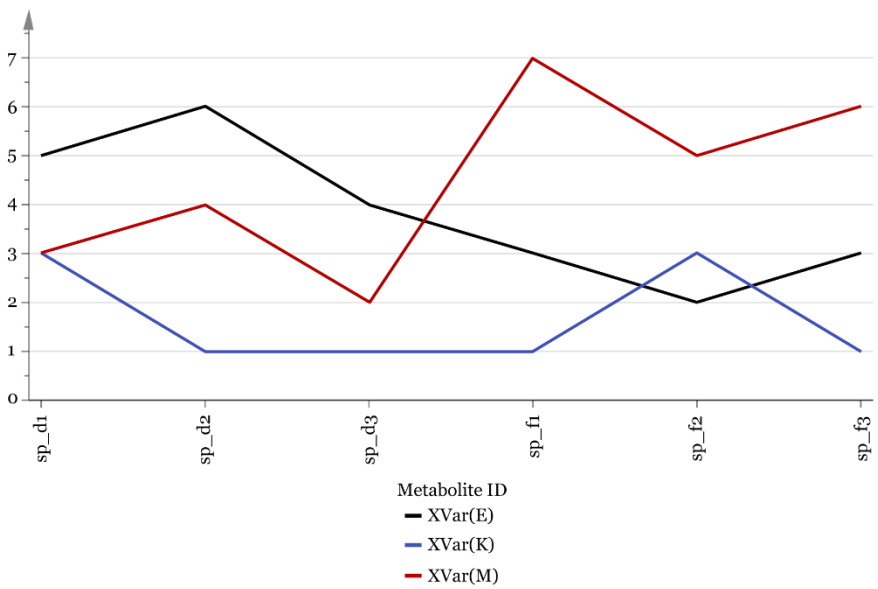

Figure 11. Variation of E (black), K (blue) and M (red) metabolite levels in sp_f and sp_d. The Y-axis describes the level of the specified metabolite (E,K or M) in a given sample (X-axis).

For this reason, it is often necessary to consult the Variable Importance in Projection score (VIP) of the PLS-DA model (**Figure 12**) (Wold, 2001). Generally, metabolites with a VIP score higher than 1 are considered important variables for discriminating between the classes in a PLS model (Bryan et al., 2008), This cut-off score depends on the type of data that is modelled. In addition, the error bar of the VIP value is also key when evaluating whether a particular metabolite is discriminatory (see metabolite P in **Figure 12**). The model can be optimized by discarding loadings with high error bars, as some measurement methods introduce noise in the data. However, this must be done with caution.

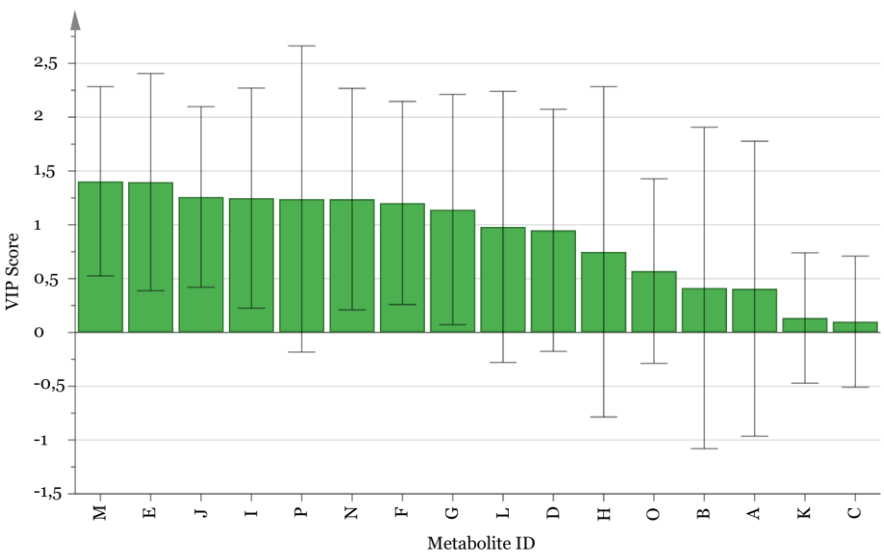

Figure 12. Variable Importance in Projection score (VIP) of the sp_f and sp_d PLS-DA model. Y-axis describes the VIP score and Metabolites (X-axis) are sorted according to the VIP score.

The R2 and Q2 values given for a PLS-DA model are good indicators of robustness. The R2 value indicates how well the model fits the data, while the Q2 value measures how well the model predicts new data. Although R2 and Q2 values are helpful for determining model robustness, use of one or more validation methods is advised for verifying the validity of the PLS-DA model obtained. The most common method used is a permutations test, which exchanges labels on variables to test whether the predictability of the model can be improved by random data alterations (Bijlsma et al., 2006). In addition, use of a machine learning algorithm such as random forest analysis to classify the observations is preferable (Chen et al., 2013).

Results, preliminary results and discussion

Paper I: Loss of ncm^5 and mcm^5 wobble uridine side chains results in an altered metabolic profile

The Elongator complex, composed of the six subunits Elp1p-Elp6p, is a conserved protein complex. Elongator complex orthologues exist in all eukaryotes tested so far (Chen et al., 2009a; Close et al., 2012; Cohen et al., 2015; Gkampeta et al., 2014; Hawkes et al., 2002; Mei et al., 2014; Nelissen et al., 2010; Nelissen et al., 2005; Okada et al., 2010; Simpson et al., 2009; Winkler et al., 2001; Xu et al., 2015b; Zhu et al., 2015). The primary role of the Elongator complex in eukaryotes is likely formation of the first-step intermediate cm^{5}U in formation of the modified nucleosides ncm^{5}U mcm^{5}U and mcm^{5}s^{2}U at the wobble position in tRNA (Esberg et al., 2006; Huang et al., 2005). Phenotypes linked to loss of Elongator complex function can be suppressed by overexpression of hypomodified $tRNA^{Lys}_{s^2UUU}$ and $tRNA^{Gln}_{s^2UUG}$ which, in wild-type *S. cerevisiae*, have the mcm^5- side chain modification (Esberg et al., 2006). These findings suggest that the primary defect in Elongator mutants is a translation defect.

Studies show that mRNAs enriched with either lysine AAA codons or glutamine CAA codons have altered protein expression (Bauer et al., 2012; Rezgui et al., 2013). The above results agree with two ribosomal profiling studies that demonstrated that ribosomal pausing occurs predominantly at the AAA and CAA codons in the absence of modified wobble uridine nucleosides (Nedialkova and Leidel, 2015; Zinshteyn and Gilbert, 2013). This increased ribosomal pausing at AAA and CAA codons in *ncs2Δ* or *elp6Δ* strains could be suppressed by overexpression of hypomodified $tRNA^{Lys}_{mcm^5/s^2UUU}$, $tRNA^{Gln}_{mcm^5/s^2UUG}$ and $tRNA^{Glu}_{mcm^5/s^2UUC}$, offering a possible explanation for the suppressive effect of elevated levels of these tRNAs on all phenotypes tested in Elongator mutants (Nedialkova and Leidel, 2015). Another ribosomal profiling study observed increased protein aggregation in the Elongator mutants. However, proteins that aggregated were not encoded by genes enriched in the AAA or CAA codons (Nedialkova and Leidel, 2015), suggesting that loss of Elongator complex function results in defects secondary to the translation defects observed.

Since many Elongator mutant phenotypes are poorly understood, we were interested in investigating perturbations in metabolism due to loss of wobble uridine modifications. We aimed to investigate metabolism by conducting untargeted GC-TOF-MS metabolic profiling of an *elp3Δ* strain strain carrying either an empty low copy *LEU2* vector, or the same vector containing the wild-

type *ELP3* gene. As Elongator mutants are temperature sensitive, we grew the prototrophic *elp3Δ* strain carrying the vectors at the permissive growth condition, 30°C, and the semi-permissive growth condition, 34°C.

Our results showed that growth of an *elp3Δ* strain at 30°C results in altered metabolism of 36% of all detected metabolites when compared to an *elp3Δ* strain containing the wild-type *ELP3* gene. We observed that metabolism of Glutamine, Beta-alanine, Ornithine and Lysine changed most significantly after loss of wobble uridine modifications when the *elp3Δ* strain was grown at 30°C. The same comparison between *elp3Δ* strains with and without the wild-type *ELP3* gene grown at 34°C exhibited altered metabolism of 46% of all metabolites detected in the latter strain. The metabolism of Lysine, Ornithine, Tyrosine and Lactic acid changed most significantly when the *elp3Δ* strain was grown at 34°C.

Thus, growth of the *elp3Δ* strain at 34°C resulted in more metabolic alterations than growth at 30°C. Further, the metabolites with altered levels from the *elp3Δ* strain grown at 34°C differed from those observed in the *elp3Δ* strain grown at 30°C. This difference may result from the effect of temperature on the extent of translation defects occurring due to loss of modified wobble uridines. Another possibility is that the elevated temperature could cause increased defects that are secondary to the translation defects in the *elp3Δ* strain.

Next we sought to investigate whether the metabolic alterations observed in the *elp3Δ* strain could be suppressed by overexpression of hypomodified $tRNA^{Lys}_{s^2UUU}$, $tRNA^{Gln}_{s^2UUG}$ and $tRNA^{Glu}_{s^2UUC}$. Our results revealed that overexpression of these aforementioned hypomodified tRNAs only partially suppress certain metabolic alterations when the *elp3Δ* strain is grown at 30°C or 34°C. The only metabolic alterations partially suppressed at both temperatures were Ornithine and Lysine, while a trend indicated that several other metabolic alterations were weakly suppressed. This result was surprising as overexpression of various combinations of these three hypomodified tRNAs suppress all Elongator mutants phenotypes tested to date (Bauer et al., 2012; Chen et al., 2011; Esberg et al., 2006; Fernandez-Vazquez et al., 2013; Nedialkova and Leidel, 2015; Tigano et al., 2015). Furthermore, the ribosomal pausing at lysine-AAA and glutamine-CAA codons in *ncs2Δ* or *elp6Δ* mutants, which lack the s^2- or the mcm^5- side chain respectively, is alleviated by overexpression of hypomodified $tRNA^{Lys}_{mcm^5/s^2UUU}$, $tRNA^{Gln}_{mcm^5/s^2UUG}$ and $tRNA^{Glu}_{mcm^5/s^2UUC}$ (Nedialkova and Leidel, 2015). Therefore, our results suggest that overexpression of hypomodified $tRNA^{Lys}_{s^2UUU}$, $tRNA^{Gln}_{s^2UUG}$ and $tRNA^{Glu}_{s^2UUC}$ in the *elp3Δ* strain suppress metabolic defects

crucial for cellular robustness. Furthermore, it does not restore metabolic alterations observed like complementation of the *elp3Δ* strain with the wild-type *ELP3* gene does. Likely explaining why overexpression of the aforementioned tRNAs in the *elp3Δ* strain does not result in complete suppression of phenotypes observed.

Preliminary results of interest for Paper I

In Paper I we observed widespread metabolic alterations in a strain lacking the *ELP3* gene. However, we did not analyse metabolism in other mutants with absent wobble uridine modifications. Thus, we performed untargeted metabolic profiling of additional strains in which genes required for formation of wobble uridine modifications were deleted. This profiling was performed to identify the metabolic alterations that are specifically associated with the loss of the modified wobble nucleosides in tRNA.

We show that mutants defective in formation of ncm^{5}U, mcm^{5}U and mcm^{5}s^{2}U modifications at the wobble position in tRNA portray a distinct metabolic profile (**Figure 13**). In addition, this distinct metabolic profile in the mutants is largely similar to the metabolic profile observed in the *elp3Δ* strain in Paper I. Random forest classification based on preliminary metabolic profiling data revealed that the *kti12Δ* strain can be misclassified as a wild-type or an Elongator mutant. After reanalyzing levels of modified wobble nucleosides in the *kti12Δ* strain, we observed trace amounts of the mcm^{5}s^{2}U nucleoside (**Figure 14**). Therefore, we speculate that these trace amounts of modified wobble nucleosides in tRNA might explain why a *kti12Δ* strain has a metabolic profile more similar to that of the wild-type and shows similar but weaker phenotypes than Elongator mutants.

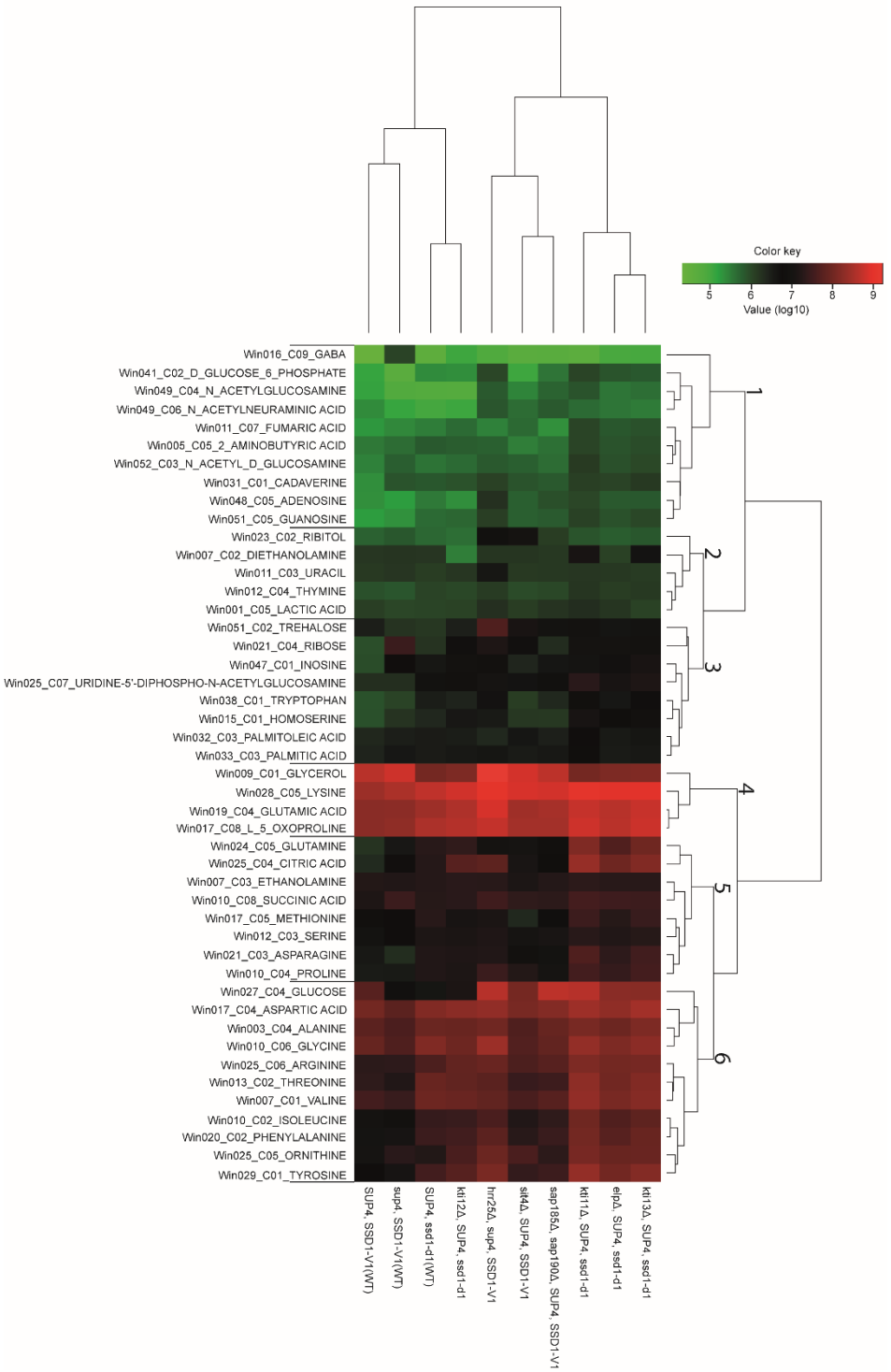
Color key
5
6
7
8
9
Value (log10)
Win016_C09_GABA
Win041_C02_D_GLUCOSE_6_PHOSPHATE
Win049_C04_N_ACETYLGLUCOSAMINE
Win049_C06_N_ACETYLNEURAMINIC ACID
Win011_C07_FUMARIC ACID
Win005_C05_2_AMINOBUTYRIC ACID
Win052_C03_N_ACETYL_D_GLUCOSAMINE
Win031_C01_CADAVERINE
Win048_C05_ADENOSINE
Win051_C05_GUANOSINE
Win023_C02_RIBITOL
Win007_C02_DIETHANOLAMINE
Win011_C03_URACIL
Win012_C04_THYMINE
Win001_C05_LACTIC ACID
Win051_C02_TREHALOSE
Win021_C04_RIBOSE
Win047_C01_INOSINE
Win025_C07_URIDINE-5'-DIPHOSPHO-N-ACETYLGLUCOSAMINE
Win038_C01_TRYPTOPHAN
Win015_C01_HOMOSERINE
Win032_C03_PALMITOLEIC ACID
Win033_C03_PALMITIC ACID
Win009_C01_GLYCEROL
Win028_C05_LYSINE
Win019_C04_GLUTAMIC ACID
Win017_C08_L_5_OXOPROLINE
Win024_C05_GLUTAMINE
Win025_C04_CITRIC ACID
Win007_C03_ETHANOLAMINE
Win010_C08_SUCCINIC ACID
Win017_C05_METHIONINE
Win012_C03_SERINE
Win021_C03_ASPARAGINE
Win010_C04_PROLINE
Win027_C04_GLUCOSE
Win017_C04_ASPARTIC ACID
Win003_C04_ALANINE
Win010_C06_GLYCINE
Win025_C06_ARGININE
Win013_C02_THREONINE
Win007_C01_VALINE
Win010_C02_ISOLEUCINE
Win020_C02_PHENYLALANINE
Win025_C05_ORNITHINE
Win029_C01_TYROSINE
1
2
3
4
5
6
SUP4, SSD1-V1(WT)
sup4, SSD1-V1(WT)
SUP4, ssd1-d1(WT)
Ktr12Δ, SUP4, ssd1-d1
hrr25Δ, sup4, SSD1-V1
slt4Δ, SUP4, SSD1-V1
sap185Δ, sap190Δ, SUP4, SSD1-V1
Ktr11Δ, SUP4, ssd1-d1
elp3Δ, SUP4, ssd1-d1
Ktr13Δ, SUP4, ssd1-d1

Figure 13. Mutants defective in formation of the modified ncm^{5}U, mcm^{5}U and mcm^{5}s^{2}U nucleosides at wobble position in tRNA show similar metabolic profiles. Strains (W303 strain background) were cultivated in YEPD media to ~0.5 OD_{600} units at 30°C before cell harvesting using an in-house-built filtration apparatus. Metabolites were extracted and quantified using GC-TOF-MS. Data was log10-transformed and processed in R-software using the heatmap.2 function in the gplots package. On the basis of metabolite abundance, 6 different subclusters were formed (denoted numerically on the dendrogram). The strains were clustered hierarchically based on their metabolic profile. The colour key signifies metabolite enrichment in red colour and metabolite reduction in green color. Random forest classification failed to discriminate between the different Elongator mutants, therefore the average metabolite levels were calculated from all the Elongator mutants when generating the heatmap, (see *elpΔ*, SUP4, ssd1-d1 in the Figure).

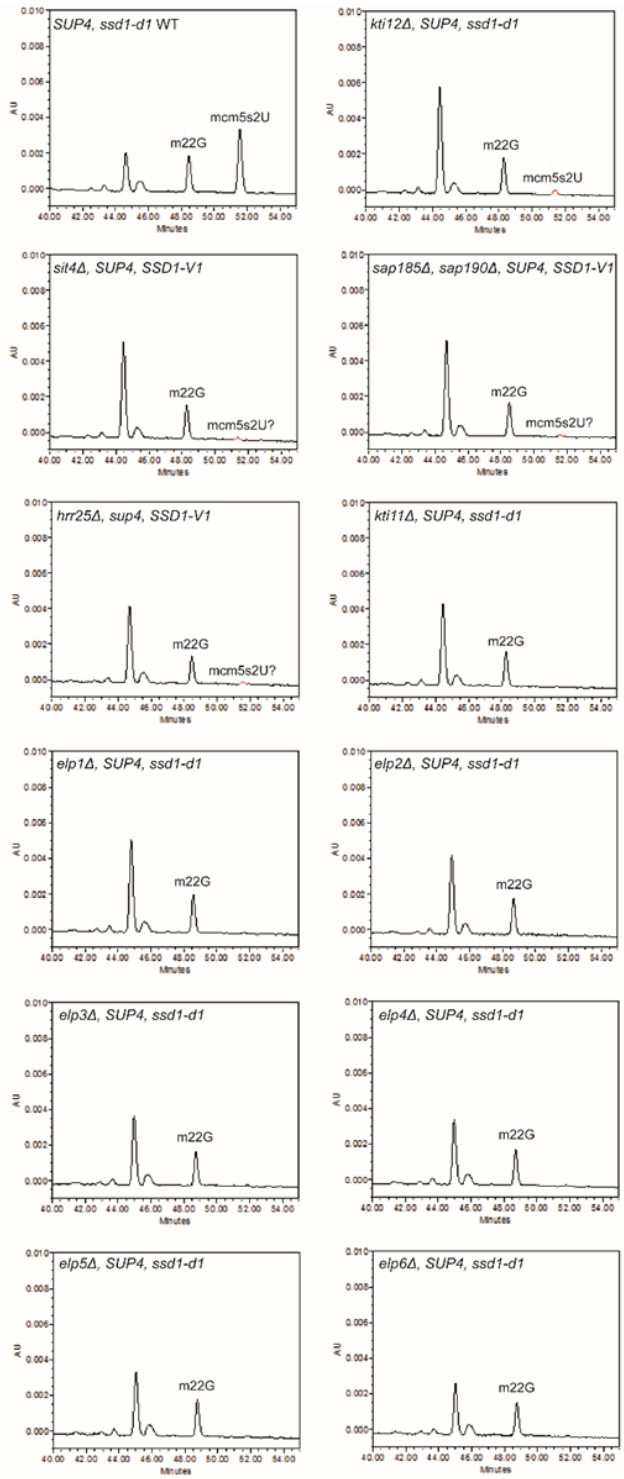
SUP4, ssd1-d1 WT
mcm5s2U
m22G
kti12Δ, SUP4, ssd1-d1
m22G
mcm5s2U
sit4Δ, SUP4, SSD1-V1
m22G
mcm5s2U?
sap185Δ, sap190Δ, SUP4, SSD1-V1
m22G
mcm5s2U?
hrr25Δ, sup4, SSD1-V1
m22G
mcm5s2U?
kti11Δ, SUP4, ssd1-d1
m22G
elp1Δ, SUP4, ssd1-d1
m22G
elp2Δ, SUP4, ssd1-d1
m22G
elp3Δ, SUP4, ssd1-d1
m22G
elp4Δ, SUP4, ssd1-d1
m22G
elp5Δ, SUP4, ssd1-d1
m22G
elp6Δ, SUP4, ssd1-d1
m22G
AU
Minutes

Figure 14. The *kti12Δ* mutant has residual amounts of the mcm^{5}s^{2}U modified wobble nucleoside. HPLC analysis of modified tRNA nucleosides in mutant and wild-type strains used for metabolic profiling. Chromatograms were monitored at 254 nm and cropped to display the peaks between the retention times 40 and 55 minutes. Abbreviations: (m^{2}2G) *N* ^{2},*N* ^{2}-dimethylguanosine; (mcm^{5}s^{2}U) 5-methoxycarbonylmethyl-2-thiouridine. A question mark (?) after mcm5s2U in the chromatograms denotes peaks with a retention time equal to mcm^{5}s^{2}U that could not be verifiably identified by analysis of peak spectra as mcm^{5}s^{2}U.

Paper II: Familial dysautonomia (FD) patients have reduced levels of the modified wobble nucleoside mcm^{5}s^{2}U in tRNA

The Elongator complex, composed of six subunits, is a conserved protein complex. Elongator complex orthologues exist in all eukaryotes tested for its presence so far, and a six-subunit complex has been purified from yeast, plants and humans (Chen et al., 2009a; Close et al., 2012; Cohen et al., 2015; Gkampeta et al., 2014; Hawkes et al., 2002; Mei et al., 2014; Nelissen et al., 2010; Nelissen et al., 2005; Okada et al., 2010; Simpson et al., 2009; Winkler et al., 2001; Xu et al., 2015b; Zhu et al., 2015). Familial dysautonomia (FD) is caused by a mutation in the human *ELP1* (*hELP1* or *IKBKAP*) gene that causes missplicing of the *hELP1* mRNA (Anderson et al., 2001; Slaugenhaupt et al., 2001). This missplicing causes skipping of exon 20 of the *hELP1* mRNA, resulting in reduced levels of the full length hELP1 (IKAP) protein (Dong et al., 2002). Reduced hELP1 protein levels result in a wide range of clinical features associated with defects in sensory and autonomic neurons in humans (Axelrod, 2004; Norcliffe-Kaufmann et al., 2013).

In yeast, the Elongator complex functions primarily in the formation of the modified ncm^{5}U, mcm^{5}U and mcm^{5}s^{2}U nucleosides at the wobble position in tRNAs (Esberg et al., 2006; Huang et al., 2005). Loss-of-function mutations in genes encoding subunits of the Elongator complex in the mouse *M. musculus*, the nematode *C. elegans*, and the plant *A. thaliana* result in the absence of wobble uridine modifications (Chen et al., 2009a; Lin et al., 2013; Mehlgarten et al., 2010). Despite the tantalizing evidence for Elongator complex involvement in formation of modified wobble nucleosides in multicellular organisms, no study has explored the possibility that FD patients have reduced levels of modified wobble nucleosides in tRNA due to reduction of hELP1 protein levels.

We quantified the levels of the mcm^{5}s^{2}U nucleoside in brain tissue and fibroblast cell lines derived from FD patients and healthy individuals using HPLC to investigate whether FD patients have reduced levels of the mcm^{5}s^{2}U nucleoside in tRNAs.

We observed that FD brain tissue has 65–71% of the mcm^{5}s^{2}U nucleoside levels observed in total tRNA extracted from non-FD brain tissue. Furthermore, FD fibroblasts had 64% of the mcm^{5}s^{2}U nucleoside levels observed in the non-FD fibroblasts. We also observed that the FD-derived fibroblasts had reduced hELP1 levels, consistent with results from previous studies. The mcm^{5}s^{2}U nucleoside levels observed in FD fibroblasts and brain tissue are similar to the reduced levels observed in studies where the yeast *KTI13* gene was deleted (Huang et al., 2008).

Deletion of the *KTI13* gene produced phenotypes that are similar to but weaker than those seen in Elongator mutants (Esberg et al., 2006; Zabel et al., 2008). Thus, partial loss of modified wobble nucleosides is sufficient to cause impairment of translation and therefore phenotypes. In addition, mouse homozygous *ikbkap*$^{-/-}$ knockouts are embryonic lethal (Chen et al., 2009b). Therefore, we did not expect a drastic reduction in levels of modified wobble nucleosides in FD patients. It is likely that impairment of translation due to reduced levels of modified wobble uridine nucleosides in tRNAs plays a decisive role in the neurodegenerative nature of FD. Moreover, studies show that reduced neuronal function is a consequence of impaired translation caused by the loss of modified wobble nucleosides in both nematodes and mice (Chen et al., 2009a; Laguesse et al., 2015; Nedialkova and Leidel, 2015).

Paper III: Elongator complex enhances Rnr1p levels in response to DNA damage by influencing Ixr1p expression

Loss of function mutations in any gene encoding an Elongator complex subunit results in loss of the modified wobble nucleosides ncm^{5}U, mcm^{5}U and mcm^{5}s^{2}U in tRNAs and consequently pleiotropic phenotypes (Karlsborn et al., 2014b). One of the many phenotypes described in Elongator mutants is sensitivity to DNA damage agents (Chen et al., 2011; Li et al., 2009). This phenotype was initially explained by an interaction between the DNA repair protein proliferating cell nuclear antigen (PCNA) and the Elongator complex (Li et al., 2009). However, this function of the Elongator complex was later questioned when overexpression of hypomodified $tRNA^{Lys}_{s^2UUU}$, $tRNA^{Gln}_{s^2UUG}$ and $tRNA^{Glu}_{s^2UUC}$, that normally have the mcm^5-side chain at wobble uridine in an wild type strain, suppressed the sensitivity of Elongator mutants to DNA damage agents (Chen et al., 2011). In addition, studies in yeast have shown that mRNAs enriched in codons decoded by tRNAs modified with mcm^{5}s^{2}U in a wild type strain display reduced protein expression in an *elp3Δ* strain lacking this modification (Bauer et al., 2012; Fernandez-Vazquez et al., 2013; Rezgui et al., 2013). These results suggest that decoding is primarily perturbed at lysine AAA, glutamine CAA and glutamic acid GAA codons in mRNAs upon

loss of tRNA wobble uridine modifications. Despite evidence suggesting that translation impairment in Elongator mutants possibly result in sensitivity to DNA damage, no molecular mechanism has been described that explain this phenotype.

Through analysis of genomic glutamine CAA codon enrichment in ORFs we discovered the *IXR1* gene. As deletion of either *IXR1* or *ELP3* results in sensitivity to DNA damaging agents (Chen et al., 2011; Li et al., 2009; Tsaponina et al., 2011), we investigated if reduced levels of Ixr1p could be observed in an *elp3Δ* strain. We observed that Ixr1p levels are reduced to 50% in an *elp3Δ* strain compared to the wild type strain. In addition, this Ixr1p reduction was partially suppressed by overexpression of hypomodified $tRNA^{Lys}_{s^2UUU}$, $tRNA^{Gln}_{s^2UUG}$ and $tRNA^{Glu}_{s^2UUC}$, indicating that translation of the *IXR1* mRNA might be impaired in an *elp3Δ* strain. The Ixr1p is important for induction of ribonucleotide reductase 1 (*RNR1*) upon DNA damage to increase dNTP production (Tsaponina et al., 2011). Therefore we investigated if the reduced levels of Ixr1p observed in the *elp3Δ* strain alters levels of Rnr1p in response to DNA damage. Our results reveal that Rnr1p expression is impaired in the *elp3Δ* strain in response to DNA damage. In addition, we show that the reduced Rnr1p levels in the *elp3Δ* strain are suppressed by overexpression of hypomodified $tRNA^{Lys}_{s^2UUU}$, $tRNA^{Gln}_{s^2UUG}$ and $tRNA^{Glu}_{s^2UUC}$. Overall, these results indicate that translational dysfunction upon loss of modified wobble nucleosides in tRNAs alter the DNA damage response in an *elp3Δ* strain. Thus, the observed sensitivity of Elongator mutants to DNA damage agents could in part be a consequence of reduced Ixr1p expression and therefore inadequate Rnr1p levels during DNA damage.

It has been suggested that impaired translation of the *RNR1* mRNA is the cause for the sensitivity to DNA damage agents observed in a *trm9Δ* strain which lack the mcm^5-side group on wobble uridines in tRNA (Begley et al., 2007; Patil et al., 2012). However, we did not observe altered levels of Rnr1p in a *trm9Δ* strain under normal growth conditions, or defects in Rnr1p induction in response to the DNA damaging agent methyl methanesulfonate (MMS). Furthermore, we observed that Rnr1p levels in *ixr1Δ* and *elp3Δ ixr1Δ* and *trm9Δ ixr1Δ* strains are reduced compared to the wild-type strain under normal growth conditions. In addition, we show that no Rnr1p induction is observed after MMS treatment of these mutant strains. Overexpression of hypomodified $tRNA^{Lys}_{s^2UUU}$, $tRNA^{Gln}_{s^2UUG}$ and $tRNA^{Glu}_{s^2UUC}$ did not improve the Rnr1p induction defect in the *ixr1Δ*, *elp3Δ ixr1Δ* or *trm9Δ ixr1Δ* strains. Therefore, we argued that the reduced Rnr1p levels in response to the DNA damage agent MMS is caused by insufficient expression of the Ixr1p in the *elp3Δ* strain.

We hypothesized that mutagenesis of the *IXR1* gene, resulting in changes of codons in the *IXR1* mRNA that circumvent decoding by tRNAs having Elongator dependent modifications would rescue Ixr1p levels in the *elp3Δ* strain. We therefore generated a mutagenized *IXR1* gene that had the AAA and CAA codons replaced by the corresponding G-ending near cognate AAG and CAG codons. Expression from this codon altered *IXR1* gene in an *elp3Δ* strain partially improved Ixr1p levels. Surprisingly, protein expression from the codon altered *IXR1* gene was fully restored by overexpression of hypomodified $tRNA^{Lys}_{s^2UUU}$, $tRNA^{Gln}_{s^2UUG}$ and $tRNA^{Glu}_{s^2UUC}$, despite that neither $tRNA^{Lys}_{s^2UUU}$ nor $tRNA^{Gln}_{s^2UUG}$ is required during translation of the codon altered *IXR1* mRNA. In addition, mutagenesis of the *IXR1* gene that results in replacement of all codons in the *IXR1* mRNA decoded by tRNAs having the Elongator complex dependent wobble uridine modifications, resulted in partial rescue of Ixr1p expression in the *elp3Δ* strain. This partial rescue was comparable to the improvement in Ixr1p expression observed when only changing the AAA and CAA codons in the *IXR1* mRNA. Moreover, overexpression of hypomodified $tRNA^{Lys}_{s^2UUU}$, $tRNA^{Gln}_{s^2UUG}$ and $tRNA^{Glu}_{s^2UUC}$ still rescued the Ixr1p levels when expressing the mutagenized version of the *IXR1* gene where all codons decoded by tRNAs having the Elongator complex dependent wobble uridine modifications were altered. These results argue that the reduced Ixr1p levels observed in the *elp3Δ* strain is only partially caused by the presence of codons read by tRNAs having the Elongator complex dependent wobble uridine modifications in the *IXR1* mRNA. Furthermore, these results suggest that Ixr1p levels are reduced in the *elp3Δ* strain due to both presence of codons requiring tRNAs having the Elongator complex dependent wobble uridine modifications in the *IXR1* mRNA, and defects in other post-transcriptional processes.

In the yeast *Schizosaccharomyces pombe* absence of the modified nucleoside mcm^5s^2U in tRNA result in reduced levels of the Cdr2p and Atf1p without altering *CDR2* and *ATF1* mRNA levels (Bauer et al., 2012; Fernandez-Vazquez et al., 2013). Changing the enriched lysine-AAA codons in the *CDR2* and *ATF1* genes to the near cognate lysine-AAG codon improved protein expression (Bauer et al., 2012; Fernandez-Vazquez et al., 2013). It was shown that codon alterations in the *ATF1* gene did not alter *ATF1* mRNA levels, therefore suggesting that changing lysine-AAA codons to Lysine-AAG codons improved translation efficiency of the *ATF1* mRNA. However mRNA levels were not measured for the codon altered *CDR2* mRNA (Bauer et al., 2012; Fernandez-Vazquez et al., 2013). Our results implicate that the events causing reduced protein expression in Elongator mutants are more complex than enrichment of codons in mRNAs requiring tRNAs having Elongator dependent wobble uridine modifications. Consequently, various secondary effects in an Elongator mutant could contribute to altered protein expression.

Conclusions

Paper I. Loss of the ncm^{5}U, mcm^{5}U and mcm^{5}s^{2}U wobble uridine nucleosides in tRNAs cause widespread metabolic alterations. Overexpression of hypomodified $tRNA^{Lys}_{s^2UUU}$, $tRNA^{Gln}_{s^2UUG}$ and $tRNA^{Glu}_{s^2UUC}$ in the *elp3Δ* strain does not result in global suppression of metabolic alterations.

Paper II. Brain tissue and fibroblasts obtained from familial dysautonomia patients display reduced levels of the mcm^{5}s^{2}U wobble uridine nucleoside in tRNAs.

Paper III. A functional Elongator complex is required for proper Ixr1p expression and consequently adequate Rnr1p levels during DNA damage. Furthermore, reduction of Ixr1p levels in the *elp3Δ* is the result of synergistic effects where both inefficient decoding of codons read by tRNAs with Elongator dependent wobble uridine modifications, and secondary effects in other post-transcriptional processes contribute.

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