University of Kassel
Faculty 10 Mathematics and Natural Science
Department of Micorbiology

Dissertation
for obtaining an academic degree
“Doktor der Naturwissenschaften (Dr. rer. nat.)”

Investigation of the functional synergy and involvement of different tRNA modifications in translational control

by

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Submitted on 2nd July, 2020

Defended on 5th October, 2020

Kassel, 2020

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„Von außen – und erst recht von innen – sieht der Prozess wissenschaftlicher Forschung ungeordnet und verwirrend aus. Man ist versucht zu glauben, Wissenschaftler seien selber unordentlich und verwirrt. In gewisser Hinsicht sind sie es – das gehört zur Forschung. Wenn man wüsste, was man tut, wäre es keine Forschung“

- Terry Pratchett: Darwin und die Götter der Scheibenwelt: Die Wissenschaft der Scheibenwelt 3, 3. Aufl., Piper Verlag, München 2012

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Bruch, **A**., Laguna, T., Butter, F., Schaffrath, R. & Klassen, R. (2020). Misactivation of multiple starvation responses in yeast by loss of tRNA modifications. *Nucleic acids research*, in press.

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Friesen, I. (2017) Expressionsanalyse der Stressmarkergene *HSP12* und *HSP82* in tRNA-Modifikationsmutanten von *Saccharomyces cerevisiae* (BSc. Biologie).

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List of Abbreviations

The bold, italic ***X*** represents any number given to the respective gene/protein appearing in this work.

A	adenosine
AA-tRNA	amino-acyl transfer RNA
ABC	ATP-binding-cassette
Acetyl-CoA	acetyl-Coenzyme A
Acp	caboxyaminopropyl
Ahp1	alkyl hydroperoxide reductase
AL	anticodonloop
Am	2`-O-methyladenosine
AMP	adenosine-monophosphate
Anb1	anaerobically induced
Arg***X***	arginine requiring
Ar(p)	2'-O-ribosyladenosine (-phosphate)
A-site	aminoacyl-site
ASL	anticodon stem and loop
Atf2	acetylTransFerase
Atg***X***	autophagy related
ATP	adenosine-triphosphate
Avo***X***	adheres voraciously
A_{37}	position 37 adenosine
BIK	BCL2 interacting Killer
BiP	binding-immunoglobulin protein
Bit61	binding partner of Tor2p
Bud32	bud site selection
C	cytidine
Cbf5	centromere Binding Factor
Cca1	tRNA CCA-pyrophosphorylase
Cdc48	cell Division Cycle
cDNA	complementary DNA
Cgi121	homolog of human CGI-121
CHOP	C/EBP homology protein
Cm	2`-O-methylcytidine
Cne1	calnexin and calreticulin homolog
Cnx1	calnexin

Cog1	conserved oligomeric golgi complex
Cox***X***	cytochrome c oxidase
Cpa1	carbamyl phosphate synthetase A
Crf1	co-repressor with FHL1
ct^6A	cyclic N6-threonylcarbamoyladenosine
C-terminus	carboxy-terminus
Cyc8	cytochrome C
D	dihydrouridine
Dal80	degradation of allantoin
DEAD-box	D-E-A-D (asp-glu-ala-asp)-box
Deg1	depressed growth rate
DENR	density regulated re-initiation and release factor
Dom34	duplication of multilocus region
DSB	double-strand break
eEF***X***	eukaryotic elongation factor
Ego***X***	exit from rapamycin-induced growth arrest
EGOC	exit from rapamycin-induced growth arrest complex
eIF***X***	eukaryotic initiation factor
Elm1	elongated morphology
Elp***X***	elongator protein
ER	endoplasmic reticulum
eRF***X***	eukaryotic release factor
E-site	exit-site
Fhl1	fork head-like
Fpr1	Fk 506-sensitive proline rotamase
Fw	forward
G	guanosine
GAAC	general amino-acid control
Gal83	galactose metabolism
Gar1	glycine arginine rich
Gcn***X***	general control nonderepressible
GDP	guanosine-diphosphate
GEF	GTP exchange factor
GFP	green fluorescence protein
GTP	guanosine-triphosphate
Glc***X***	glycogen
Gln	glutamine

Gln3	glutamine metabolism
Glu	glutamate
Gm	2′-O-methylguanosine
GO	gene ontology
Gsy2	glycogen synthase
Gtr***X***	GTP binding protein resemblance
h	hour
HAC1^{i}	homologous to Atf/Creb1 induced
HAC1^{u}	homologous to Atf/Creb1 uninduced
Hbs1	Hsp70 subfamily B suppressor
Hrr25	HO and radiation repair
Hom***X***	homoserine requiring
Hsf1	heat shock transcription factor
Hsp***X***	heat shock protein
Hxk***X***	hexokinase
I	inosine
Ifh1	interacts with fork head
Ire1	inositol requiring
IXR1	intrastrand cross (X)-link recognition
i^{6}A	N6-isopentenyladenosine
Kti***X***	*Kluveromyces lactis* toxin insensitive
Kae1	kinase-associated endopeptidase
Kar2	karyogamy
KEOPS	kinase, putative endopeptidase and other proteins of small size
KlLEU2	*Kluveromyces lactis* leucine biosynthesis
Kog1	kontroller of growth
Los1	loss of suppression
Lph1	tRNA Ligase
Lst8	lethal with sec thirteen
Ltn1/Rkr1	RING domain mutant Killed by Rtf1 deletion
mcm^{5}U	5-methoxycarbonylmethyluridine
mcm^{5}s^{2}U	5-methoxycarbonylmethyl-2-thiouridine
MCT-1	MCTS1 re-initiation and release Ffactor
Met***X***	methionine requiring
Mex67	messenger RNA export factor of 67 kDa
Mig1	multicopy inhibitor of GAL gene expression

mL	millilitre
Mod5	tRNA modification
MoTT	modification tuneable transcript
mRNA	messenger RNA
Msn***X***	multicopy suppressor of *SNF1* mutation
Mtr***X***	mRNA transport
m^1A	1-methyladenosine
m^3C	3-methylcytidine
m^5C	5-methylcytidine
m^1G	1-methylguanosine
$m^{2,2}G$	N2,N2-dimethylguanosine
m^7G	7-methylguanosine
ncm^5U	5-carbamoylmethyluridine
ncm^5Um	5-carbamoylmethyl-2′-O-methyluridine
NCR	nitrogen catabolite repression
Ncs***X***	needs Cla4 to survive
Nfs1	NiFS-like
NGD	no-go decay
Nhp2	non-histone protein
NMD	nonsense mediated decay
Nop10	nucleolar protein
NRG1	negative regulator of glucose-repressed genes
NSD	non-stop decay
nt	nucleotide
N-terminus	amino-terminus
OD	optical density
Pab1	poly(A) binding protein
PAS	phagophore assembly site/pre-autophagosomal structure
Pcc1	polarized growth chromatin-associated controller
PE	phosphatidylethanolamines
PERK	protein kinase RNA-like ER kinase
PIC	pre-initiation complex
PKA	protein kinase A
POL5	polymerase
Pop***X***	processing of precursor RNAs
PP2A	protein phosphatase 2A
P-Site	peptidyl-site

PTC[1]	peptidyl-transferase center
PTC[2]	premature stop codon
Pus***X***	pseudouridine synthase
qRT-PCR	quantitative real-time polymerase chain reaction
RAC	ribosome-associated complex
RBA50	RNA polymerase II (B) associated protein
Rex1	RNase H
Rgt1	restores glucose transport
Ribi	ribosome biogenesis
Rim15	regulator of IME2
Rit1	ribosylation of initiator tRNA
Rli1	RNase L inhibitor
RNP	ribonucleic particle
RNQ1	rich in asparagine (N) and glutamine (Q)
Rnr1	ribonucleotide reductase
RP	ribosomal protein
*RPC**X***	RNA polymerase C
RPN4	regulatory particle non-ATPase
Rpp1	ribonuclease P protein
Rpr***X***	RNase P ribonucleoprotein
RQC	ribosome-associated quality-control
Rqc2	ribosome quality control complex
rRNA	ribosomal RNA
Rrn***X***	regulation of RNA polymerase I
rT	ribothymidine
RTD	rapid tRNA decay
RT-PCR	reverse transcriptase polymerase chain reaction
Rv	reverse
S	Svedberg
S^{2-}	sulphide
Sah1	S-adenosyl-l-homocysteine hydrolase
Sak1	Snf1 activating kinase
SAM	S-adenosylmethionine
Sam***X***	S-adenosylmethionine requiring
Sap***X***	Sit4 associated protein
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sen***X***	splicing endonuclease
Sfp1	split finger protein
SGA	synthetic genetic array
Sip***X***	SNF1-interacting protein
Sit4	suppressor of initiation of transcription
Snf1C	Snf1-complex
Snf***X***	sucrose nonfermenting
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
SpHIS5	*Schizosaccharomyces pombe* histidine requiring
SRP21	signal recognition particle
*SSA**X***	stress-seventy subfamily A
*SSB**X***	stress-seventy subfamily B
SSE1	stress-seventy subfamily E
Sua5	suppressor of upstream AUG
T	thymidine
t^6A	N6-threonylcarbamoyladenosine
Tap42	two A phosphatase associated protein
TC	ternary complex
TC-AMP	threonyl-carbamoyl-adenylate
*TCD**X***	tRNA threonylcarbamoyladenosine dehydratase
Tco89	Tor complex one
Thr***X***	threonine requiring
Tip41	Tap42 interacting protein
TM	tunicamycin
Tma***X***	translation machinery associated
TORC1	Tor1 complex
*TOR**X***	target of rapamycin
Tos3	target of sbf
Tpt1	tRNA 2'-phosphotransferase
TRAMP	Trf4/Air2/Mtr4p polyadenylation complex
Trl1	tRNA ligase
Trm***X***	tRNA methyltransferase
tRNA	transfer RNA
$tRNA_i^{Met}$	initiator tRNA-methionine
Trz1	tRNase Z
TSL1	trehalose synthase long chain

Tum1	thiouridine modification
TUP1	dTMP-uptake
U	uridine
U_{34}	position 34 uridine
Uba4	ubiquitin-Activating
UBL	ubiquitin-like protein
Um	2′-O-methyluridine
uORF	upstream open reading frame
Upf***X***	up frameshift
UPR	unfolded protein response
UPS	ubiquitin proteasome system
URE2	ureidosuccinate transport
Urm1	ubiquitin related modifier
Vam6	vacuolar morphogenesis
WT	wildtype
Yak1	yet another kinase
YAP6	yeast homolog of AP-1
YEF3	yeast elongation factor
YPD	yeast extract peptone dextrose media
yW	wybutosine
ZUO1	zuotin
°C	degree celsius
µg	microgram
Ψ	pseudouridine

1. Abstract

Transfer RNAs are essential adapter molecules employed in the translational process and are highly modified all over their structure. Specific modification of the four canonical ribonucleotides (A, U, C, G) is accomplished by various proteins and/or protein complexes, ranging from simple isomerisations (e.g. Ψ) and methylations (e.g. m^5C) up to complex chemical structures (e.g. mcm^5s^2U, ct^6A). The functions of tRNA modifications are linked to the specific position and may influence translation by supporting the native RNA folding, amino-acylation efficiency and fidelity or anticodon-codon interaction. Nonetheless, since tRNA is modified at multiple motives, it is possible that specific groups of modifications collaboratively support function of the adapter molecule, enforcing the idea of a functional interactome. This network might further modulate translation in response to various extra- and intracellular triggers, bearing the possibility of a new layer of gene expression regulation.

Accordingly, the aim of this work was to establish and investigate a network of different modifications located in the anticodon stem and loop (ASL) for their role in gene expression regulation. Combined modification mutants lacking mcm^5/s^2U_{34} (*elp3* or *urm1*) together with either $\Psi_{38/39}$ (*deg1*) or ct^6A_{37} (*tcd1*) were generated and examined for their physiology, morphology, translational capacity and other regulatory abnormalities. Interestingly, all double mutants displayed comparable cytological defects despite the presumed different majorly defective $tRNA^{Lys}_{UUU}$ (*urm1*/*elp3 tcd1*) and $tRNA^{Gln}_{UUG}$ (*urm1*/*elp3 deg1*) normally bearing the unique modification combinations. The anticipated translational deficiencies applied to the reading frame accuracy in the investigated cases and biogenesis of proteins consisting of high amounts of either lysine or glutamine and also involved the general accumulation of protein aggregates. Surprisingly, the unfolded protein response known to cope with protein aggregation in the endoplasmic reticulum appeared to be suppressed under non-stressed and stressed conditions in all double mutants. Moreover, the regulome controlling the starvation stress response and stationary phase transcriptome emerged to be corrupted since various pathways like glucose repressed carbohydrate metabolism, nitrogen catabolite repression and autophagy were inappropriately activated. Since all deficiencies could be rescued by either $tRNA^{Lys}_{UUU}$ or $tRNA^{Gln}_{UUG}$ overexpression in the respective mutants, the data in sum implies that various anticodon-loop modifications of different tRNAs fulfil critical roles in mRNA translation and protein homeostasis protection which in absence consequentially evoke transcriptional stress responses.

2. Abstract in German/Zusammenfassung

Transfer RNAs stellen essenzielle Adaptermoleküle dar, welche in der Translation zum Einsatz kommen und über die gesamte Sekundär- und Tertiärstruktur umfassender postranskriptioneller Modifikationen unterliegen. Unterschiedlichste Proteine und Proteinkomplexe übernehmen dabei die spezifische Modifikation der vier kanonischen Ribonukleotide (A, U, C, G), wobei diese von simplen Isomerisierungen (z.B. Ψ) und Methylierungen (z.B. m^5C) bis hin zum Anfügen von komplexen chemischen Seitengruppen (z.B. mcm^5s^2U, ct^6A) reichen können. Die Funktion von tRNA Modifikationen hängt teilweise von ihrer Lokalisation ab und unterstützt demnach unterschiedlichste Aspekte wie die dreidimensionale Faltung, effiziente und korrekte Aminoacylierung oder die Anticodon-Codon Interaktion zur Erhaltung der Translationseffizienz. Da jede tRNA nichtsdestotrotz mehrfach an unterschiedlichsten Positionen modifiziert wird, ist es vorstellbar, dass spezifische Modifikationsgruppen bestimmte Aufgaben des Adaptermoleküls unterstützen und damit die Vorstellung von einem funktionellen Interaktom bestärken. Dieses Netzwerk könnte weiterhin die Translation in Reaktion auf verschiedene extra- und intrazelluläre Signale modulieren und damit eine neue Variante der Genexpressionsregulation darstellen.

Ziel dieser Arbeit war es dementsprechend, diese Modifikationsnetzwerke in der Anticodonschleife und -stamm nachzuweisen und ihre Rolle in der Genexpressionsregulation zu untersuchen. Zu diesem Zweck wurden verschiedene kombinierte Modifikationsmutanten, denen mcm^5/s^2U_{34} (*elp3* oder *urm1*) zusammen mit $\Psi_{38/39}$ (*deg1*) oder ct^6A_{37} (*tcd1*) fehlten, erstellt und auf ihre Physiologie, Morphologie, Translationskapazität und andere Regulationsabnormalitäten hin überprüft. Interessanterweise zeigten dabei alle Doppelmutanten vergleichbare zytologische Defekte, obwohl diese hauptsächlich von zwei verschiedenen defizitären tRNAs, der $tRNA^{Lys}_{UUU}$ (*urm1*/*elp3 tcd1*) und $tRNA^{Gln}_{UUG}$ (*urm1*/*elp3 deg1*) verursacht wurden und die einzigen sind, die normalerweise die oben genannten Modifikationskombinationen tragen. Die translationalen Defekte betrafen in diesen Mutanten unter anderem die Leseraster-Genauigkeit und die Biogenese von Lysin- oder Glutamin-reichen Proteinen, wobei auch eine generelle Aggregation von Proteinen nachgewiesen werden konnte. Überraschenderweise führten diese hervorgerufenen Fehler oder andere etwaige Stresskonditionen in den Doppelmutanten zu keiner Aktivierung, sondern Suppression des *unfolded protein response*, welcher normalerweise für die Bewältigung von Proteinaggregaten im Endoplasmatischen Retikulum zuständig ist. Darüber hinaus schien das regulatorische Netzwerk des Transkriptoms in den Mutanten beeinträchtigt zu sein, da unnötigerweise verschiedenste Signalwege zur Hungerantwort und stationären Phase wie beispielsweise die Glukose supprimierten Kohlenstoffstoffwechsel, *nitrogen catabolite repression* und Autophagie induziert wurden. Da alle Defekte durch die Überexpression der $tRNA^{Lys}_{UUU}$ oder $tRNA^{Gln}_{UUG}$ in den entsprechenden Mutanten unterdrückt werden konnten,

implizieren die Daten zusammenfassend, dass verschiedenste Modifikationen der Anticodonschleife in unterschiedlichen tRNAs ähnliche wichtige Funktionen in der Translation und dem Schutz der Protein Homöostase erfüllen und bei Verlust transkriptionelle Stressantworten hervorrufen.

3. List of Original Publications

This thesis is based on the below listed publications. They will be referred throughout the text by their roman numerals:

I. Klassen, R., Ciftci, A., Funk, J., **Bruch**, **A**., Butter, F., & Schaffrath, R. (2016). tRNA anticodon loop modifications ensure protein homeostasis and cell morphogenesis in yeast. *Nucleic acids research*, *44*(22), 10946-10959.

II. Klassen, R., **Bruch**, **A**., & Schaffrath, R. (2017). Independent suppression of ribosomal+ 1 frameshifts by different tRNA anticodon loop modifications. *RNA biology*, *14*(9), 1252-1259.

III. **Bruch**, **A**., Klassen, R., & Schaffrath, R. (2018). Unfolded protein response suppression in yeast by loss of tRNA modifications. *Genes*, *9*(11), 516.

IV. **Bruch**, **A**., Laguna, T., Butter, F., Schaffrath, R. & Klassen, R. (2020). Misactivation of multiple starvation responses in yeast by loss of tRNA modifications. *Nucleic acids research*, in press.

The authors contributions of the four publications are detailed in section 9.

4. Introduction

4.1 The translational process: steps and regulation

Protein biosynthesis or translation is a well-controlled process which is regulated at different levels in all living organisms. It can be subdivided into four steps, which begin with initiation, proceed with elongation and end with termination after which the ribosome is recycled. These steps are accomplished by the ribosome and different translation factors which fulfil various tasks throughout translation (**Figure 1**) (Dever and Green, 2012; Dever et al., 2016, 2018). These factors as well as the biogenesis of the ribosomal subunits can be targeted by different regulatory signals (e.g. nutrition supply, stress situations) and therefore influence the translational capacity or more broadly the gene expression in a eukaryotic cell (Warringer et al., 2010; De Nadal et al., 2011; Dever et al., 2016). In this regard, the expression, processing, amino acylation (charging) and post-transcriptional modification of tRNAs strongly modulate the translational process/efficiency (Phizicky and Hopper, 2010; Ranjan and Rodnina, 2016; Hopper, 2013; Torrent et al., 2018). In the following, an overview will be given about the four key steps and translation factors necessary for the translational process.

4.1.1 Translation initiation and elongation

The initiation of translation involves the formation of the 80S ribosome which consists of the small (40S) and large ribosomal subunit (60S) and is loaded with the initiator methionine-tRNA ($tRNA_i^{Met}$) which interacts with the AUG-start codon at the ribosomal P-site. To form this complex 11 initiation factors are necessary to recruit the $tRNA_i^{Met}$ and the 40S subunit for subsequent association with the activated messenger RNA (mRNA) (**Figure 1**). In a first step, Met-$tRNA_i^{Met}$ and GTP are bound by the GTPase eIF2, forming the ternary complex (TC). The TC binds to the 40S subunit, which is additionally associated to eIF1, eIF1A, eIF3 and eIF5 to accomplish the 43S pre-initiation complex (PIC). The assembly of the PIC is primarily conducted by the eIF3 complex (eIF3a, b, c, g, I, j) which seems to be capable to interact with all necessary components (Dever et al., 2016). Furthermore, the A-site of the small subunit is occupied by eIF1A while eIF1 is situated nearby the P-site. Both factors promote conformational changes of the 40S, presumably to enable TC and later on mRNA association (Passmore et al., 2007; Hussain et al., 2014; Dever et al., 2016). Subsequently after 43S PIC formation, the complex is transported to the designated mRNA at the far 5' end. For activation, the 7-methylguanosine (m^7G)-cap of the transcript is bound by the initiation factors eIF4E, eIF4A, eIF4G and eIF4B. The first factor is necessary to bind the mRNA cap, while the second one is a DEAD-box RNA helicase likely unwinding the mRNA for secondary structure prevention throughout scanning for the translational start site (Rajagopal et al., 2012; Dever et al., 2016). Among the latter two factors, eIF4G consists of different factor binding domains which are needed to simultaneously interact with eIF4E and Pab1, a poly A-tail binding protein,

to support the formation of the ring like architecture of the mRNA. The interaction of eIF4G, the mRNA, 43S PIC and eIF4A is supported by eIF4B which also enhances helicase activity of the latter initiation factor (Dever et al., 2016). The assembly of all these components establishes the 48S initiation complex which scans the mRNA for the AUG start codon (**Figure** 1).

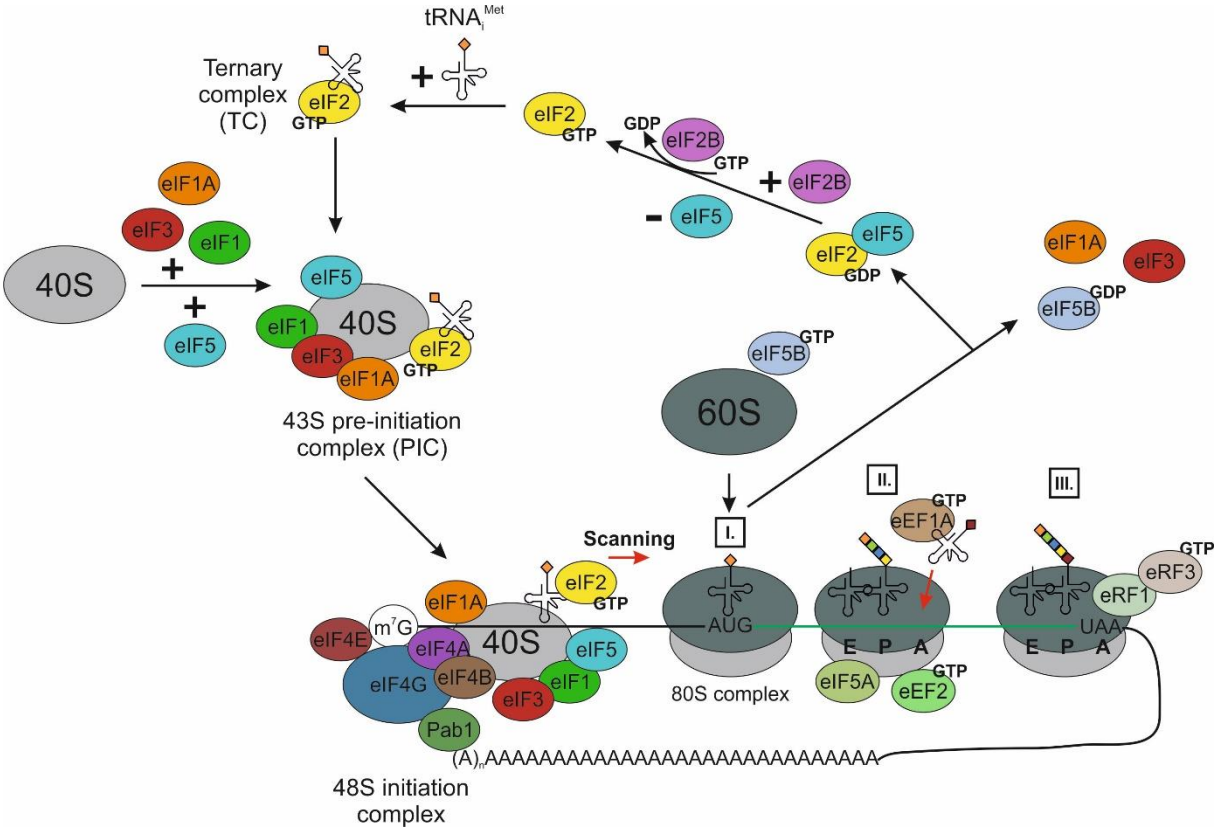

Figure 1 Schematic overview of the translation process in *Saccharomyces cerevisiae*. Depicted are the different ribozymes (40S and 60S subunit) and translation factors involved in the translation process. eIF2-GTP forms together with Met-tRNA$_i^{Met}$ the ternary complex (TC). The TC interacts with the 40S subunit together with eIF1, eIF1A, eIF3 and eIF5 which completes the 43S pre-initiation complex (PIC). This structure associates with the activated mRNA, aided by the cap bound translation initiation factors eIF4A, eIF4B, eIF4E, eIF4G and Pab1, which connects also to the poly-A-tail of the transcript. After scanning for and recognition of the start codon and the association of the 60S subunit (aided by eIF5B-GTP) the 80S ribosome is completed and starts with translation (**Box I**=initiation, **II**=elongation, **III**=termination). Elongation (eEF1A, eEF2 and eIF5A) and termination factors (eRF1 and eRF3) are indicated and needed for specific tasks. Recycling of eIF2 is depending on eIF2B. Every process step of translation is detailed in the text. Adapted from (Dever and Green, 2012; Dever et al., 2016, 2018; Karlsborn, 2016)

As soon as the initiation complex has found the translation start site (i.e. AUG codon recognition) eIF2 hydrolyzes the bound GTP which is additionally stimulated by the GTPase activating protein eIF5. Upon hydrolysis of GTP, phosphate (Pi), eIF5, eIF2-GDP and eIF1 are released. This leads to the reorganization of the 43S PIC and repositioning of the Met-tRNA$_i^{Met}$ into the P-site of the small subunit. Afterwards, the 60S subunit is associated to the PIC aided by eIF5B-GTP leading to the formation of the 80S ribosome. Subsequently, GTP of eIF5B is hydrolyzed and eIF5B-GDP as well as eIF3 and eIF1A are released by the complex (**Figure**

1, **Box I**). The A-site of the 80S ribosome is now accessible by amino-acyl-tRNAs (aa-tRNA) which leads to the elongation step of translation (Dever et al., 2016).
In contrast to the high number of different factors needed for the translation initiation, the 80S ribosome is only accompanied by eEF1A-GTP, eIF5A, eEF2-GTP and eEF1B for the elongation step (**Figure 1**, **Box II**, eEF1B is not depicted). Thus, eEF1A-GTP forms a ternary complex with charged tRNAs complementary to the mRNA-codon in the A-site of the ribosome. The TC interacts with this site of the ribosome promoting the anticodon-codon contact and after GTP hydrolysis, eEF1A-GDP releases the tRNA which is included into the A-site (Dever et al., 2016, 2018). The recycling of eEF1A-GDP is conducted by eEF1B which exchanges GDP with GTP enabling eEF1A to again bind aa-tRNAs for further elongation steps.
During peptide bond formation, the aa-tRNA and the peptidyl-tRNA, placed in the A-site and P-site, respectively, are positioned by the ribosomal peptidyl-transferase center (PTC) to enable a so called hybrid state in which the respective acceptor stem is pointing into the P- or E-site (Dever et al., 2016, 2018). This step is aided by eEF5A which binds to the E-site of the ribosome. The elongation factor is post-translationally hypusinated by the addition of a 4-aminobutyl moiety at the ε-aminogroup of a conserved lysine residue (Kim et al., 1998; Park et al., 2010). Hypusine has been shown to be essential for the elongation step but is involved in the synthesis of polyproline stretches (Gutierrez et al., 2013) or functioning in translation termination (Schuller et al., 2017). Finally, after peptide bond formation, translocation of the 80S complex is achieved by eEF2-GTP. The elongation factor blocks the A-site of the ribosome and enhances the translocation step, accompanied by GTP hydrolysis, leading to the new positioning of the tRNAs into the P- and E-site, respectively (Dever and Green, 2012; Dever et al., 2016, 2018). Interestingly, eEF2 is also post-translationally modified at the histidine 699 by attachment of a diphthamide side chain. The diphtamide modification is a well conserved modification in most eukaryotic and archaeal systems and is thought to support the translocation during translation elongation and is involved in maintaining the mRNA reading frame (Ortiz et al., 2006; Hawer et al., 2018). The deacylated tRNA is now released from the E-site and the 80S complex is ready for the next round of translation (Dever and Green, 2012; Dever et al., 2016, 2018).

4.1.2 Translation termination and ribosome recycling

Translation termination is initiated as soon as the ribosomal A-site reached one of the three stop codons UAA, UAG or UGA which are recognized by eRF1 (**Figure 1**, **Box III.**). This termination factor binds the A-site and directly interacts with the stop codon via its N-terminal domain (Dever and Green, 2012; Bertram et al., 2000; Dever et al., 2016). The middle domain of eRF1 resembles structurally the acceptor stem of tRNAs and is capable to interact with the PTC which is necessary to engage polypeptide release (Song et al., 2000). This process is facilitated by eRF3-GTP, which interacts with the middle and C-terminal domain of eRF1

(Merkulova et al., 1999; Kononenko et al., 2008; Cheng et al., 2009). eRF3-GTP promotes the reposition of the eRF1-middle domain into the PTC enforcing the dissociation of the polypeptide from the peptidyl-tRNA. During this process GTP hydrolyses and eRF3-GDP is released from eRF1 (Dever and Green, 2012).

This step is facilitated by Rli1 that interacts with eRF1 and is also necessary for ribosome recycling. Rli1 belongs to the ABC-ATPase family and (i) stimulates ribosome release activity of eRF1 (ii) but is also critical for the separation of the mRNA, P-site bound uncharged tRNA, 60S and 40S subunit (Shoemaker and Green, 2011; Dever and Green, 2012; Pisarev et al., 2010; Dever et al., 2016). Rli1 is hydrolyzing ATP for the 80S ribosome recycling process (Pisarev et al., 2010). Notably, mRNA and tRNA are still bound to the 40S subunit after ribosome separation. In mammalian cells, they are released by Ligatin or MCT-1 in complex with DENR, respectively (Skabkin et al., 2010). Accordingly, homologs of all three proteins exist in yeast, termed Tma20 (MCT-1), Tma64 (Ligatin) and Tma22 (DENR). While they all associate with the ribosome, neither has been characterized mechanistically so far (Fleischer et al., 2006). Nevertheless, according to the resemblances of the yeast and mammalian “recycling factor” proteins, they are thought to be functionally conserved (Dever and Green, 2012).

4.2 Transfer RNAs: maturation and modification

The translation of mRNA information into a protein necessitates transfer RNAs (tRNA). One of the twenty canonical amino acids is transported by a tRNA to the translating ribosome. The tRNA is always charged with the matching amino acid according to its anticodon, which is monitored by the ribosome for codon complementarity and consequently accepted or rejected. These adapter molecules are single stranded RNAs of 70-90 nucleotides (nt) length that form clover-leaf-structures due to intramolecular base-pairing. Hence, every tRNA consists of an acceptor stem, variable loop, D-arm, TΨC-arm and the anticodon-stem-loop (ASL) (**Figure 3**). This secondary structure is accomplishing an L-shaped three-dimensional architecture through tertiary base-pairing, representing the native conformation in all living cells.

In yeast, 41 main tRNA families can be grouped according to their respective anticodon and are expressed to translate the degenerative genetic code consisting of 61 possible codons (Hani and Feldmann, 1998; Hopper, 2013). Interestingly, 274 tRNA genes have been identified in *S. cerevisiae* and most tRNA species are encoded by more than one gene (Hani and Feldmann, 1998; Hopper, 2013; Goffeau et al., 1996; Bloom-Ackermann et al., 2014). Posttranscriptional maturation of a tRNA involves complex, tightly regulated steps and can be roughly subdivided into (i) 5’ leader and 3’ trailer trimming, (ii) splicing (not necessary for all), (iii) 3’-CCA addition, (iv) nucleotide modification and finally (v) amino acylation at the 3’ end (Hopper, 2013).

4.2.1 Posttrancriptional processing of tRNAs in *Saccharomyces cerevisiae*

The transcription of tRNAs is achieved by the RNA-polymerase III (PolIII) and they are in general expressed as precursor molecules (pre-tRNA). The transcripts consist of a ~12 nt 5' leader, followed by the tRNA sequence, an intron 3'adjacent of one base after the anticodon for 59 of 274 tRNA species and the ~12 nt 3' trailer (**Figure 2**) (Hopper and Phizicky, 2003; Chan and Lowe, 2009; Hopper, 2013).

A first step of tRNA maturation is the removal of the 5' leader and 3' trailer nucleotides. Accordingly, the multiprotein endonuclease Rnase P, consisting of the proteins Pop1, Pop3-Pop8, Rpp1 and Rpr2 as well as the RNA *RPR1*, respectively, recognizes the 5' leader sequence and detaches it (**Figure 2**, blue) (Hopper, 2013; Xiao et al., 2002). The elimination of the 3' trailer is more complicated and involves the exonuclease Rex1, the endonuclease RNase Z (Trz1) and the RNA binding protein Lph1 (**Figure 2**, green) (Phizicky and Hopper, 2010; Maraia and Lamichhane, 2011; Hopper, 2013). If Lph1 binds to the 3' end of the tRNA, it prevents the interaction with Rex1 which enables RNase Z to associate and remove the 3' extension (Yoo and Wolin, 1994; Maraia and Lamichhane, 2011). Otherwise, Rex1 exonucleolytically degrades the 3' trailer.

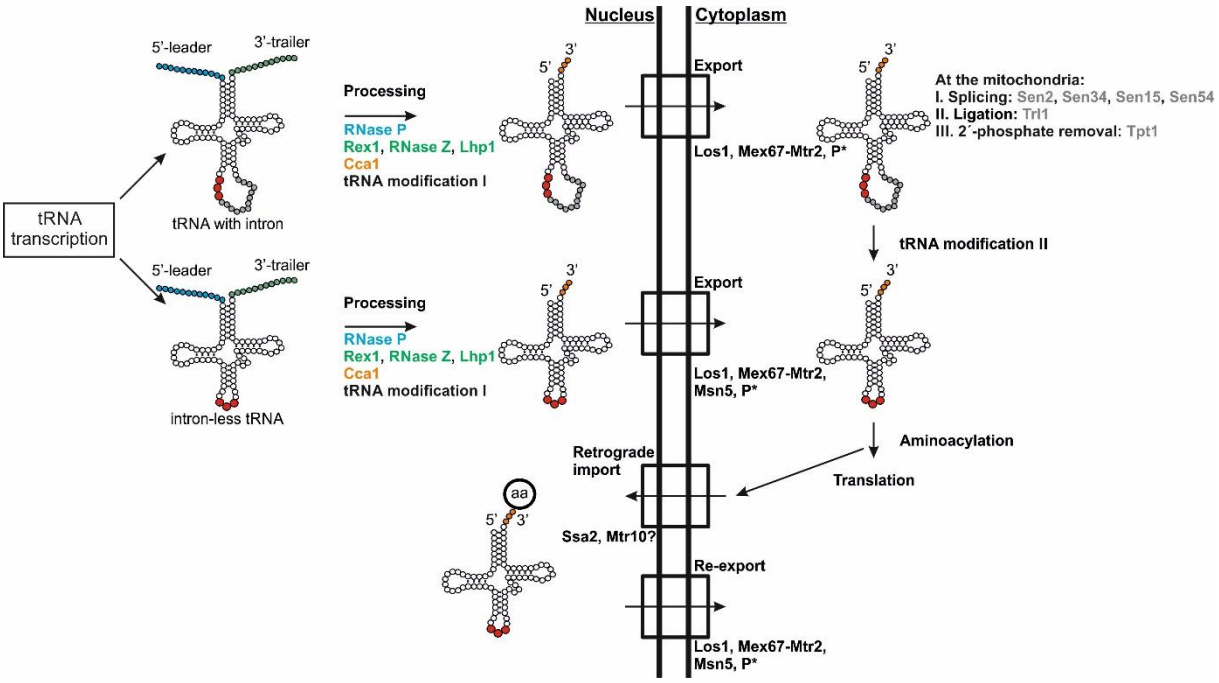

Figure 2 Depiction of the different tRNA maturation steps in *S. cerevisiae*. This scheme displays the various processing steps of yeast intron-containing/intron-less tRNAs occuring in the nucleus and the cytoplasm. Participating enzymes and protein complexes are coloured the same way as the nucleotides (circles) they add, remove or edit in the tRNA: blue=RNase P, 5´ leader; green=Rex1, RNase Z, Lhp1, 3´ trailer; orange=Cca1, 3´ CCA, grey= Sen2, Sen34, Sen15, Sen54, Trl1, Tpt1, intron. The anticodon is coloured in red and the amino acids are abreviated in an open circle (aa). Proteins necessary for the tRNA nucleus export, retrograde import and re-export are specified at the arrows indicating the transport direction. Different circuits of tRNA modification are given in their respective compartment (nucleus, cytoplasm) and will be detailed in **Figure 3**. Taken and adapted from (Hopper, 2013; Chatterjee et al., 2018)

After the maturation of the 5' and 3' ends, the tRNA must be prepared for the amino acylation. While all prokaryotic tRNA genes already encode the 3' CCA end, eukaryotic systems must

add the three nucleotides posttranscriptionally by a nucleotidyl transferase. In yeast, this task is achieved in the nucleus by Cca1 (**Figure 2**, orange) (Hopper, 2013; Aebi et al., 1990), which occurs also in two additional isoforms either responsible for tRNA 3' end repair necessary to deal with partially degraded CCA ends (cytoplasmic localization) or fulfills both functions in the mitochondrion (Martin and Hopper, 1994). After tRNA end processing, a first set of tRNA modifications are installed (**Figure 2**, also detailed in the next chapter). Some of these modifications depend on the non-spliced pre-tRNA like $\Psi_{34\text{-}36}$, m^5C_{34} and m^5C_{40} while others are introduced subsequently to intron removal (e.g. Gm_{18}, i^6A_{37}, m^1G_{37}) (Phizicky and Hopper, 2010; Grosjean et al., 1997; Hopper, 2013). Another group of modifications can be found on tRNAs independently of an intron (e.g. $\Psi_{38,39}$, $D_{16,17}$) (Phizicky and Hopper, 2010; Hopper, 2013). Intriguingly, while the first modification group is added strictly in the nucleus, the latter two modification groups are installed either in the nucleus or the cytoplasm. This is only possible because tRNAs (either if splicing is necessary or not) are dynamically trafficking between the nucleus and the cytosol (see below) (Shaheen and Hopper, 2005; Chatterjee et al., 2018). Hence, primary nuclear export of intron-containing processed tRNAs is executed by Los1, Mex67-Mtr2 and possibly by other unknown proteins while the intron-less tRNA species are recognized by the same transporters in addition to Msn5 (Shaheen and Hopper, 2005; Murthi et al., 2010; Chatterjee et al., 2017, 2018).

After the export, processing of intron-containing tRNAs continues in the cytoplasm, since the splicing machinery (SEN complex), ligase (Trl1) and 2' phosphotransferase (Tpt1) in *S. cerevisiae* are localized to the outer surface of the mitochondrion, in the cytoplasm or in the cytoplasm and nucleus, respectively (Dhungel and Hopper, 2012; Huh et al., 2003; Yoshihisa et al., 2003). The SEN endonuclease complex in yeast is a heterotetramer and consists of Sen2, Sen34, Sen15 and Sen54. Together, they remove the 12 to 60 nt long intron at the 5'-3' splice site (Phizicky and Hopper, 2010; Trotta et al., 1997; Hopper, 2013). Subsequently, the remaining two tRNA fragments are recognized by the ligase Trl1, ligating both halves. This is achieved by (i) opening the 2', 3' cyclic phosphate of the 5'-half generating 2'-PO_4 and 3'-OH, (ii) phosphorylation of the 5'-OH group of the 3'-half under GTP consumption, (iii) subsequentially adenylating the added phosphate group and (iv) finally connecting the two halves releasing AMP. Additionally, the reaction generates a splice junction consisting of a 3', 5' phosphodiester bond and the residual 2' phosphate, a remnant of the 2', 3' cyclic phosphate conversion (Greer et al., 1983; Abelson et al., 1998). The phosphate is finally removed by Tpt1 (**Figure 2**) (Spinelli et al., 1997).

Both, the initial intron-less tRNAs and the processed intron-containing tRNAs are now accessible to a second round of nucleotide modifications (detailed in the next chapter) as well as amino acylation of the 3' CCA end with the matching amino acid (Phizicky and Hopper, 2010; Hopper, 2013). As a result, the matured tRNAs are applicable for translation or can be

also transported back into the nucleus by Ssa2 and either directly or indirectly by Mtr10 (Shaheen and Hopper, 2005; Takano et al., 2015; Chatterjee et al., 2018). This retrograde tRNA nuclear import is thought to occur constitutively, possibly to allow tRNA quality control via rapid tRNA decay (RTD) or the TRAMP complex/nuclear exosome (Parker, 2012; Hopper, 2013; Vanácová et al., 2005; Chatterjee et al., 2018), tRNA modification after splicing for instance by Trm7 (Murthi et al., 2010; Guy et al., 2012), amino acylation to promote tRNA re-export (Sarkar et al., 1999; Grosshans et al., 2000; Azad et al., 2001; Chatterjee et al., 2018) and is increased upon starvation stress probably to diminish protein biosynthesis (Whitney et al., 2007). Transfer RNAs retrogradely situated in the nucleus are re-exported by Los1, Mex67-Mtr2, Msn5 and presumably other participants, whereas amino acylation in the nucleus of the matured tRNAs seems to be one major driver for this process (**Figure 2**) (Sarkar et al., 1999; Grosshans et al., 2000; Azad et al., 2001; Chatterjee et al., 2018).

4.2.2 The Epitranscriptome: tRNA modifications in focus

As pointed out above, tRNAs are essential adapter molecules for translation and are heavily processed (**Figure 2**). In this regard, every tRNA species is modified and harbours an individual combination of 7-17 modifications (in yeast) distributed all over the sequence (Phizicky and Hopper, 2010). Until now, 111 tRNA modifications have been identified in all three domains of life of which 25 are conserved in *S. cerevisiae* (summarized in **Figure 3**) (Phizicky and Hopper, 2010; Ranjan and Rodnina, 2016; Cantara et al., 2011; Lorenz et al., 2017; Boccaletto et al., 2018; McCown et al., 2020). Notably, some modifications are conserved throughout different organisms and can be found in the same tRNA species and sequence position (Björk et al., 2001).

In general, tRNA modifications are distributed all over the tRNA body whereas the anticodon loop represents a modification hotspot, especially concerning the positions 34 (*wobble* position) and 37 displaying the highest diversity of possible modifications (**Figure 3**) (Phizicky and Hopper, 2010). Modifications in the ASL are thought to support translational fidelity by stabilizing the anticodon loop (e.g. ct^6A, Ψ) and/or codon-anticodon interaction (e.g. mcm^5s^2U, m^5C) (Lecointe et al., 1998; Huang et al., 2005, 2008; Leidel et al., 2009; El Yacoubi et al., 2012; Miyauchi et al., 2012) while other posttranscriptional modifications are situated in the tRNA body supposedly ensuring structure and stability. For instance, Trm8 methylates G_{46} (7-methylguanosine=m^7G) and Trm4 modifies $C_{34,40,48,49}$ (5-methylcytosine=m^5C), respectively. The corresponding double mutant *trm4 trm8* misses the methyl groups at position 46 and 40 of the $tRNA^{Val}_{AAC}$ which promotes its degradation at higher temperatures by rapid tRNA decay (Alexandrov et al., 2006; Chernyakov et al., 2008). Furthermore, methylation of adenosine 58 (m^1A_{58}) of the $tRNA_i^{Met}$ is essential, since it is not only necessary for the maturation of the pre-initiator tRNA but also for its stability (Anderson et al., 2000). Both genes, *TRM6* and *TRM61*, are essential but partial loss of function mutations of *TRM6* were demonstrated to reduce the

amount of pre-tRNA$_i^{Met}$ at higher temperatures due to the absence of m^{1}A$_{58}$ and the active degradation by the TRAMP complex/nuclear exosome (Kadaba et al., 2004; Anderson, 2005; LaCava et al., 2005).

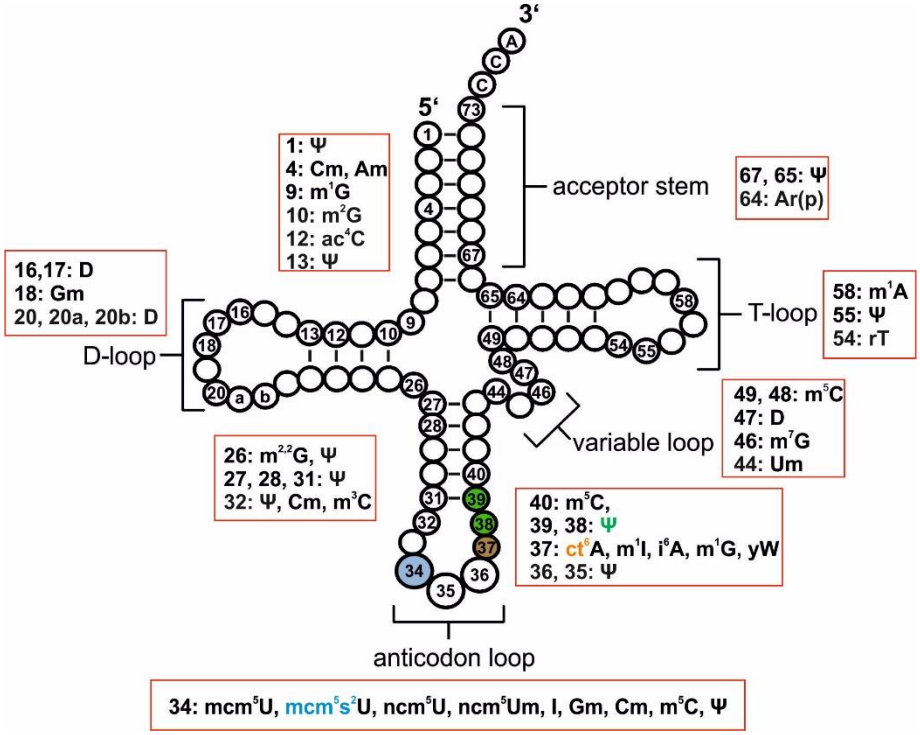

Figure 3 Nucleotide modifications on cytoplasmic tRNAs in *Saccharomyces cerevisiae.* Nucleotides are displayed as circles and numbered if they are modified. The different tRNA modifications are given in the red boxes. The obligate added 3´ CCA-Triplet is indicated and all arms/loops of the secondary tRNA structure are labelled. Following abbreviations for tRNA modifications were used: (Ψ) pseudouridine; (Am) 2`-O-methyladenosine; (Cm) 2`-O-methylcytidine; (m^{1}G) 1-methylguanosine; (m^{2}G) 2-methylguanosine; (ac^{4}C) 4-acetylcytidine; (D) dihydrouridine; (Gm) 2`-O-methylguanosine; (m$^{2,2}$G) N2, N2-dimethylguanosine; (m$^3$C) 3-methylcytidine; (I) inosine; (m$^5$C) 5-methylcytidine; (mcm$^5$U) 5-methoxycarbonylmethyl-uridine; (mcm$^5$s$^2$U) 5-methoxycarbonylmethyl-2-thiouridine; (ncm$^5$U) 5-carba-moylmethyl-uridine; (ncm$^5$Um) 5-carbamoylmethyl-2`-O-methyluridine; (m^{1}I) 1-methylinosine; (i^{6}A) N6-isopentenyl-adenosine; (yW) wybutosine; (t^{6}A) cyclic N6-threonylcarbamoyladenosine; (Um) 2`-O-methyluridine; (m$^7$G) 7-methylguanosine; (rT) ribothymidine; [Ar(p)] 2`-O-ribosyladenosine (phosphate). The positions 34, 37, 38 and 39 as well as the relevant assigned modifications are coloured in blue (34, mcm^{5}s^{2}U), orange (37, ct^{6}A) and green (38,39, Ψ), since they were focused in this work. Taken and adapted from (Phizicky and Hopper, 2010; Ranjan and Rodnina, 2016).

Beside the support of translational fidelity and tRNA stability, nucleotide modifications are also thought to define tRNA identity for amino acylation or translation initiation. Regarding this, *RIT1* encodes a 2'-O-ribosyl phosphate transferase which modifies the adenosine at position 64 to generate 2'-O-ribosyladenosine (-phosphate) (Ar(p)$_{64}$). This modification ensures that the tRNA$_i^{Met}$ is only utilised for translation initiation and prevents its application for the elongation step (Åström and Byström, 1994).

Although these examples represent different functional groups to which tRNA modifications can be sorted, the function of most modifications remain elusive and must be clarified in future studies. This appears to be difficult, since the deletion of many modifier genes in yeast is tolerated and leads if at all to mild phenotypes (Phizicky and Alfonzo, 2010; Phizicky and Hopper, 2010). However, combined modification defects may result in more severe

phenotypes. It was demonstrated that the combined absence of specific modifications like mcm^5 and s^2 holds severe implication onto the cells physiology indicating a functional cooperativity of distinct tRNA modifications (Nedialkova and Leidel, 2015; Klassen et al., 2015).

4.2.3 Modification cascade of the *wobble* uridine 34

As previously described, position 34 of tRNAs is frequently modified. This also includes tRNA species harbouring a uridine at the *wobble* position (Helm and Alfonzo, 2014). Of 13 tRNAs with an U$_{34}$ in *S. cerevisiae*, the tRNA$^{Leu}_{UAG}$ *wobble* position remains unmodified while the one in tRNA$^{Ile}_{UAU}$ is pseudouridylated by Pus1 (Simos et al., 1996; Motorin et al., 1998) and 11 other tRNAs harbour 5-methoxycarbonylmethyluridine (mcm^{5}U), 5-methoxycarbonylmethyl-2-thiouridine (mcm^{5}s^{2}U), 5-carbonylmethyluridine (ncm^{5}U) and 5-carbonylmethyl-2'-O-methyluridine (ncm^{5}Um) (**Figure 4**) (Johansson et al., 2008; Chen et al., 2011a).

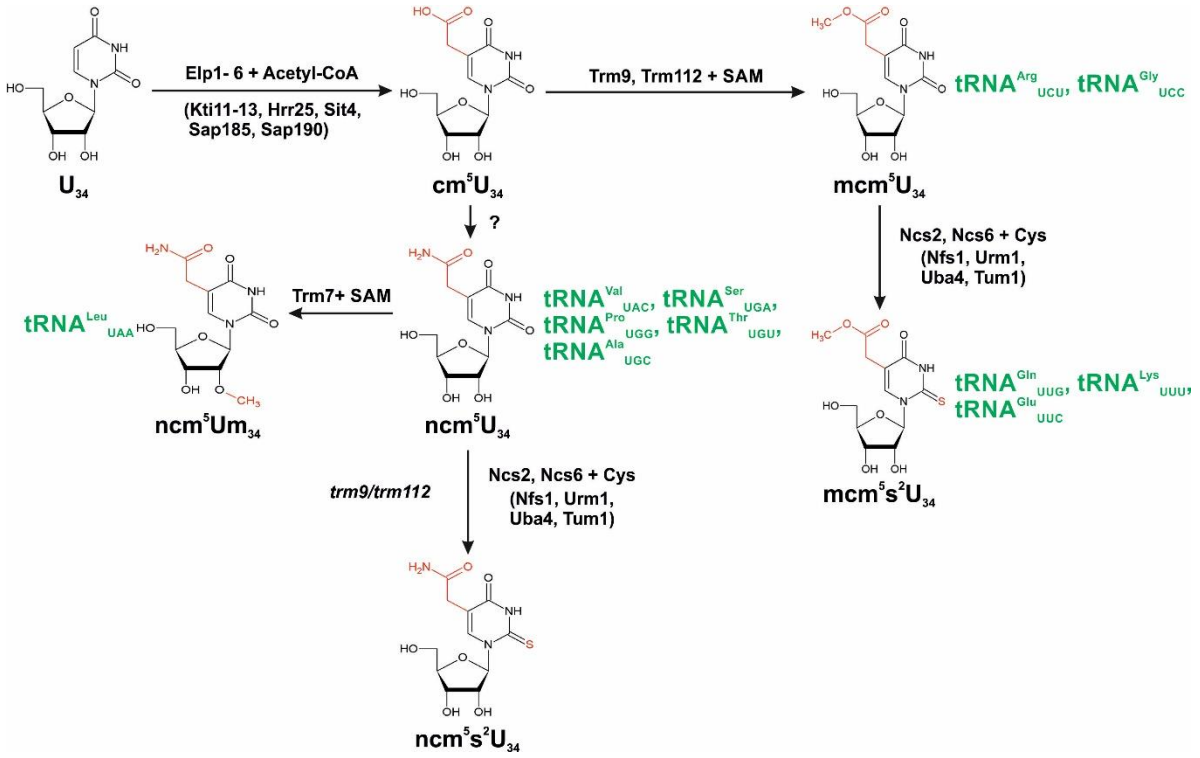

Figure 4 Generation of ncm^{5}U, ncm^{5}Um, mcm^{5}U and mcm^{5}s^{2}U on the *wobble* uridine of 11 different tRNAs. The proteins required for modification formation are indicated on the black arrows and if necessary, depicted with the appropriate co-factor (Acetyl-CoA, S-adenosylmethionine=SAM, Cysteine). Cytoplasmic transfer RNAs harbouring the modifications are coloured in green. No enzyme so far has been discovered generating ncm^{5}U (**?**). Deletion of *TRM9* and/or *TRM112* has been shown to lead to an accumulation of ncm^{5}s^{2}U instead of mcm^{5}s^{2}U. Taken and adapted after (Kalhor and Clarke, 2003; Huang et al., 2005, 2008; Johansson et al., 2008; Nakai et al., 2008; Leidel et al., 2009; Noma et al., 2009; Chen et al., 2011a; Boccaletto et al., 2018).

The latter depend on a complex modification cascade which starts with the addition of a carbonyl-methyl-moiety on the C5 of the uridine base (cm^{5}U) catalyzed by the Elongator complex (Huang et al., 2005; Chen et al., 2011a). This reaction consumes Acetyl-CoA and the complex is tightly regulated by Kti11-13, Hrr25, Sit4, Sap185 and Sap190 via its

phosphorylation status (Fichtner et al., 2003; Huang et al., 2005, 2008; Bär et al., 2008; Abdel-Fattah et al., 2015; Glatt et al., 2015; Krutyhołowa et al., 2019). The cm^5U moiety is then converted into ncm^5U or mcm^5U depending on the tRNA species. It is unknown, which proteins are necessary in addition to the Elongator complex to generate ncm^5U on 6 tRNAs (**Figure 4**) but the ncm^5U modified wobble base of $tRNA^{Leu}_{UUA}$ is further ribose-methylated by Trm7 resulting in the hypermodified ncm^5Um (Pintard et al., 2002). The 5 other tRNA species are equipped with mcm^5U which is generated from cm^5U or ncm^5U by Trm9 and Trm112 (Kalhor and Clarke, 2003) and 3 of them, $tRNA^{Gln}_{UUG}$, $tRNA^{Lys}_{UUU}$ and $tRNA^{Glu}_{UUC}$, are additionally thiolated (**Figure 4**). The thiolation is achieved by Ncs2 and Ncs6, both putatively coordinating an iron-sulfur cluster (Liu et al., 2016; Nakai et al., 2017), upon consumption of a sulfur. The sulfur is extracted from cysteine by Nfs1 and transported further through a sulfur relay system involving Tum1, Uba4 and the ubiquitin like protein (UBL) Urm1 (Termathe and Leidel, 2018; Nakai et al., 2008; Leidel et al., 2009; Noma et al., 2009).

4.2.4 Pseudouridine synthesis on transfer RNAs

The conversion of uridine to pseudouridine is one of the most prevalent modifications that can be found in different non-coding and coding RNAs including snRNAs, mRNAs, rRNAs and tRNAs (Sun et al., 2016; Penzo and Montanaro, 2018). The general modification procedure is executed via rotation of the uridine base by 180° and replacing the N-glycosidic bond by a C-glycosidic bond to the C1` of the ribose (**Figure 5**) (Charette and Gray, 2000; Spenkuch et al., 2014).

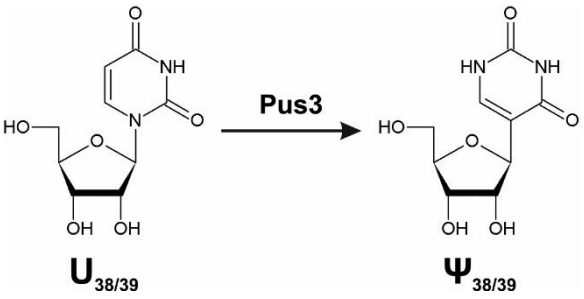

Figure 5 Isomerization of uridine to Ψ by Pus3. Pseudouridine generation is achieved by rotation of the uridine base changing the N-C bond to a C-C bond to the C1` of the ribose without aid of any guide RNA. Pus synthetases are capable of this modification variant and are represented by Pus3/Deg1 together with the target positions in tRNAs. Taken and adapted from (Charette and Gray, 2000; Spenkuch et al., 2014; Boccaletto et al., 2018).

This process can be achieved in a RNA-dependent manner at which a small nucleolar (sno) RNA, termed H/ACA RNA, guides the ribonucleic particle (RNP) to the target nucleotide or by stand-alone enzymes (Ganot et al., 1997; Ni et al., 1997; Spenkuch et al., 2014). In yeast, Cbf5, Nop10, Nhp2 and Gar1 complete the RNP which can be targeted by specific snoRNAs to different substrates including various sites of the 18S and 28S rRNA as well as at least position 42 of the snRNA U2 (Ma et al., 2005; Ge and Yu, 2013; Spenkuch et al., 2014). However, this mode of catalysis is not employed for the modification of tRNAs which rather

depend on various pseudouridine synthases (Pus) (Phizicky and Hopper, 2010; Spenkuch et al., 2014). For *S. cerevisiae* 9 *PUS* genes have been described and nearly all of them (except for Pus5) are responsible for Ψ modifications found on cytoplasmic (Pus1, Pus3, Pus4, Pus6, Pus7, Pus8) and/or mitochondrial (Pus2, Pus3, Pus4, Pus6, Pus9) tRNAs (Decatur and Schnare, 2008; Becker et al., 1997; Motorin et al., 1998; Lecointe et al., 1998; Massenet et al., 1999; Ansmant et al., 2000, 2001; Behm-Ansmant et al., 2003, 2004, 2006, 2007; Ma et al., 2003).

Many of these synthases proved to act promiscuously on their substrate or target multiple nucleotides leading to various positions and RNA species to be modified by only one enzyme. Pus1 for instance converts 8 different uridines to Ψ in various tRNAs but is also responsible for the pseudouridylation of position 44 in snRNA U2 (Phizicky and Hopper, 2010; Simos et al., 1996; Motorin et al., 1998; Massenet et al., 1999; Behm-Ansmant et al., 2006). Thus, Pus3 (Deg1) represents no exception to this rule since it modifies position 38 or 39 in roughly 20 different tRNAs (Lecointe et al., 1998; Boccaletto et al., 2018). Deletion of *DEG1* perturbs growth and translational fidelity whereas the single deletion of the other tRNA specific Pus genes causes no or more moderate growth defects at elevated temperature (Gustavsson and Ronne, 2008; Phizicky and Hopper, 2010; Lecointe et al., 2002; Han et al., 2015). These findings show that Deg1 fulfils a specific and more important role in translation compared to the most other members of Pus-family.

4.2.5 Adenine 37 is modified by the KEOPS and Tcd1/Tcd2 complexes

In addition to position 34, the 37th nucleotide in tRNAs is also a modification hotspot. A_{37} is modified with a N6-threonyl-carbamoyl moiety (t^6A) in nearly all tRNAs with an ANN anticodon if U_{36} appears beforehand in the sequence (Elkins and Keller, 1974; Körner and Söll, 1974; Ishikura et al., 1969; Sibler et al., 1985; Grosjean et al., 1987; Morin et al., 1998; Thiaville et al., 2014b). The discovery of this modification dates back more than 50 years (Schweizer et al., 1969) but the catalysis of t^6A as well as the participants were resolved in the last two decades for all 3 domains of life (Thiaville et al., 2014b). The eukaryotic t^6A pathway is summarized below.

Generally, t^6A is installed by the action of the Kinase, putative Endopeptidase and Other proteins of Small size (KEOPS)-complex consisting in *S. cerevisiae* of the 5 proteins Bud32, Kae1, Gon7, Pcc1 and Cgi121 as well as Sua5 for cytoplasmic tRNAs (Daugeron et al., 2011; El Yacoubi et al., 2011; Srinivasan et al., 2011; Perrochia et al., 2013a; Thiaville et al., 2014b). In contrast, only Qri7 and Sua5 are necessary to equip mitochondrial tRNAs with t^6A (Sibler et al., 1986; Huh et al., 2003; Oberto et al., 2009; Thiaville et al., 2014a) and both proteins are sufficient to modify tRNAs *in vitro* (Wan et al., 2013). However, cytoplasmic t^6A formation seems to rely on the joint functionality of the complex components since single deletion of the different “KEOPS”-genes had always impacted the modification status. Deleterious mutation

of *BUD32*, *GON7* and *KAE1* leads to a total abolishment of t^6A while *pcc1* and *cgi121* mutants display a diminished t^6A level of 30% and 60%, respectively (El Yacoubi et al., 2011; Wan et al., 2013; Thiaville et al., 2014b). Hence, all parts of the KEOPS-complex seem to fulfill a specific role in the generation of N6-threonyl-carbamoyladenosine which have not been elucidated so far for all of them.

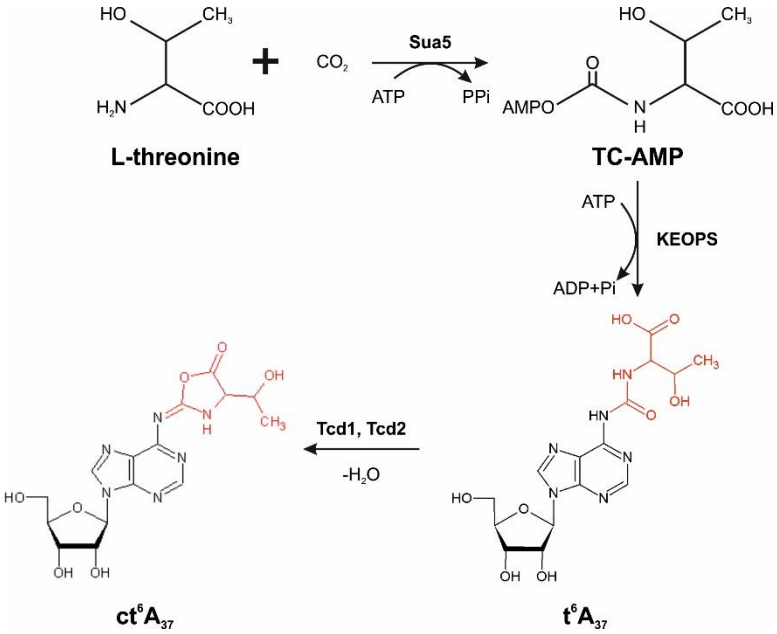

Figure 6 Modification pathway of cyclic N6-threonyl-carbamoyl-adenosine. The modification cascade starts upon consumption of L-threonine, bicarbonate and ATP by Sua5 generating a threonyl-carbamoyl-adenylate (TC-AMP) which is further transferred onto the target adenine of the destined tRNA by the KEOPS-complex (components are detailed in the text). During this reaction, AMP of TC-AMP is released, and ATP is hydrolyzed. The t^6A moiety can be further processed by Tcd1 and Tcd2 to generate ct^6A under the release of water. Taken and adapted from (Miyauchi et al., 2012; Wan et al., 2013; Thiaville et al., 2014b; Boccaletto et al., 2018).

The modification procedure starts with Sua5 carboxylating the amino-group of L-threonine upon bicarbonate consumption forming N-carboxythreonine which is subsequently adenylated utilizing one ATP to generate threonyl-carbamoyl-adenylate (TC-AMP, **Figure 6**) (Wan et al., 2013). TC-AMP is recruited together with the target tRNA (e.g. $tRNA^{Lys}_{UUU}$) by the KEOPS complex to transfer the activated carboxythreonine onto adenine N6 for t^6A formation upon ATP hydrolysis (**Figure 6**) (Wan et al., 2013). The exact biochemical mechanism occurring in the complex is still under investigation and it is also not clear what role all the different compartments play during the modification procedure. However, Kae1 seems to be the core component catalyzing the reaction and dimerizes with Pcc1 for functionality (Mao et al., 2008; Wan et al., 2013) while KEOPS-embedded Bud32 appears to have ATPase activity despite its actual protein kinase behavior (Perrochia et al., 2013b). Cgi121 in this regard seems to be an allosteric regulator of Bud32 (Perrochia et al., 2013b). The function of Gon7 is not clear yet but seems to be critical to the t^6A modification pathway not only for yeast but also for the human system (El Yacoubi et al., 2011; Wan et al., 2013, 2017; Thiaville et al., 2014b).

It was established in 2012 that t^6A is not the final modification and can be further converted into a cyclic derivative (ct^6A). This reaction is executed by Tcd1 and Tcd2 in yeast (Miyauchi et al., 2012). Since ct^6A can be only detected utilizing neutral nucleotide preparation conditions, it is possible that in many cases t^6A is formed during tRNA isolation and thus represents a derivative of the naturally occurring ct^6A modification (Miyauchi et al., 2012; Thiaville et al., 2014b).

4.2.6 Functional implications of tRNA modifications

Modifications in the tRNA anticodon-stem-loop are shown to support different facets of translational fidelity. In the previous sections the generation of $\Psi_{38/39}$, $mcm^5s^2U_{34}$ and ct^6A_{37} were described and all can be found in specific areas of the ASL. Whereas $\Psi_{38/39}$ is either located in the loop (position 38) or in the stem (position 39), mcm^5s^2U is installed on the last nucleotide of the anticodon and 3'-adjacent ct^6A can be found on position 37. The arrangement and the nature of the modifications has direct consequences for the architecture of the ASL but also for the efficiency of anticodon-codon interaction (Väre et al., 2017) as explained below. Structural integrity of the ASL adds on to the functionality of the tRNA tertiary structure since alterations can affect the general translational capacity of tRNA and ultimately perturb interaction with the ribosome (Väre et al., 2017). Sequences and tertiary base interactions necessary to form the L-shape of tRNAs can strongly vary and it is obligate to modulate nucleotide interaction by tRNA modifications at critical positions (Sprinzl and Vassilenko, 2005; Klug et al., 1974; Grosjean et al., 1996). Pseudouridine is one modification frequently found in tRNAs to structurally support the stability of the tRNA core but also in the ASL (Spenkuch et al., 2014; Väre et al., 2017). Pseudouridylation has been shown to promote so called sugar puckering of the RNA strand by facilitating the 3'*endo* conformation of the ribose's of adjacent nucleotides (Davis, 1995; Sipa et al., 2007; Spenkuch et al., 2014). This sugar conformation enhances the RNA stacking and stabilizes helices and loops, respectively (Davis, 1995; Sipa et al., 2007).

The hydrophobic t^6A fulfills a similar role and (i) stabilizes the anticodon-loop (AL) but also (ii) positively influences anticodon orientation for codon interaction (Väre et al., 2017). It was shown for human $tRNA^{Lys}_{UUU}$ that the modification enhances stacking of the 3' AL-part while it prevents the formation of cross hydrogen bonds e.g. between U_{33} and A_{37} by ribosome interaction (Stuart et al., 2000). This effect ensures the open conformation of the anticodon-loop and allows the anticodons to freely interact with the codons in a Watson-Crick manner during translation (Stuart et al., 2000; Vendeix et al., 2012). However, it is not clear what impact the conversion of t^6A to ct^6A has on the above given functions.

The anticodon-codon interaction is modulated by extensive chemical modification of the AL 34^{th} position to expand or narrow codon recognition (Agris, 1991; Väre et al., 2017). This is necessary, since there are too many possible codons that have to be decoded by a lesser

number of tRNAs and is only feasible if the first anticodon base can bind to non-canonical binding partners termed *wobbling* (Crick, 1966). Nonetheless, the non Watson-Crick base interaction of the *wobble* base is not limited to only modified nucleotides, mitochondrial $tRNA^{Gly}$ with hypomodified uridine for instance has been shown to read all 4 possible glycine codons on the expense of translational fidelity (Lagerkvist, 1978, 1981; Rogalski et al., 2008). Contrastingly, *wobble* position modifications like the $mcm^5s^2U_{34}$ modification was reported to enhance decoding of A- and G-ending codons (Johansson et al., 2008) and to support reading frame maintenance (Tükenmez et al., 2015). In addition, these modifications improve the anticodon-codon interaction in the ribosomal A-site and prevent ribosomal tRNA rejection (Agris, 2008; Bauer et al., 2012; Fernandez-Vazquez et al., 2013; Rezgui et al., 2013).

4.3 Physiological role of tRNA modifications in bakers' yeast

The biochemical properties of $mcm^5s^2U_{34}$, ct^6A_{37} and $\Psi_{38/39}$ outlined in the previous section show the importance of the ASL modifications for structural integrity, anticodon-codon interaction, reading-frame maintenance and tRNA-ribosome interaction. Nonetheless, many studies in the last two decades not only investigated mechanistical but also physiological functions of tRNA modifications. Indeed, loss of $\Psi_{38/39}$, (c)t^6A_{37} and mcm^5/s^2U_{34} can induce pleiotropic consequences which affect the translational capacity of the corresponding mutants (Lecointe et al., 2002; Thiaville et al., 2016; Johansson et al., 2018). Surprisingly, those modification defects are also linked to a variety of other deficiencies e.g. proteome, transcriptome and metabolome homeostasis aberrations as well as premature activation of starvation responses and nutrient signaling abnormalities (reviewed in e.g. Phizicky and Hopper, 2010; Schaffrath and Leidel, 2017; Gupta and Laxman, 2019; Johansson et al., 2018; Sokołowski et al., 2017; Spenkuch et al., 2014; Thiaville et al., 2014b). Hence, the following sections summarize the role of tRNA modifications for the physiology of the eukaryotic system and explain their possible interconnection to extra- and intracellular signals by environmental triggers.

4.3.1 Importance of tRNA modifications during translation

The loss of mcm^5U, mcm^5s^2U, ncm^5U and ncm^5Um can have pleiotropic negative effects on the translational capacity of yeast cells. Hence, the deletion of one of the elongator subunits (e.g. *ELP3*) or regulatory proteins like *KTI11-13*, *SIT4, HRR25* (only loss of function mutations are viable) or *SAP185* together with *SAP190* lead to a slow growth phenotype and vulnerability against different stressors (e.g. high temperature, oxidative reagents) (Phizicky and Hopper, 2010; Ranjan and Rodnina, 2016; Schaffrath and Leidel, 2017; Huang et al., 2005, 2008; Chen et al., 2011b; Sokołowski et al., 2017; Johansson et al., 2018). Further investigations on mcm^5/mcm^5s^2U deficient mutants revealed that modification absence clearly affect the translation process (**Figure 7**) through reduced tRNA-ribosome A-site binding (Rezgui et al.,

2013), ribosomal frame shifting (Tükenmez et al., 2015) and codon specific ribosome pausing (Zinshteyn and Gilbert, 2013; Nedialkova and Leidel, 2015). Notably, ASL-modification loss beside the *wobble* position also affects the translational capacity of tRNA. Deletion of *DEG1* could be demonstrated to promote temperature and drug sensitivity as well as inflicting translational inaccuracy (Bekaert and Rousset, 2005; Lecointe et al., 1998, 2002). On the other hand, loss of t^6A by deletion of essential KEOPS-components causes similar, however, more drastic physiological defects but also impairs ribosome assembly and translational speed in a codon-specific manner (Thiaville et al., 2016).

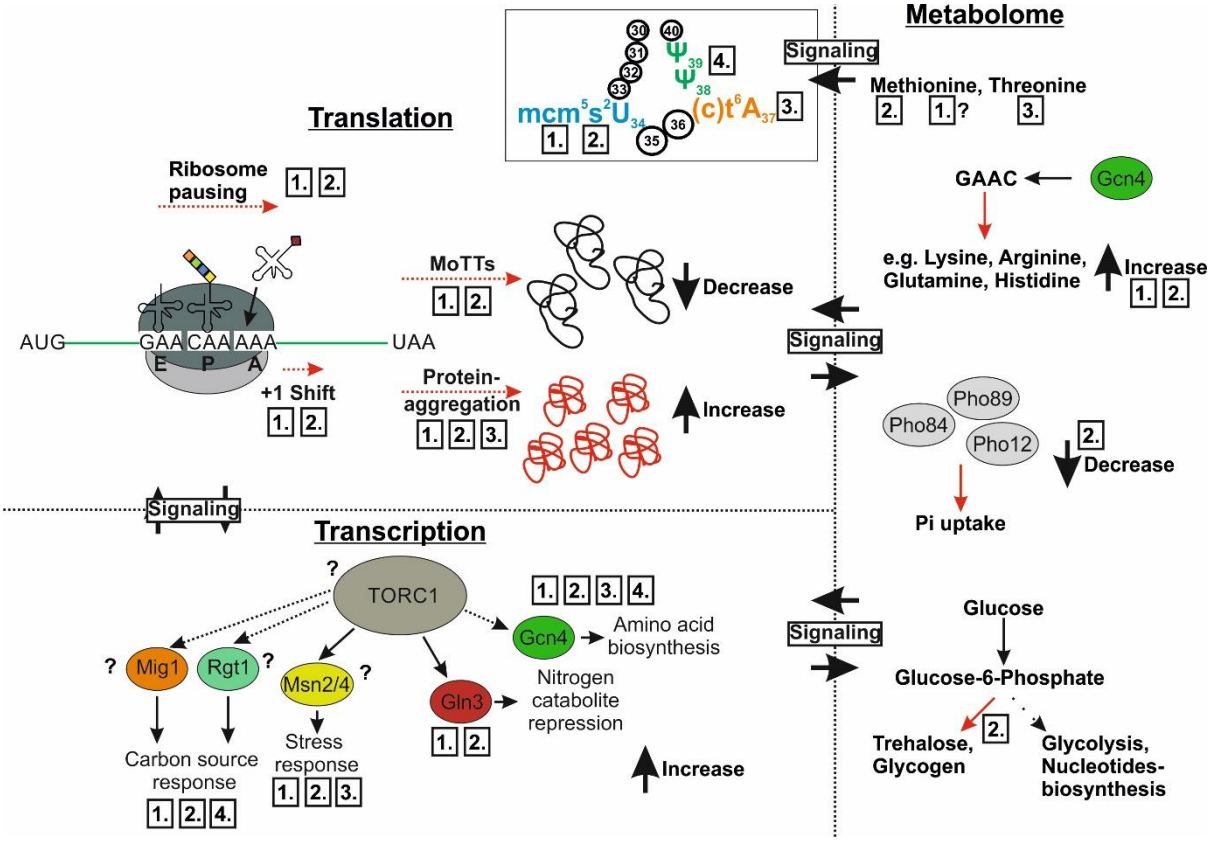

Figure 7 Impact of the individual losses of mcm^5/s^2U_{34}, $(c)t^6A_{37}$ or $\Psi_{38/39}$ on various physiological aspects. Depicted are the ASL-modifications focused in this thesis (top box) which are numbered for easier assignement to specific reported physiological defects upon modification loss (**1.**= mcm^5U_{34}, **2.**=s^2U_{34}, **3.**=$(c)t^6A_{37}$, **4.**=$\Psi_{38/39}$). Various studies describe the involvement of the individual modifications in translational fidelity but also transcriptional and metabolic regulation (three seperated squares) which seem to affect signalling of other regulatory levels (bi-directional arrows and signalling in boxes). Hence, outcome of respective modification loss can result in increased amounts of protein aggregates or impeded translation of MoTTs (translation panel), influencing transcription probably involving TORC1 and/or transcription factor deregulation (**?**, transcription panel) and alteration of metabolite homeostasis (e.g. in the metabolome panel). (Zinshteyn and Gilbert, 2013; Nedialkova and Leidel, 2015; Tyagi and Pedrioli, 2015; Lecointe et al., 2002; Huang et al., 2005; Nakai et al., 2008; Johansson et al., 2008; Leidel et al., 2009; Noma et al., 2009; Patil et al., 2012; de Virgilio, 2012; Rezgui et al., 2013; Laxman et al., 2013; Damon et al., 2014; Scheidt et al., 2014; Gu et al., 2014; Tükenmez et al., 2015; Han et al., 2015; Thiaville et al., 2016; Karlsborn, 2016; Chou et al., 2017; Gupta et al., 2019)

Accordingly, negative influences on translation by hypomodified tRNA impacts protein homeostasis in at least two ways: reduced protein biosynthesis of so-called modification tunable transcripts (MoTTs) and/or protein aggregation. The first event applies to proteins which are encoded by genes enriched for codons that are efficiently translated via modified

tRNAs (**Figure 7**) (Dedon and Begley, 2014; Gu et al., 2014; Endres et al., 2015). Different examples showed that there are various gene transcripts depending on the presence of $mcm^5/mcm^5s^2U_{34}$ (e.g. *YEF3*, *IXR1*) to be properly translated (Begley et al., 2007; Chen et al., 2011b; Patil et al., 2012; Gu et al., 2014; Karlsborn, 2016). It is unclear, what is the exact regulatory task of MoTTs but it is assumed that different modifications are critical during distinct stress situations to translate modification dependent transcripts. For instance, investigations on the m^5C_{34} modification installed among others on $tRNA^{Leu}_{CAA}$ by Trm4 revealed that the modification is necessary to translate oxidative stress response transcripts whereas absence leads to sensitivity against H_2O_2 (Gu et al., 2014). Another report showed the necessity of Elongator dependent modifications for the translation of TORC1 and TORC2 key regulators indicating a more pronounced role of tRNA modifications in nutrient signalling (detailed in the following chapter) (Candiracci et al., 2019). On the opposite, much less is known about the formation of protein aggregates during translation in terms of a possible (in)dependence on mRNA codon composition (i.e. MoTTs) or translational speed stagnation in tRNA modification mutants. However, individual loss of mcm^5/s^2U_{34} or $(c)t^6A_{37}$ promotes the accumulation of protein aggregates (Thiaville et al., 2016; Pollo-Oliveira et al., 2020). Surprisingly, in *S. cerevisiae* all the above-mentioned defects can be rescued by higher than normal doses of hypomodified tRNAs which seem to be malfunctional in the respective mutant (except for mutants lacking t^6A_{37}, see below). In mcm^5/s^2U deficient mutants combined overexpression of $tRNA^{Gln}_{UUG}$ and $tRNA^{Lys}_{UUU}$ is sufficient to compensate negative phenotypes although other tRNA species are also Elongator and/or Urm1-pathway dependent modified (Esberg et al., 2006; Leidel et al., 2009). Hence, one might suggest a native hierarchy of tRNA species/isoacceptors regarding their importance for the translational process in yeast. This is even more pronounced in the *deg1* mutant where $tRNA^{Gln}_{UUG}$ is the only known tRNA to rescue reported negative phenotypes despite of the high amount of Deg1 modified tRNA species (Klassen and Schaffrath, 2017; Han et al., 2015). KEOPS-mutants do represent an exception to this rule since overexpression of various tRNAs normally harbouring t^6A_{37} could not rescue any growth phenotypes (Thiaville et al., 2016). Thus, the complex might fulfil other tasks beside tRNA modification which was supported by experiments indicating that KEOPS can recognize and bind double-strand breaks (DSB), implicating a role in DNA-damage repair (He et al., 2019).

4.3.2 Essential co-factors for the modification of tRNAs

As already described (see **4.2.3**-**4.2.5**), 3 of 4 modifications (mcm^5U, s^2U, t^6A) depend on co-factors to be synthesized which either provide the chemical group to be added onto the tRNA (sulfur, carbonyl-methyl or methyl groups) or are completely utilized for modification (L-threonine) (**Figure 7**, up right). The mcm^5U_{34} installation is achieved under consumption of acetyl-CoA and S-adenosylmethionine (SAM) (Selvadurai et al., 2014; Lin and Glatt, 2018;

Dauden et al., 2019; Lin et al., 2019), whereas the subsequent thiolation requires cysteine or methionine as sulphur source (Nakai et al., 2008; Leidel et al., 2009; Noma et al., 2009; Laxman et al., 2013; Jüdes et al., 2016). Uridine 34 thiolation is the best investigated example in this regard since methionine depletion was demonstrated to diminish s^2U_{34} formation which ultimately affected translational efficiency (Laxman et al., 2013; Jüdes et al., 2016). The composition of sulphur containing amino acids (i.e. methionine and cysteine) starts with the uptake of sulphate from the environmental media and further processing to form sulphide (S^{2-}) (Ljungdahl and Daignan-Fornier, 2012). Met17 incorporates S^{2-} and O-acetylhomoserine, which is synthesized on basis of homoserine, resulting in homocysteine formation (Thomas and Surdin-Kerjan, 1997). This molecule is fed among others into the two branches of L-methionine and/or L-cysteine biosynthesis (Ljungdahl and Daignan-Fornier, 2012).

Importantly, L-methionine can be further processed by Sam1 and Sam2 resulting in SAM formation which is a well-established co-factor for various pathways (Cherest and Surdin-Kerjan, 1978; Thomas and Surdin-Kerjan, 1997). In fact, SAM can act as methyl-donor for a multitude of reactions including, as mentioned above, elongator dependent tRNA modification (Kalhor and Clarke, 2003; Pintard et al., 2002) but can in this regard also provide other RNA modification processes like the wybutosine-formation (yW) with a caboxyaminopropyl-moiety (acp) (Umitsu et al., 2009). Resulting by-products of these SAM-consuming reactions (e.g. S-adenosylhomocysteine) are recycled by Sah1 and Met6 to again provide L-methionine to the methyl cycle (Thomas and Surdin-Kerjan, 1997; Ljungdahl and Daignan-Fornier, 2012).

Notably, L-aspartate derived homoserine serves as branch point leading into L-methionine (as described above) or L-threonine biosynthesis. The latter process requires Hom2, Hom3 and Hom6 to convert L-aspartate into this serine derivative which is then recognized by the homoserine kinase Thr1 (Jones and Fink, 1985; Ramos and Calderón, 1994; Schultes et al., 1990). The kinase phosphorylates homoserine forming O-phospho-homoserine that is further processed by Thr4 resulting in L-threonine (Ramos and Calderón, 1994; Mannhaupt et al., 1990). This amino acid represents, as described earlier, the core molecule for the synthesis of the N6-threonylcarbamoyl moiety (Wan et al., 2013).

Thus, the different modification cascades of the tRNA modifications subject to this study depend on the supply of specific amino acids and/or nutrients (**Figure 7**, up right). On the opposite, individual abolishment of the mcm^5/s^2U_{34}, t^6A_{37} or also $\Psi_{38/39}$ is reported to affect the transcriptome and metabolome activating various metabolic relevant pathways like nitrogen catabolite repression (NCR) or general amino acid controlled genes (GAAC) (**Figure 7**, transcription and metabolome panel) (Zinshteyn and Gilbert, 2013; Nedialkova and Leidel, 2015; Tyagi and Pedrioli, 2015; Patil et al., 2012; Laxman et al., 2013; Rezgui et al., 2013; Damon et al., 2014; Scheidt et al., 2014; Han et al., 2015; Thiaville et al., 2016; Karlsborn et al., 2016; Chou et al., 2017; Gupta et al., 2019). Specifically the Gcn4 dependent induction of

GAAC controlled genes appears to be a general symptom linked to the loss of tRNA modifications and might suggest a more intimate relationship between tRNA modifications and amino acid synthesis or to the global metabolic state of the cell (Chou et al., 2017). This possible regulatory network will be detailed in the following chapters.

4.4 Interconnection between the regulome and tRNA modifications?

The modification of transfer RNAs depends on the supply of nutrients, amino acids and other metabolites which either function as co-factors or are incorporated in the modification. Knowing that, it can be assumed that the extra- and intracellular amount of nutrients might influence the modification status of a tRNA, diminishing one or more specific modifications. Such an effect was already described for the thiolation process of wobble uridines (Laxman et al., 2013; Jüdes et al., 2016; Gupta et al., 2019). Conversely, artificial abolishment of modifications by deleting key modifier genes (e.g. *elp3*, *urm1*) have been reported to not only affect translation but also the regulation of the transcriptome and metabolome (see previous section). How the loss of tRNA modifications precisely affect the different physiological aspects and what regulatory mechanisms are controlling those effects is only emerging to be understood. Nevertheless, the following chapters try to give an overview of what is known so far about the interconnection of tRNA modifications and the different regulatory layers in the eukaryotic system.

4.4.1 Regulation of transcriptional and translational adaptation in response to nutrient limitation

Adaptation of cellular growth in response to nutrient depletion is controlled by the activity of different kinases which pass on starvation signals onto downstream effectors. In this regard, TORC1 and TORC2 are two complexes which are either necessary for the control of the nitrogen and carbon metabolism (first complex) or three-dimensional cell growth (second complex), respectively (de Virgilio, 2012; Smets et al., 2008; Conrad et al., 2014). *S. cerevisiae* is the only eukaryote known so far to possess two mostly identical (67%) kinases encoded by *TOR1* and *TOR2* of which the expressed proteins are differentially employed in the two mentioned complexes (de Virgilio, 2012; Smets et al., 2008; Conrad et al., 2014). Whereas Tor1 or Tor2 can associate with Kog1, Lst8 and Tco89 to form TOR complex 1, only Tor2 is found together with Avo1-3, Lst8 and Bit61 to compound TORC2 (de Virgilio, 2012; Loewith et al., 2002; Wedaman et al., 2003; Reinke et al., 2004; Smets et al., 2010; Conrad et al., 2014). Despite the well-known composition of both complexes, only the function of TORC1 has been studied in great detail.

TORC1 is localized to the membrane of the vacuole (or lysosome for mammals) which is necessary to monitor the nutrient availability (i.e. nitrogen and/or amino acids) by the amino acid flux between the organelle and cytoplasm (**Figure 8**) (Zoncu et al., 2011). Good nitrogen availability is measured by the generation of glutamine (Gln) and glutamate (Glu) since only

preferred nitrogen sources can easily be converted into these amino acids (Magasanik and Kaiser, 2002). Importantly, both Glu and Gln also represent the basis for the synthesis of all other amino acids (Magasanik and Kaiser, 2002; Ljungdahl and Daignan-Fornier, 2012) and accordingly Glu and Gln levels provide information about the cellular nutrient status (at least for nitrogen consuming metabolites). These signals are passed to TORC1 by interaction with various other protein complexes also located at the organelle membrane (de Virgilio, 2012; Smets et al., 2010; Conrad et al., 2014) like the EGO complex (EGOC) comprised of Ego1, Ego3, Gtr1 and Gtr2 (Dubouloz et al., 2005). EGOC keeps TORC1 in an active state according to the amino acid level which is facilitated by Gtr1-GTP binding to Kog1 and Tco89 (Binda et al., 2009; Conrad et al., 2014). As soon as GTP is replaced by GDP via the guanine nucleotide exchange factor (GEF) Vam6, the interaction of the complexes diminishes and TORC1 is inactivated (Binda et al., 2009). This mode of action is one way to inhibit the regulatory complex activity and many more have been discovered in recent years, among them the inactivation of the kinase complex by treatment with rapamycin that binds to Fpr1 which then inhibits TORC1 (**Figure 8**) (Schreiber, 1991).

Nevertheless, various effector proteins are subsequently either dephosphorylated or indirectly affected which can influence their localization, activity, interaction pattern and target priority. Modulating the activity of the (master-regulator) TOR complex 1 can be therefore assumed as a first layer of regulation that engages a signaling cascade. Downstream communication is among others facilitated by two main TOR-pathway participants Sch9 and Tap42 which are both direct phosphorylation targets of the complex (Di Como and Arndt, 1996; Jiang and Broach, 1999; Urban et al., 2007). This posttranslational modification keeps Sch9 active, leading to the phosphorylation of different target proteins and thereby affects their localization in response to stress and starvation (Smets et al., 2010; Conrad et al., 2014). For instance, Maf1 represents one target that is frequently phosphorylated by Sch9 upon nutrient replete conditions which avoids the inhibition of the RNA polymerase III (RNA pol III, **Figure 8**) (Huber et al., 2009).The same mode of action blocks the activation of general stress responses under optimal growth conditions by hindering Msn2, Msn4 (transcription factors) and Rim15 (kinase) to translocate into the nucleus (Urban et al., 2007; Wanke et al., 2008).

On the opposite acts phosphorylated Tap42, assisted by Tip41, as an inhibitor of the phosphatase complex by binding the catalytic subunits of Sit4 and PP2Ac (**Figure 8**) which promotes association to TORC1 (Di Como and Arndt, 1996; Jiang and Broach, 1999). Upon starvation conditions, TORC1 inactivation leads to a decline of Tap42 phosphorylation followed by the release of the phosphatase complex into the cytoplasm (Yan et al., 2006). This event also prevents the nuclear export of at least one Sch9-phosphorylation target Msn2 (Santhanam et al., 2004) and possibly also Msn4. Moreover, nuclear localized Msn2/4 are also functionally controlled by Rim15 by a yet unknow mechanism, as soon as the kinase is also situated in the

nucleus (see above) (Pedruzzi et al., 2003; Cameroni et al., 2004; Roosen et al., 2005). Canonically, nutrient- or more specifically nitrogen-depleted conditions also promote the dephosphorylation of the transcription factor Gln3 and anchor protein Ure2 by the phosphatase complex, which under good nutrient supply are both hyperphosphorylated by TORC1 (**Figure 8**) (Beck and Hall, 1999; Cardenas et al., 1999; Hardwick et al., 1999; Shamji et al., 2000). The extensive phosphorylation of Gln3 and Ure2 forces the association of both proteins to each other and blocks nuclear entrance of the transcription factor and with that the induction of NCR genes (Coschigano and Magasanik, 1991; Beck and Hall, 1999; Blinder et al., 1996; Feller et al., 2013).

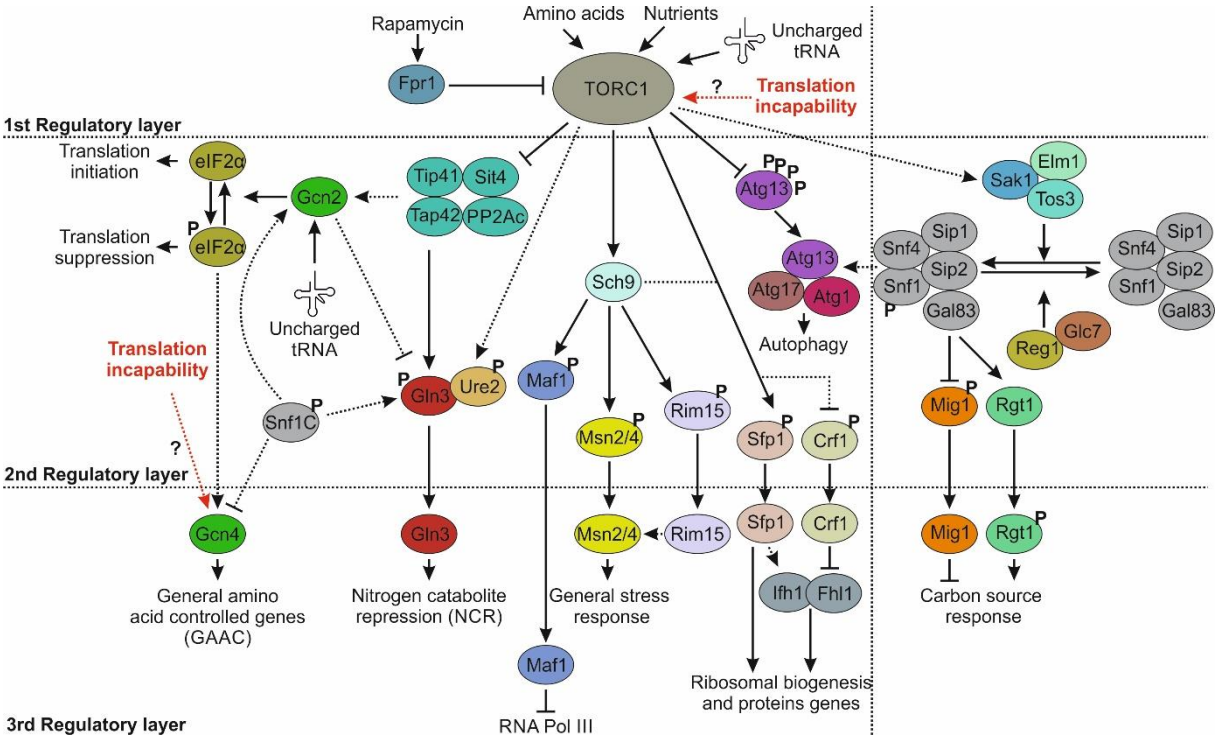

Figure 8 Scheme of the different regulatory layers influencing starvation responses controlled in-/directly by TORC1. The *target of rapamycin complex 1* activity is influenced by a wide variety of signals e.g. amino acids, nutrients, uncharged tRNA and negative regulator Fpr1 upon Rapamycin treatment (**first regulatory layer**). These occasions consequently inactivate TORC1 kinase function impacting phosphorylation/regulation of multiple downstream targets. Among these are represented the phosphatase complex PP2A (including Sit4, Tip41 and Tap42), Ure2, Sch9, Sfp1, Crf1, Atg13 and the Snf1 regulation. Accordingly, the **second regulatory layer** represents a complicated regulatory network in which abolishment of TORC1 dependent phosphorylation/regulation activates a cascade of downstream regulatory units affecting e.g. translation, transcription factor localisation and consequently stress responses (for more details see text). Transcriptional activation/repression of the indicated pathways requires the re-localization of transcription factors like Gln3, Maf1, Msn2/4 or Mig1 (**third regulatory layer**) which critically depend on the phosphorylation status of these proteins also modulating in some cases the interaction with regulatory binding partners (e.g. Gln3 and Ure2). Translational incapability caused by tRNA modification loss (indicated in red) might influence TORC1 activity or Gcn4 translation, respectively, in tRNA modification mutants by a until now unknown mechanism. The Snf1 complex depicted on the right half (with interaction partners and also represented by Snf1C) is regulated by its phosphorylation status balanced by kinases Sak1, Elm1 and Tos3 on the one hand and the phosphatases Reg1 and Glc7 on the other which depends on the glucose supply. Arrows and bars indicate negative and positive interaction, respectively. Indirect or putative interactions are represented by dashed lines. Taken and adapted from (Hinnebusch, 2005; Santangelo, 2006; Beck and Hall, 1999; Cherkasova and Hinnebusch, 2003; Düvel and Broach, 2004; Chen and Powers, 2006; Hedbacker and Carlson, 2009; Ljungdahl and Daignan-Fornier, 2012; Cardenas et al., 1999; Hofman-Bang, 1999; Gasch et al., 2000; Crespo et al., 2002; Schawalder et al., 2004; Rohde et al., 2008; Smets et al., 2010; de Virgilio, 2012; Chou et al., 2017)

Additionally, a decline in nutrients requires the cell to adjust protein biosynthesis to spare energy and metabolites which also lies in the responsibility of TORC1. Hence, the complex controls the expression of genes necessary for ribosome biogenesis (Ribi), ribosomal proteins (RP) and rRNA. RP gene expression by RNA polymerase II is induced via Fhl1 binding to the respective promoters whereas for that the transcription factor have to be in complex with its co-activator Ifh1 (Lee et al., 2002; Martin et al., 2004; Schawalder et al., 2004; Wade et al., 2004; Rudra et al., 2005). The co-repressor Crf1 is competing with Ifh1 for Fhl1 interaction upon nutrient depleted conditions and in consequence suppresses RP biosynthesis (**Figure 8**) (Martin et al., 2004; Zhao et al., 2006). Active TORC1 is believed to indirectly promote Fhl1-Ifh1 complex formation via PKA and Yak1 dependent phosphorylation of Crf1 to sequester the co-repressor in the cytoplasm (Zhao et al., 2006; Smets et al., 2010). However, the mechanism of this regulatory link has not been elucidated and furthermore Crf1 is not present in all eukaryotes raising the need for alternative RP gene regulation mechanisms (Zhao et al., 2006; Smets et al., 2010). Regarding this, Sfp1 represents another transcription factor necessary for RP biosynthesis and Ribi gene expression which seems to support by a so far not elucidated process Fhl1-Ifh1 complex localization to the nucleus and with that transcriptional activation (Jorgensen et al., 2004). Sfp1 is thought to be directly phosphorylated by TORC1 and re-localizes into the nucleus supporting RP and Ribi gene expression during exponential growth as long as the Tor1 complex is not inactivated upon general or nutrient stress conditions (**Figure 8**) (Jorgensen et al., 2004; Marion et al., 2004; Lempiäinen et al., 2009).

Besides its involvement in regulation of RNA pol II for RP and Ribi gene expression, TORC1 influences RNA Pol I and III activity for the synthesis of 35S rRNA and tRNA. This is achieved by modulating the interaction of activators (e.g. Rrn3 for RNA Pol I) or repressors (e.g. Maf1 for RNA Pol III, see above) with the RNA polymerases, or involves the direct interaction of Tor1 to the 35S and 5S rDNA loci which supports the recruitment of both polymerases to the respective rRNA and tRNA genes (Claypool et al., 2004; Li et al., 2006; Huber et al., 2009; Wei et al., 2009). Moreover, the target of rapamycin 1 complex is involved in the regulation of the translational machinery. Different reports indicate that TORC1 indirectly promotes interaction of eIF4G and eIF4E therefore supports translational initiation (Berset et al., 1998). It is also indirectly involved in the Ser577 phosphorylation of Gcn2 achieved by an unknown kinase, preventing the phosphorylation of eIF2α and thereby inhibition of translation initiation. In addition, dephosphorylation and activation of Gcn2 upon TORC1 inactivation is assumed to be executed by Tap42-unblocked Sit4 (Cherkasova and Hinnebusch, 2003; Kubota et al., 2003; Hinnebusch, 2005). This mode of action is thought to cooperatively enhance the canonical GAAC pathway induction together with the binding of uncharged tRNA by Gcn2 (**Figure 8**, see also following chapter) (Cherkasova and Hinnebusch, 2003; Garcia-Barrio et al., 2002; Staschke et al., 2010; Conrad et al., 2014). Interestingly, the amino-acylation status

as well as the loss of critical modification/s of tRNAs also seem to represent immanent signals influencing the activity of the TORC1 (Kamada, 2017; Scheidt et al., 2014). This was in part supported by investigations of the *elp3* mutant that increased various metabolites indicative for the induction of the regulatory kinase complex controlled anabolic pathways (Karlsborn, 2016; Karlsborn et al., 2016). Additionally, loss of uridine thiolation in this regard uncovered metabolic adaptations depending on the induction of the GAAC, storage carbohydrate synthesis and nitrogen uptake which was suggested to balance the phosphate household of the respective mutants (Gupta and Laxman, 2019; Gupta et al., 2019). Moreover, the Elongator dependent modification cascade is also regulated by the PP2A phosphatase Sit4 (see chapter **4.2.3**) and appears to regulate the activation of TORC1 in *Schizosaccharomyces pombe* (Mehlgarten et al., 2009; Candiracci et al., 2019). These findings support a more intricate network between the Tor1 complex and translational regulation expanding the repertoire of how the kinase complex influences and is influenced by the translational machinery. Thus, tRNA modifications might not only affect translational capability (see chapter **4.2.6** and **4.3.1**) but could also serve as another unappreciated regulator of nutrient dependent expression adaptation. Regarding this, the mechanism, however, how tRNA modification defects might influence the activity of TORC1 or other regulatory pathways still remains elusive (**Figure 8**, see also the following chapter).

Besides downregulation of translation cells also enforce recycling of amino acids, fatty acids and other metabolites upon nutrient limitation by degrading different compartments in the vacuole which was termed autophagy. TORC1 is intimately regulating activation of this process through distinct direct and indirect signals, respectively (Noda and Ohsumi, 1998). For instance, the complex directly phosphorylates the autophagy protein Atg13 which inhibits association with Atg1 and Atg17 (**Figure 8**) (Kamada, 2010; Kamada et al., 2000, 2010; Cheong et al., 2005; Kabeya et al., 2005). This interaction is necessary for the kinase activation of Atg1 and leads to formation of the Atg1 complex (Kamada, 2010; Kamada et al., 2000, 2010; Cheong et al., 2005; Kabeya et al., 2005). Upon activation, this complex associates with Atg31 and Atg29 bridged by Atg17 and later on with various autophagy core proteins which is necessary to initiate autophagosome formation promoting the organization of the essential phagophore assembly site/pre-autophagosomal structure (PAS) (Kamada, 2010; Kamada et al., 2000, 2010; Kawamata et al., 2005, 2008; Kabeya et al., 2007). In addition to TOR-dependent regulation, autophagy is also controlled by protein kinase A (PKA) and AMP activated kinase Snf1. Various reports indicate that PKA, in cooperation with Sch9, negatively regulates the autophagy induction in which PKA-dependent Atg1 and Atg13 phosphorylation seems to be relevant for the inhibition by impeding the association of both proteins to the PAS (Papinski and Kraft, 2016; Budovskaya et al., 2005; Yorimitsu et al., 2007; Stephan et al., 2009;). On the opposite, Snf1 was demonstrated to positively regulate autophagy by an

unknown mechanism since deletion of the kinase completely abolished the induction of this process (**Figure 8**) (Wang et al., 2001).

Besides its participation in autophagy, Snf1 is found to be involved in the regulation of various stress responses (e.g. respiratory metabolism, gluconeogenesis, glycogen accumulation) which are majorly activated upon glucose deprivation (Hedbacker and Carlson, 2009; Usaite et al., 2009; Smets et al., 2010; de Virgilio, 2012; Conrad et al., 2014). For that, Snf1 functions in a complex composed of itself, Snf4 and one of the three Sip1, Sip2 or Gal83 subunits probably responsible for substrate specificity (**Figure 8**) (Celenza and Carlson, 1989; Jiang and Carlson, 1997; Hedbacker and Carlson, 2009; Celenza et al., 1989; Yang et al., 1994). The complex is activated by the redundantly operating kinases Sak1, Elm1 and Tos3 phosphorylating threonine 210 of Snf1 and conversely blocked via dephosphorylation by the Glc1-Reg7 phosphatase complex (**Figure 8**) (McCartney and Schmidt, 2001; Estruch et al., 1992; Wilson et al., 1996; Ludin et al., 1998; Sanz et al., 2000; Sutherland et al., 2003; McCartney et al., 2005). This balance of phosphorylation-status dependent activation of the Snf1 complex ensures a precise transcriptional reaction according to the glucose supply. Snf1 activation results in phosphorylation of different downstream targets by the kinase complex, including the transcriptional repressor Mig1 and the activator Rgt1 (**Figure 8**) (Smith et al., 1999; Palomino et al., 2006; Zaman et al., 2009). Interestingly, Snf1 has also been shown to be involved in the regulation of Gln3, the transcriptional activator of NCR upon glucose depletion (Bertram et al., 2002) and negatively modulates GAAC transcription factor Gcn4 via its translation and/or transcriptional induction activity (Shirra et al., 2008). Moreover, Snf1 promotes Gcn2 activity and in consequence eIF2α phosphorylation supporting thereby GAAC induction (**Figure 8**) (Cherkasova et al., 2010). Besides that, phosphorylation of Snf1 is affected by nitrogen starvation and accordingly by a TORC1 dependent manner (Orlova et al., 2006). That probably indicates a regulatory network in which the different major kinases are directly or indirectly interconnected.

4.4.2 Modification defects: implications on Gcn2/Gcn4 regulation

The above described mechanism, exemplified for the Snf1 and Tor1 complexes, might be necessary to finetune activation of stress and starvation responsive pathways. Modification of transfer RNAs may in this regard play a vital role either by modulating translational capability, representing signals for the nutrient availability or a mix of both. Hence, a common effect reported upon loss of tRNA modifications is the activation of the general amino acid control (GAAC) pathway (Zinshteyn and Gilbert, 2013; Thiaville et al., 2016; Chou et al., 2017).

Canonically, it is activated upon reduced intracellular levels of amino acids which is recognized due to increasing amounts of non-amino-acylated (uncharged) tRNAs (Zaborske et al., 2009, 2010). Gcn2 monitors for and binds to uncharged tRNAs (**Figure 8**) unblocking its kinase activity. Subsequently, the kinase phosphorylates the initiation factor 2 subunit α (eIF2α) and

thereby prevents the formation of the ternary complex (eIF2-GTP, Met-$tRNA_i^{Met}$) necessary for translation initiation (Ljungdahl and Daignan-Fornier, 2012; Wek et al., 1989; Dong et al., 2000; Qiu et al., 2001). Global translation consequently drops allowing efficient expression of the transcription factor Gcn4. This regulatory mechanism involves four upstream open reading frames (uORF) located in the 5′-UTR of the *GCN4* mRNA which blocks the translation of the main ORF (Mueller and Hinnebusch, 1986). Upon amino acid starvation and decreased translation initiation the ribosome skips the uORFs allowing efficient translation of the *GCN4* main ORF (Abastado et al., 1991; Hinnebusch, 2005). Subsequently, Gcn4 activates genes involved in the GAAC and NCR as well as encoding for various protein kinases and other targets (Hinnebusch and Natarajan, 2002; Ljungdahl and Daignan-Fornier, 2012; Natarajan et al., 2001; Tate et al., 2017).

Surprisingly, different *elp* mutants, Urm1-pathway related deletions as well as t^6A or $\Psi_{38/37}$ deficient strains activate GAAC-controlled genes (Zinshteyn and Gilbert, 2013; Nedialkova and Leidel, 2015; Thiaville et al., 2016; Chou et al., 2017). Interestingly, however, these mutants display no decrease in the cellular levels of charged tRNAs *in vivo* (Johansson et al., 2008; Han et al., 2015) or the isolated hypomodified tRNA was found to exhibit wild type-like charging efficiencies *in vitro* (Thiaville et al., 2015). Moreover, and in correlation to this observation, anticodon-loop modification mutants enhanced translation of Gcn4 (Zinshteyn and Gilbert, 2013; Nedialkova and Leidel, 2015; Thiaville et al., 2016; Chou et al., 2017; Gupta et al., 2019). However, Gcn2 appeared to be not involved in the amino acid starvation response of mcm^5/s^2U and t^6A deficient mutants since disruption of *GCN2* did not abolish the induction of *GCN4* mRNA translation (Zinshteyn and Gilbert, 2013; Thiaville and de Crécy-Lagard, 2015), pointing to a non-canonical GAAC induction.

This data in summary indicates a correlation between the non-canonical Gcn4-dependent induction of the GAAC and the translational incapability of several tRNA modification mutants (**Figure 8**) (Chou et al., 2017). Nevertheless, it is not clear what mechanism might induce such a transcriptional response and why tRNA modification defects are a predominant driver of general amino acid-controlled gene induction.

5. Aims

Studies in the last decades focused on the individual loss of specific tRNA modifications to examine their function in the translation context. However, tRNAs are modified on multiple positions by different pathways, creating a unique pattern of modifications on every tRNA species (Phizicky and Hopper, 2010; El Yacoubi et al., 2012; Lorenz et al., 2017; McCown et al., 2020). First indications of a functionally cooperation of these modifications were obtained in studies on the $mcm^5s^2U_{34}$ modification, which is installed by two partially independent pathways (Nedialkova and Leidel, 2015; Huang et al., 2005, 2008; Johansson et al., 2008; Nakai et al., 2008; Leidel et al., 2009; Noma et al., 2009). Loss of both modifications aggravated the growth phenotypes of the corresponding double mutants and severely compromised the translation process (Nedialkova and Leidel, 2015). At the same time, other studies on the absence of two tRNA methylations in a *trm8 ncl1* mutant lead to the destabilization of a specific tRNA and triggered its degradation upon heat stress by a newly established tRNA surveillance pathway (Alexandrov et al., 2006; Chernyakov et al., 2008). Those examples conclusively implied a modification network on transfer RNAs provoking the idea of functionally cooperating modifications to ensure different aspects of a tRNAs features and task, and may therefore have strong regulatory power in eukaryotic organisms like *Saccharomyces cerevisiae* (Sokołowski et al., 2017). Hence, this project pursues the idea by establishing new cooperation networks based on a synthetic genetic array (SGA) analysis utilizing genes of four tRNA anticodon-loop modification pathways as a hub for the generation of double mutants (**Publication I**). These tRNA modification mutants lack different combinations of the well-studied mcm^5/s^2U_{34} together with $\Psi_{38/39}$ (*elp3*/*urm1 deg1*) or ct^6A_{37} (*elp3*/*urm1 tcd1*) and are used for subsequent studies. Those are orienting on the previous reports and involve different aspects of the physiological role of the presumed cooperating tRNA modifications:

- Investigation on the phenotypic consequences induced by combined modification defects
- Identification of the majorly dysfunctional tRNA species and translational complications like decreased translational fidelity, weakly translated mRNAs (modification tunable transcripts=MoTTs) or the induction of protein aggregation (Dedon and Begley, 2014; Nedialkova and Leidel, 2015; Lecointe et al., 2002; Gu et al., 2014; Tükenmez et al., 2015; Thiaville et al., 2016; Joshi et al., 2018)
- Examination of the induction of proteotoxicity preventive pathways like the unfolded protein response (Patil et al., 2012)
- RNAseq analysis to uncover transcriptome aberrations presumably promoted by translational corruption and establishing a possible link between both events (Zinshteyn and Gilbert, 2013; Nedialkova and Leidel, 2015; Laxman et al., 2013; Thiaville et al., 2016; Chou et al., 2017)

6. Publications

6.1 Publication I

Klassen, R., Ciftci, A., Funk, J., **Bruch**, **A**., Butter, F., & Schaffrath, R. (2016). tRNA anticodon loop modifications ensure protein homeostasis and cell morphogenesis in yeast. *Nucleic acids research*, *44*(22), 10946-10959.

6. Publications

6.1.1 Transition between publication I and II

Investigations on the function of tRNA modifications in the last decades focused on single modifications, their role in translation regulation and cell physiology. Functional cooperation of specific tRNA modifications and consequences upon their loss were only elusively studied and represent a mostly untouched aspect of the epitranscriptome. Hence, this project aimed to find indications of a functional cooperation network of modifications and characterize their physiological role by concentrating on the anticodon-stem and loop (ASL) of tRNAs. While the ASL represent a modification hotspot, the nucleotides specifically at the *wobble* position 34 as well as position 37 are strongly modified by a huge variety of chemical side chains (Phizicky and Hopper, 2010; El Yacoubi et al., 2012; Lorenz et al., 2017; McCown et al., 2020). The mcm^5s^2U moiety for instance is installed on a position 34 uridine of three tRNAs ($tRNA^{Glu}_{UUC}$, $tRNA^{Lys}_{UUU}$, $tRNA^{Gln}_{UUG}$) by two partially independent pathways, the Elongator complex and the Urm1-modification cascade (Huang et al., 2005, 2008; Johansson et al., 2008; Nakai et al., 2008; Leidel et al., 2009; Noma et al., 2009). Based on a synthetic genetic array (SGA) analysis, genes of the two pathways appeared to negatively interact with three other tRNA modifiers. One gene coded for *DEG1*, a pseudouridine synthetase known to isomerize uridines to Ψ at position 38/39 in the ASL and the other two were *TCD1* and *TCD2* which are converting t^6A_{37} to ct^6A_{37} (Lecointe et al., 1998; Miyauchi et al., 2012).

Combined deletion of essential tRNA modification genes resulted in the four double mutants *urm1 tcd1*, *elp3 tcd1*, *urm1 deg1* and *elp3 deg1* which were phenotypically characterised and examined for their majorly defective tRNAs. All double mutants displayed aggravated sensitivity against mild and strong heat stress compared to the wild type or the corresponding single mutants whereas *elp3 deg1* showed the severest phenotypes. Surprisingly, bioinformatics indicated that only two tRNAs appeared to be decorated with the considered modification combinations: the $tRNA^{Gln}_{UUG}$ (mcm^5/s^2U and Ψ_{38}) and $tRNA^{Lys}_{UUU}$ (mcm^5/s^2U and ct^6A_{37}). The *in silico* analysis was challenged by tRNA overexpression experiments in the respective mutants which resulted in the rescue of the growth phenotypes by the two mentioned tRNAs upon heat stress. Thus, the experiment confirmed that the $tRNA^{Gln}_{UUG}$ appeared to be majorly defective in the *deg1* combinations while the defects of the *tcd1* combinations seemed to be caused by the hypomodified $tRNA^{Lys}_{UUU}$. Further morphological characterisations revealed severe cytological deficiencies affecting bud site selection, nuclear segregation, cytokinesis and the actin cytoskeleton which led to an accumulation of worm-like cell clusters among the double mutants.

Since the growth defects of the double mutants were rescued by the two above mentioned tRNAs, the cytological abnormalities were assumed to be caused by compromised translation upon tRNA modification loss. Hence, the translational capacity of the different mutants was

investigated. Indeed, the mRNA encoding glutamine rich Rnq1 emerged to be weakly translated specifically in the *deg1* combinations and this effect was rescuable by higher than normal doses of $tRNA^{Gln}_{UUG}$. Moreover, all examined double mutants accumulated protein aggregates which together with the Rnq1 results indicated a truly corrupted proteome probably responsible for the cytological defect. This hypothesis was tested on morphological inconspicuous WT and *deg1* yeast cells by overexpressing a non-aggregating and an aggregation-prone huntingtin variant. The latter indeed induced the formation of morphological defects comparable to the double mutants. In sum, these results implied a link between translation defects, protein aggregation and the cytological deficiencies all caused by the loss of critical tRNA modifications.

According to this first report, it seemed to be justified to intensify the investigations on the nature of the defects occurring during translation. Earlier studies on the loss of mcm^5/s^2U already indicated a lack of translational reading frame maintenance, ribosomal pausing at AAA and CAA codons and ribosomal tRNA rejection at the A-site hinting to a plethora of deleterious events happening upon modification defects (Zinshteyn and Gilbert, 2013; Nedialkova and Leidel, 2015; Ranjan and Rodnina, 2017; Rezgui et al., 2013; Tükenmez et al., 2015; Joshi et al., 2018). Moreover, deletion of *DEG1* or components of the KEOPS complex and consequently loss of $\Psi_{38/39}$ or t^6A_{37}, respectively, also negatively influenced the translation process indicating that individual absence of critical tRNA modifications already account to the perturbation of protein biosynthesis (Lecointe et al., 2002; Thiaville et al., 2016).However, only little was known about the contribution of cooperating tRNA modifications on the translation accuracy specifically of anticodon loop localized groups. Hence, the following study focused on mutants lacking individually or combined the ct^6A_{37} and $mcm^5s^2U_{34}$ modifications to characterize their benefit on the translation process.

6.2 Publication II

Klassen, R., **Bruch**, **A**., & Schaffrath, R. (2017). Independent suppression of ribosomal+ 1 frameshifts by different tRNA anticodon loop modifications. *RNA biology*, *14*(9), 1252-1259.

6. Publications

6.2.1 Transition between publication II and III

In principle, two models were investigated which aim to describe ribosomal frame shifting events due to the loss of critical tRNA modifications. The first one emanates from the findings that the hypomodified $tRNA^{Lys}_{UUU}$ is rejected from the ribosomal A-site and the ribosome pausing at the AAA codon (Zinshteyn and Gilbert, 2013; Nedialkova and Leidel, 2015; Rezgui et al., 2013;). This would give the tRNA in the P-site the time to move one nucleotide in the +1 frame direction. That A-site effect would therefore be responsive to higher doses of the defective tRNA which would increase the possibility that the right tRNA is loaded in due time to the ribosome leading to a suppression of frame shifting. A P-site effect at the same time would assume that the malfunctional $tRNA^{Lys}_{UUU}$ is switching frame when occupying the P-site. In contrast to the A-site effect, this event would rely on an already accepted hypomodified tRNA which conversely would not be rescuable by overexpression of the respective tRNA.

In order to test both models and the contribution of mcm^5s^2U and ct^6A (alone or together) on frame accuracy, a *lacZ* based assay was conducted with an integrated frame shift site to monitor AAA lysine-codon-anticodon interaction (Belcourt and Farabaugh, 1990; Tükenmez et al., 2015). First, the corresponding construct was transformed in mutants deficient for either mcm^5/s^2U alone (*elp3*, *urm1*) or in combination (*elp3 urm1*) to (i) confirm previous findings on the importance of the individual modifications on frame maintenance (ii) but also characterize the impact of the combined loss on frame shifting events (Tükenmez et al., 2015). As expected, the measured *lacZ* activity indicative for enhanced frame shifting increased in *elp3* and *urm1* compared to the WT. Additive effects in the double mutant were observable supporting the idea of a cooperation of both modifications to ensure codon-anticodon interaction as well as frame accuracy. Interestingly, $tRNA^{Lys}_{UUU}$ overexpression was sufficient to reduce frame shifting in all mutants significantly which underlined the first model.

In a second experiment, the influence of heat induced tRNA thiolation inhibition was tested with the same assay. The sulphur provided by a cysteine is extracted, transported and attached on tRNAs by Urm1-pathway proteins which were revealed to be heat sensitive in the BY4741 background leading to a decline in tRNA as well as protein modification (Tyagi and Pedrioli, 2015; Alings et al., 2014; Damon et al., 2014; Jüdes et al., 2016). Thus, wild type cells cultivated at 37°C indeed displayed increased frame shifting events comparable to the *urm1* mutant and indicated an abolishment of uridine thiolation. The decline of the essential sulphur-carrier Urm1 was confirmed by an Ahp1-urmylation assay at the same growth conditions. Increased levels of the $tRNA^{Lys}_{UUU}$ again were sufficient to suppress the frame shifting. The heat stress experiment was repeated with a *tcd1* mutant that showed no ct^6A_{37} modification anymore. Under permissive growth conditions *tcd1* displayed no decrease in frame accuracy. Nonetheless, upon heat induced decline of U_{34} thiolation, the frame shifting rate increased up

to eight-fold and exceeded the values of the heat stressed WT or *urm1*, respectively, implying a cooperativity of s^2U_{34} and ct^6A_{37} in frame maintenance.

In sum, this paper found evidence that mcm^5U_{34} and s^2U_{34} individually contribute to frame shift suppression involving $tRNA^{Lys}_{UUU}$ during translation. Combined abolishment of both compromised frame maintenance and led to additive translation defects which might be responsible or at least account to the deficiencies observed for the double mutant in previous reports (Nedialkova and Leidel, 2015; Klassen et al., 2015). $tRNA^{Lys}_{UUU}$ overexpression in the tested cases was sufficient to rescue or at least diminish the frame shift events and underlined the hypothesis suspecting an A-site effect to be responsible for frame inaccuracies. This finding however adds on to the understanding why higher than normal doses of the malfunctional tRNA are capable to supress modification-defect induced deficiencies in the corresponding mutants. Interestingly, the ct^6A_{37} modification appears to also support frame accuracy but only in cooperation with s^2U_{34} considering the deletion of *TCD1* alone had no effect on the *lacZ* level. Meanwhile, the Tcd1 and Tcd2 dependent modification might be more important at higher temperatures like 40 °C, since the single mutants only displayed slight growth defects at this condition which were rescuable by $tRNA^{Lys}_{UUU}$. However, it can be assumed that similar effects as detailed above might occur due to abolishment of other critical anticodon modifications (e.g. mcm^5U) which all together may point to a general decline in translational efficiency in the *urm1 tcd1* and *elp3 tcd1* mutants and add on to growth, cytological and proteostasis deficiencies documented in **Publication I**. Ongoing translational aberrations leading to protein aggregation might trigger several stress responsive pathways of which the unfolded protein response was already found to be activated in $mcm^5s^2U_{34}$ deficient mutants in various eukaryotic systems (Patil et al., 2012; Rojas-Benítez et al., 2013; Laguesse et al., 2015; Freeman et al., 2019). Accordingly, the following study involved the investigation of the UPR activity in aggregation prone tRNA modification double mutants.

6.3 Publication III

Bruch, **A**., Klassen, R., & Schaffrath, R. (2018). Unfolded protein response suppression in yeast by loss of tRNA modifications. *Genes*, *9*(11), 516.

6. Publications

6.3.1 Transition between publication III and IV

The unfolded protein response (UPR) is a conserved mechanism localised to the endoplasmic reticulum and activated upon accumulation of misfolded proteins and aggregates in the organelle (Mori, 2009). The activation in yeast relies on the non-canonical Ire1 dependent splicing of the *HAC1* transcript which is then translated and induces transcription of different UPR client genes (Cox and Walter, 1996; Sidrauski and Walter, 1997; Mori et al., 1993, 1996). Different studies on tRNA modification mutants (e.g. $mcm^5s^2U_{34}$) in various eukaryotic systems found evidence for the activation of UPR presumably by compromised translation and consequential proteostasis deficiencies (Patil et al., 2012; Rojas-Benítez et al., 2013; Laguesse et al., 2015; Freeman et al., 2019). Since the latter was identified to play a key role on the development of neuropathies, tRNA modifications may positively impact translational efficiency and protein homeostasis and therefore contribute to neurogenesis (Warren et al., 2013). This would explain, why an increasing number of neuropathies is linked to the decline of tRNA modifications including t^6A_{37} or $\Psi_{38/39}$ (Shaheen et al., 2016; Braun et al., 2017; Edvardson et al., 2017).

In this regard, the combined abolishment of mcm^5/s^2U_{34} together with ct^6A_{37} or $\Psi_{38/39}$ also induced severe deficiencies in yeast considering the cytology, translation efficiency and protein homeostasis (**Publication I** and **Publication** II). The double mutants (i.e. *elp3 tcd1*, *elp3 deg1*) were found to accumulate protein aggregates similar to the well-studied *elp6 ncs2* mutant and therefore an UPR induction appeared plausible (Nedialkova and Leidel, 2015). In order to investigate this, RT-PCR and qRT-PCR based approaches were utilized to monitor the *HAC1* splicing in the double mutants by quantifying the resulting $HAC1^i$ splice product. Interestingly, none of the investigated tRNA modification mutants displayed UPR activation and surprisingly, *elp3 deg1* and *urm1 deg1* even displayed a decrease of the $HAC1^i$ level in unstressed and tunicamycin (TM) stressed conditions. Accordingly, it was of interest to test whether this effect might be caused by the translational deficiencies of these mutants which was examined with *elp3 deg1* either transformed with an empty vector or a $tRNA^{Gln}_{UUG}$ overexpressing plasmid. The results indicated an improvement of the $HAC1^i$ transcript level under tunicamycin untreated and treated conditions upon $tRNA^{Gln}_{UUG}$ overexpression. This suggested a suppression of the UPR mechanism in the tRNA modification mutants was likely caused by translational incapabilities. Inhibition of the UPR leads to hypersensitivity against any trigger (e.g. TM) of the mechanism which may also apply to the double mutants (Mizuno et al., 2015; Halbleib et al., 2017; Sarkar et al., 2017). Surprisingly, the cultivation of *urm1 deg1* and *elp3 deg1* with different concentrations of TM revealed strong resistance against the UPR-inducing agent in both mutants. Moreover, deletion of *IRE1* in the *elp3 deg1* background produced no alteration to this phenotype, only overexpression of $tRNA^{Gln}_{UUG}$ in both mutants was sufficient to restore a nearly wild type growth behaviour upon tunicamycin stress.

Conclusively, the combined absence of mcm^5/s^2U_{34} together with $\Psi_{38/39}$ (or ct^6A_{37}, see section **7.2**) in yeast seem to impede the activation of the unfolded protein response which appeared to be caused by the translational incapacity of the mutants. This contrasted previous findings of a mcm^5s^2U deficient mutant (*trm9*) providing evidence of an increase of the *HAC1*^{i} level and consequently UPR induction (Patil et al., 2012). Nonetheless, the corruption of translation due to tRNA modification defects and the substantial plethora of physiological consequences including the UPR system inhibition implicated a general regulatory disturbance of maybe various other pathways (**Publication I-Publication** III). In regard of this hypothesis, a line of evidence was presented in different studies displaying a significant alteration of the transcriptome and of the metabolome in different tRNA modification mutants (Zinshteyn and Gilbert, 2013; Nedialkova and Leidel, 2015; Scheidt et al., 2014; Karlsborn et al., 2016; Thiaville et al., 2016; Chou et al., 2017; Gupta et al., 2019). Hence, it seemed to be justified to investigate and characterize similar transcriptional aberrations in the different double mutants to study a potential connection between translational corruption and downstream regulome deficiencies.

6.4 Publication IV

Bruch, **A**., Laguna, T., Butter, F., Schaffrath, R. & Klassen, R. (2020). Misactivation of multiple starvation responses in yeast by loss of tRNA modifications. *Nucleic acids research*, in press.

6. Publications

7. Discussion

7.1 Functional synergy of specific tRNA modifications

The majority of epitranscriptomic studies addressed single transfer RNA modifications and their physiological role. However, tRNAs are modified at multiple sites across their structure and these might cooperate synergistically in terms of tRNA function (see also chapter **4.2** and the following) (Phizicky and Hopper, 2010; Ranjan and Rodnina, 2016; Cantara et al., 2011; Lorenz et al., 2017; Sokołowski et al., 2017; Boccaletto et al., 2018; McCown et al., 2020). Shedding light on this possibility, the aim of this work was to identify tRNA modifications that functionally cooperate during translation and to investigate their physiological and presumed regulatory role in *S. cerevisiae*.

Hence, the general phenotypes of mutant backgrounds deficient in mcm^5/s^2U_{34} together with ct^6A_{37} or $\Psi_{38/39}$ were characterized including the identification of the functionally impaired tRNAs upon loss of these modifications (**Publication I**). Expression decrease of distinct proteins (MoTTs) or accumulation of protein aggregates among other severe defects indicated translational complications of the double mutants. This prompted continuative examinations of the presumed abnormal translation process and how the mutant cells adapt to the stress situations. Accordingly, in depth investigations employing a *lacZ* reporter construct (Tükenmez et al., 2015) responsive to the above mentioned modification losses revealed interesting anomalies of the frame accuracy during translation for a subset of double mutants (**Publication II**). These findings underlined the previous results of **Publication I**, revealing another layer of mechanistical defects during translation, probably adding on to the protein aggregation phenotype of the studied mutants.

Based on these data, it was also necessary to dissect possible responses of the tRNA modification mutants upon the accumulation of proteotoxic clusters. Previous reports on higher eukaryotic cells as well as other yeast tRNA modification mutants suggested an induction of the unfolded protein response (UPR) to cope with the aggregation stress (Patil et al., 2012; Laguesse et al., 2015). The tRNA modification double mutants focused on in this study were therefore examined for a possible activation of UPR by using a combination of reverse transcriptase (RT)-, quantitative real time (qRT)-PCR and phenotypic approaches (**Publication III**).

Finally, **Publication IV** dealt with the question what happens to the regulome of the *elp3 deg1*, *urm1 deg1*, *elp3 tcd1* and *urm1 tcd1* due to combined modification loss and if there is any interdependency identifiable between the observed defects. Various modification losses of transfer RNAs were already found to not only affect the translational process but also to modulate transcriptional and probably in consequence metabolic regulation (Karlsborn, 2016; Zinshteyn and Gilbert, 2013; Nedialkova and Leidel, 2015; Karlsborn et al., 2016; Thiaville et

al., 2016; Chou et al., 2017; Gupta et al., 2019). Accordingly, a combination of RNA sequencing, qRT-PCR, protein aggregates isolation, tRNA rescue and other experiments confirmed similar findings for the double mutants. Moreover, it expanded the insights on the molecular changes occurring in tRNA modification mutants and revealed a possible link between the emerging predominant defects.

7.1.1 Physiological and functional implications of tRNA modifications

Transfer RNAs are highly modified adapter molecules necessary for the delivery of appropriate amino acids to the ribosome and decoding of the genetic information imbedded in the translated mRNA. Despite the knowledge about these essential molecules and tasks, the need and function of the modifications remained less well understood. Nevertheless, every organism including bakers yeast employs an extensive repertoire of proteins and pathways to create an individual modification pattern on every tRNA species indicating the importance of these posttranscriptional changes (Phizicky and Hopper, 2010; Ranjan and Rodnina, 2016; Cantara et al., 2011; Lorenz et al., 2017; Boccaletto et al., 2018; McCown et al., 2020). As detailed in chapter **4.3** and the following, initial investigations focused on particular modifications like mcm^5/s^2U_{34}, t^6A_{37} or $\Psi_{38/39}$ and their role in translational but also physiological processes (Lecointe et al., 2002; Thiaville et al., 2016; Johansson et al., 2018). These studies revealed a plethora of mutant phenotypes including increased stress sensitivity, decline in ribosome association, frame shifting events, proteome aberrations and many more (Zinshteyn and Gilbert, 2013; Nedialkova and Leidel, 2015; Klassen and Schaffrath, 2017; Lecointe et al., 1998, 2002; Huang et al., 2005, 2008; Björk et al., 2007; Nakai et al., 2008; Johansson et al., 2008; Leidel et al., 2009; Noma et al., 2009; Miyauchi et al., 2012; Laxman et al., 2013; Rezgui et al., 2013; Damon et al., 2014; Scheidt et al., 2014; Tükenmez et al., 2015; Han et al., 2015; Thiaville et al., 2016; Karlsborn, 2016; Karlsborn et al., 2016; Chou et al., 2017; Gupta et al., 2019).

Since the mcm^5 and s^2 moieties are sequentially installed on the same *wobble* uridine (U34) by two different pathways, first functional cooperation studies focused on these well-studied modifications (Huang et al., 2005, 2008; Johansson et al., 2008; Nakai et al., 2008; Leidel et al., 2009; Noma et al., 2009). Hence, combined loss of mcm^5s^2U (e.g. *elp6 ncs2*) appeared to be lethal in some yeast genetic backgrounds (W303) (Björk et al., 2007; Xu et al., 2019) whereas other strains were able to compensate this modification failure (S288c) but displayed more severe physiological abnormalities and ribosome pausing in comparison to the corresponding single mutants (Nedialkova and Leidel, 2015; Klassen et al., 2015). These findings suggested a novel cooperative modulation of tRNA function by a subset of posttranscriptional modifications, implying an interactive network (Sokołowski et al., 2017). Accordingly, investigations based on synthetic genetic arrays (SGA) indicated a negative interactome not only between all Elongator and Urm1-pathway relevant genes but also *DEG1*

or *TCD1*/*TCD2*, both necessary to generate either $\Psi_{38/39}$ or ct^6A_{37} in the ASL, respectively (**Publication I**). Combining deleterious mutations of the corresponding genes (i.e. *elp3 tcd1*, *urm1 tcd1*, *elp3 deg1*, *urm1 deg1*) and sub-sequential phenotypic examination revealed severe morphological and growth defects. All double mutants displayed abnormal nuclear segregation defects alongside cell cluster formation i.e. cytoskeleton breakdown, aberrant bud site selection and decline in daughter cell separation (in sum cytokinesis defects). Heat or other forms of environmental stressors impacted the growth of the *tcd1* and *deg1* combinations whereas *urm1 deg1* and especially *elp3 deg1* appeared to be highly vulnerable compared to *urm1 tcd1* and *elp3 tcd1*, nevertheless confirming the SGA analysis (**Publication I**; Klassen and Schaffrath, 2017, 2018).

Since the loss of specific modifications seemed to affect the translational capacity of the respective mutant/s, proteostasis was examined in the respective mutants. Individual loss of mcm^5/s^2U or t^6A was already shown to increase the amount on protein aggregates (Nedialkova and Leidel, 2015; Thiaville et al., 2016). The absence of these modifications induced the accumulation of insoluble protein clusters indicating severe translational complications which additionally aggravated upon combination of the defects (Nedialkova and Leidel, 2015; Pollo-Oliveira et al., 2020). This event was also observed for the most severely affected modification mutants *elp3 tcd1* and *elp3 deg1*, each displaying increased insoluble protein clusters than the corresponding single mutants (**Publication I**). Moreover, depending on what tRNA modifications were together abolished in the respective mutants a variation in the abundance of protein aggregates was observed (**Publication I**; **Publication IV**). This could be seen in a direct comparison of the double mutants *elp6 ncs2*, *elp3 tcd1* and *elp3 deg1.* The latter mutant which exhibits the most severe growth defect contained an increased amount of aggregates than the other two mutants (**Publication IV**, **Figure S7**). These findings indicated a hierarchy ranging from more to less critical tRNA modifications. Accordingly, many individual modification losses can be compensated to a certain extent but are in combination strongly deleterious to the organism. For example, absence of one of the $mcm^5s^2U_{34}$ moieties (mcm^5 or s^2U) can already negatively influence the codon-anticodon interaction of $tRNA^{Gln}_{UUG}$ and $tRNA^{Lys}_{UUU}$ leading to a slow-down of the ribosome at the respective A-ending codons (Zinshteyn and Gilbert, 2013; Ranjan and Rodnina, 2017). The remaining modification (either mcm^5 or s^2) appeared to partially compensate the individual loss in these cases but combined deficiencies aggravated the ribosome pausing events and negatively influenced more aspects of the translational process (detailed in the following chapter) (**Publication II**; Nedialkova and Leidel, 2015). Thus, besides single tRNA modifications clearly fulfilling an important role for translational capacity (e.g. m^1G_{37}, $\Psi_{26\text{-}28}$, t^6A_{37}) more evidence points to a critical relevance of a modification interactome. This network was expanded and includes not only the anticodon loop (e.g. i^6A_{37}, Ψ_{35}) but also the extended ASL or variable loop (e.g. $\Psi_{26\text{-}28}$, m^5C_{48}, D_{47})

localized modifications (Klassen and Schaffrath, 2018; Khonsari and Klassen, 2020; Björk et al., 1989, 2001; Lee et al., 2007; Thiaville et al., 2016;). This notion was supported by the increased abundance of protein aggregates in single and combined modification mutants either defective for m^1G_{37} (*trm5*, **Figure A1 A**) or mcm^5/s^2U together with $\Psi_{26\text{-}28}$ (*pus1 urm1*, *pus1 elp3*, **Figure A1 B**) indicating the accumulation of protein aggregates, possibly as a consequence of protein folding defects during translation (**Publication IV**). Moreover, protein aggregation might be responsible for some defects observed for the combined tRNA modification mutants (**Publication I**). Plasmid based overexpression of an aggregating huntingtin variant artificially induced cytokinesis deficiencies and cell clusters in the WT or the *deg1* mutant, neither displaying any morphological abnormalities under normal growth conditions nor when expressing a non-aggregating huntingtin variant (**Publication I**; Mason and Giorgini, 2011). Since all investigated double mutants (*elp3 tcd1*, *urm1 tcd1*, *elp3 deg1*, *urm1 deg1*) formed similar cell clusters, one might assume that the protein aggregation due to weak translational competence represents a key driver of the observed defects which was tested in following experiments (**Publication IV**).

Interestingly, searching for the majorly defective tRNAs in this set of mutants revealed that the specific modification combinations can only be found in the $tRNA^{Gln}_{UUG}$ and $tRNA^{Lys}_{UUU}$, respectively. Both tRNAs are equipped with mcm^5s^2 at the U34 and carry either Ψ at position 38 of the $tRNA^{Gln}_{UUG}$ or the adenine conversion ct^6A_{37} at $tRNA^{Lys}_{UUU}$ (Johansson et al., 2008; Miyauchi et al., 2012; Han et al., 2015; Boccaletto et al., 2018). Accordingly, overexpression of the hypomodified tRNAs in *urm1 tcd1* and *elp3 tcd1* ($tRNA^{Lys}_{UUU}$) or *urm1 deg1* and *elp3 deg1* ($tRNA^{Gln}_{UUG}$) suppressed the above described defects (not efficiently for *elp3 deg1*, see below) (**Publication I** and **Publication** IV). Notably, overexpression of $tRNA^{Gln}_{UUG}$ was only partially sufficient in suppressing the negative phenotypes of *elp3 deg1* indicating more deficient tRNAs adding on to the defects detailed above. This might be explained by the sheer number of tRNAs documented to be modified by the Elongator complex (11 (Johansson et al., 2008)) or Deg1 (21 cytotRNAs (Han et al., 2015; iimcb.genesilico.pl/modomics/)), six of which (including $tRNA^{Gln}_{UUG}$) reported or predicted to be modified by both pathways. Accordingly, phenotypes of a similar modification deficient strain background (*kti12 deg1*) displayed improved growth due to $tRNA^{Gln}_{UUG}$ and $tRNA^{Pro}_{UGG}$ overexpression at elevated temperatures supporting the assumption of additional dysfunctional tRNAs in a mutant lacking $ncm^5U/mcm^5U/mcm^5s^2U$ and $\Psi_{38/39}$ (Han et al., 2015). Nonetheless, these results suggested the two error-prone $tRNA^{Lys}_{UUU}$ and $tRNA^{Gln}_{UUG}$ to be majorly responsible for the physiological and translational abnormalities (**Publication I**). Accordingly, this hypothesis was further challenged by various translational and transcriptional experimental approaches which will be detailed in the following chapters.

7.1.2 Cooperating modifications ensure translational competence of specific tRNAs

As described in the previous section, the double mutants displayed various physiological defects which could be tracked back to either $tRNA^{Lys}_{UUU}$ (*urm1 tcd1*, *elp3 tcd1*) or $tRNA^{Gln}_{UUG}$ (*urm1 deg1*, *elp3 deg1*). Hence, further experiments were conducted to further investigate the assumed tRNA dependent translational defects. An attempt to analyse this was to test a possible decline in translational efficiency of AAA- or GAA-codon enriched genes. Those mRNAs expression presumably rely on the presence of specific tRNA modifications in either $tRNA^{Lys}_{UUU}$ or $tRNA^{Gln}_{UUG}$ as they represent the natural decoders of the AAA and GAA codons, respectively. The concept of a regulatory translation mechanisms involving dynamic tRNA modification is referred to as modification tuneable transcripts (MoTTs) and describes a possible dependency of mRNAs with a high number of specific codons on the presence of tRNA modifications for an efficient decoding process (Dedon and Begley, 2014; Gu et al., 2014; Endres et al., 2015). Hence, the tRNA modification status may bear implications on the translation efficiency of specific transcripts, potentially affecting abundance of the respective protein as well as various signalling pathways downstream of those translation products (Dedon and Begley, 2014; Gu et al., 2014; Endres et al., 2015).

Thus, the yeast genome was examined for glutamine- and lysine rich proteins to be investigated in either the *deg1* ($tRNA^{Gln}_{UUG}$ defect) or *tcd1* ($tRNA^{Lys}_{UUU}$ defect) combinations. The naturally occurring prion Rnq1 (Sondheimer and Lindquist, 2000) was found to be rich in asparagine (12.35%) and glutamine (19.01%). Glutamines in Rnq1 are encoded by 53 A-ending and 24 of the alternative G-ending codons (**Publication I**; Cherry et al., 2012). Hence, the translational efficiency of *RNQ1* mRNA should heavily depend on functional $tRNA^{Gln}_{UUG}$. Indeed, expressing a Rnq1-GFP fusion (Nakayashiki et al., 2005) revealed decreased translation of the *RNQ1* mRNA in *urm1 deg1* and *elp3 deg1* which could be improved by overexpressing the hypomodified $tRNA^{Gln}_{UUG}$ but not the isoacceptor $tRNA^{Gln}_{CUG}$ (**Publication I**). A similar approach was applied for *SRP21* assumed to be a lysine rich (19%) MoTT employing a Lys-codon ratio of 21/11 (AAA/AAG) (Begley et al., 2007; Cherry et al., 2012). The translation efficiency of the *SRP21* mRNA declined in the *urm1 tcd1* and *elp3 tcd1* double mutants and could be rescued by higher than normal doses of the hypomodified $tRNA^{Lys}_{UUU}$ (**Publication IV**). Both experiments demonstrated that high amounts of AAA or CAA codons of the respective mRNA are indeed less efficiently translated in the investigated mutants ultimately leading to lower protein levels. Other reports dealing with a mutant deficient for the methylation step in mcm^5/mcm^5s^2U synthesis (*trm9*) additionally demonstrated reduced translatability of *RNR1* and *YEF3* (Begley et al., 2007; Patil et al., 2012). Notably and in contrast to the above described lysine and glutamine rich proteins, Rnr1 and Yef3 are not rich on the respective amino acids (or glutamate) but employ a high amount of codons decoded by

two of three normally $mcm^5s^2U_{34}$ modified tRNAs (Begley et al., 2007; Cherry et al., 2012). Thus, *RNR1* Gln and Glu amino acids are encoded by a 25/7 (CAA/CAG) and 44/14 (GAA/GAG) codon ratio, respectively. *YEF3* utilizes even more of the corresponding A-ending codons (Gln= 27/2 (CAA/CAG), Glu= 91/1 (GAA/GAG)) (Begley et al., 2007). This, however, indicates that the translation of MoTTs not only relies on the general amount of the respective codons/amino acids but might also depend on the codon ratio that is employed in the transcript sequence (Gu et al., 2014). Both attributes might individually or in combination account for the "definition" of modification tuneable transcript.

Moreover, even these aspects might only be one part of the mode of action since the discovery of another potential MoTT Atg8 prompted new questions on this issue (**Publication IV**). *ATG8* encodes a ubiquitin like protein (UBL) which plays a vital role in the propagation of autophagy by the modification of membrane-situated phosphatidylethanolamines (PE) of phagophores (Huang et al., 2000; Ohsumi et al., 2000). The UBL is lysine rich (11.1 %) and accordingly a GFP-Atg8 fusion was weakly expressed in the *urm1 tcd1* and *elp3 tcd1* mutants malfunctional for $tRNA^{Lys}_{UUU}$ which fitted the previous findings on *SRP21* (**Publication IV**). Contrastingly, screening for the utilization of lysine codons revealed a 3/10 (AAA/AAG) ratio for *ATG8*. However, since so far, no quantification of the *GFP-ATG8* transcript level was performed in the mutants offering the possibility that GFP-Atg8 biogenesis may decline transcriptionally rather than translationally. Hence, further work is required to determine whether Atg8 may represent another MoTT-candidate despite differing in the expected codon composition from established MoTTs (Begley et al., 2007).

Thus, all the presented data point to different options how the codon composition of a mRNA sequence might influence its translation. Regarding this, different theoretical and practical studies imply that the codon usage bias of an organism is clearly affecting the accuracy and efficiency of translation and that the abundance of frequently used codons correlate with the respective tRNA level apparently influencing the translational speed (Plotkin and Kudla, 2011; Shah and Gilchrist, 2011; Novoa and Ribas de Pouplana, 2012; Varenne et al., 1984; Dong et al., 1996; Ghaemmaghami et al., 2003; Tuller et al., 2010; Frumkin et al., 2018). Specific tRNA modifications or modification patterns might therefore account to this conserved system by modulating translation accuracy and frequency in a stress adaptive manner (Novoa and Ribas de Pouplana, 2012; Pollo-Oliveira and de Crécy-Lagard, 2019). Hence, modification tuneable transcripts might critically depend on these regulatory layers consisting of post-transcriptional modification, mRNA codon composition and tRNA abundance. This would imply that (i) the mRNA sequence context of the imbedded modification dependent codon may be as important as (ii) its abundance or (iii) ratio to the alternative codon which in sum are at least three principles adding on to the translatability of a MoTT.

Accordingly, loss of critical modifications clearly must promote mechanistical consequences during the translation process as soon as the ribosome reaches the codon that is read by the hypomodified tRNA. Loss of mcm^5/s^2U in this regard already strongly influences the tRNA-ribosome A-site interaction and promotes +1 frame shifting as well as ribosomal retention at the AAA, CAA and GAA codons (Zinshteyn and Gilbert, 2013; Rezgui et al., 2013; Tükenmez et al., 2015). Combined absence of the two modifications appeared to aggravate the frame inaccuracy and ribosome pausing events again underlining a synergistic role of both on tRNA functionality (**Publication II**; Nedialkova and Leidel, 2015). These defects could be rescued via overexpression of the respective hypomodified $tRNA^{Gln}_{UUG}$ and $tRNA^{Lys}_{UUU}$ whereas the latter was already sufficient to significantly reduce frameshifting of the double mutant (**Publication II**). Since the $tRNA^{Lys}_{UUU}$ also represents the majorly defective tRNA in the *tcd1* combinations, the same assay was employed which confirmed +1 frame shifting events in the *urm1 tcd1* mutant. These findings identified ct^6A as a major factor that together with s^2U (and presumably mcm^5U) ensures the frame maintenance of the translating ribosome likely by enforcing efficient codon-anticodon interaction (**Publication II**). A similar mode of action for *elp3 deg1* and *urm1 deg1* could be anticipated since pseudouridylation is thought to stabilize the tertiary structure of tRNAs and ASL to support codon-anticodon interaction (Davis, 1995; Sipa et al., 2007; Spenkuch et al., 2014; Väre et al., 2017). The $\Psi_{38/39}$ formation was in this regard already reported to be important for frame maintenance during translation (Lecointe et al., 2002). Later studies focusing on the readthrough capability of modified tRNAs found no significant contribution of $\Psi_{38/39}$ alone on the codon-anticodon interaction (Klassen and Schaffrath, 2018). The same study, however, demonstrated that as soon as the *deg1* mutation was combined with the deletion of another tRNA modification relevant gene (e.g. *MOD5* necessary for i^6A_{37}), the readthrough capacity of the mutant drastically dropped (Klassen and Schaffrath, 2018). This indicates that the pseudourydilation at position 38/39, comparable to modifications at position 37 (e.g. ct^6A_{37}), may engage in a supportive role to prevent frame inaccuracies and probably also ribosomal pausing at specific codons.

In sum, the presented data imply a vital role of the four modifications in either supporting the translation accuracy (codon-anticodon interaction) and/or frequency (ribosomal A-site interaction). Absence of the modifications therefore leads to the above observed defects whereas it is not clear which of these translational deficiencies or a combination of them are affecting the expression of MoTTs or may even promote protein aggregation.

7.2 The unfolded protein response remains dysfunctional in tRNA modification mutants

Translational complications caused by tRNA modification defects were demonstrated to trigger protein aggregation in different eukaryotic systems (**Figure A1** and **Figure** A3) (**Publication I** and **IV**; Nedialkova and Leidel, 2015; Freeman et al., 2019; Pollo-Oliveira et al., 2020; Thiaville

et al., 2016; Tuorto et al., 2018). Thus, this proteotoxicity is presumed to force a cell to activate different pathways to cope with this stress. Among them, the most prominent are the ubiquitin proteasome system (UPS), autophagy (**Publication IV**, see also following chapters) and unfolded protein response (UPR) which each contribute to the clearance of protein aggregates (Patil and Walter, 2001; Chen and Klionsky, 2011; Fichtner et al., 2003; Nandi et al., 2006; Mori, 2009; Finley et al., 2012). Especially individual loss of tRNA modifications investigated in higher eukaryotic organisms and bakers yeast appeared to not only induce the formation of protein aggregates but was also sufficient to activate the UPR (Patil et al., 2012; Rojas-Benítez et al., 2013; Laguesse et al., 2015; Freeman et al., 2019). Interestingly, these studies dealt with the decline or loss of the t^6A_{37} or mcm^5/s^2U_{34} moieties indicating a link between these specific modification defects and the activation of the endoplasmic reticulum (ER) resident UPR system. This also included the *trm9* mutant displaying no final methylation step of the cm^5U/cm^5s^2U moieties to create $mcm^5/mcm^5s^2U_{34}$ and clearly induced the response by the obligate splicing of *HAC1* mRNA (Patil et al., 2012). In yeast, upon accumulation of unfolded proteins in the ER the UPR system is induced by uncanonical Ire1 dependent splicing of the immature transcript denoted $HAC1^u$ to generate $HAC1^i$. The latter can be translated into the Hac1 transcription factor that induces expression of UPR-client genes (Cox and Walter, 1996; Sidrauski and Walter, 1997; Mori et al., 1993, 1996).

The data obtained from the above given studies on mcm^5s^2U and t^6A losses, however, implied a conserved role of the unfolded protein response in coping with translational incapabilities and consequently proteotoxic stress. Thus, since the combined modification mutants analysed in this thesis were shown to accumulate protein aggregates (**Publication I** and **Publication** IV), one might also assume an induction of the UPR in this strain backgrounds. Astonishingly, a first RT-PCR based screening for the splicing of the *HAC1* mRNA revealed no such event (**Publication III**, **Figure A1 A**). Moreover, specific quantification of $HAC1^i$ by qRT-PCR confirmed the first examination and even revealed a general decline of the mature mRNA under non-stressed conditions (**Publication III**, **Figure A2 A**). This might be the case either because there is no accumulation of protein aggregates in the ER, or because there is a general dysfunctionality of the UPR due to combined modification defects. This idea prompted the investigation of the general inducibility of this system by treatment with tunicamycin (TM). This agent blocks the N-linked glycosylation and thereby promotes UPR activation (Elbein, 1987). In general, TM treatment induced the splicing of *HAC1* mRNA in the wildtype (WT) as expected and in all four double mutants, implying the functionality of the unfolded protein response (**Publication III**, **Figure A2 A**). Surprisingly, the *elp3 tcd1* mutant displayed a reduced intensity of the faster migrating band ($HAC1^i$) compared to the WT which was also the case for *urm1 deg1* and *elp3 deg1* whereas the latter showed the lowest signal. In contrast, the intensity of the $HAC1^u$ band was far stronger for the double mutants than the wild type

which further underlined a reduced splicing rate of *HAC1* mRNA (**Publication III**, **Figure A2 A**). This observation was confirmed by the more sensitive qRT-PCR approach revealing even a reduced *HAC1*^{i} level for *urm1 tcd1* (**Publication III**, **Figure A2 B**). These experiments in sum demonstrated no activation of the UPR in protein aggregation prone double mutants and conversely suggested a severe obstacle of activating this system.

Hence, different reasons are conceivable for these results and might either involve a decline of protein transport into the ER preventing an accumulation of protein aggregates through TM treatment or impeding of the UPR by a general decline in translation rate. In support for the first case, the double mutants are suffering of severe translational defects regarding the frame maintenance, translation of lysine and glutamine rich proteins (**Publication I**, **Publication** II and **Publication** IV; see previous chapter) and probably also ribosome pausing according to the findings about mcm^5/s^{2}U deficiencies (Zinshteyn and Gilbert, 2013; Nedialkova and Leidel, 2015). Thus, a reduced translation rate of all four tRNA modification mutants could lead to a diminished protein content in the cells including the ER and would limit protein aggregation to the cytoplasm avoiding the necessity to activate the UPR (Abdullah and Cullen, 2009). In this regard, the in part similar *elp6 ncs2* double mutant was already shown to indeed display a reduced protein level compared to other single tRNA modification mutants and its aggregates consisted mainly of cytosolic than ER proteins (**Publication III**; Nedialkova and Leidel, 2015). Moreover, overexpression of the hypomodified tRNA$^{Gln}_{UUG}$ in *elp3 deg1*, capable to improve translation, also rescued TM-induced UPR activation which might support a link between the proteostasis defect and this system (**Publication III**).

On the other hand, an earlier report indicated that translational deficiencies caused by deletion of various ribosomal protein genes rendered protection against ER stress. In essence, disruption of 14 non-essential ribosomal protein genes induced slower growth and a reduced translation rate which conversely led to tunicamycin resistance presenting a link between these two occasions (Steffen et al., 2012). Moreover, the resistance was not depending on functional UPR contrasting other findings that illustrated hyper-sensibilities against TM caused by a dysfunctional response system of mutants lacking key proteins (e.g. Ire1, Hac1) of the pathway (Mizuno et al., 2015; Halbleib et al., 2017; Sarkar et al., 2017). Nonetheless, reproducing these experiments with the *tcd1* and *deg1* combinations (*urm1 tcd1*, *elp3 tcd1*, *urm1 deg1*, *elp3 deg1*) treated with different concentrations of TM to induce ER stress surprisingly revealed all double mutants to display enhanced resistance against this agent (**Publication III**, **Figure** A2 **C**). Single deletion of *IRE1* on the other hand led to tunicamycin hypersensitivity but had no effect if the disruption was introduced in the double mutants exemplified on *elp3 deg1* (**Publication III**). This, however, underlined the findings of Steffen et al., (2012) implying that a generally reduced translational rate and/or capacity also reported for the tRNA modification

double mutants indeed mediates resistance against ER stress and might therefore impede UPR activity.

Intriguingly, a previous study regarding ongoing ribosomal mistranslation in human cell lines (HEK293) and consequently accumulation of misfolded proteins pointed to a new mechanism. Mistranslation supresses UPR to prevent ER-stress (UPR^{ER}) mediated apoptosis which may similarly account for the here investigated tRNA modification mutants (Shcherbakov et al., 2019). The UPR^{ER} is as in all eukaryotic organisms necessary to prevent and resolve protein aggregation that might occur in the endoplasmic reticulum. In human cells, ongoing mistranslation or folding incapability in the organelle triggers UPR^{ER} which induces apoptosis via two ways: either by the PERK (protein kinase RNA-like ER kinase) regulated transcription factor CHOP (C/EBP homology protein, GADD153) activating mitochondria-dependent apoptosis or via BiP-suppressing p53 induced pro-apoptotic BIK (Oyadomari and Mori, 2004; Ohoka et al., 2005; López et al., 2017; Shcherbakov et al., 2019). Despite the knowledge about these apoptotic cascades, the precise molecular mechanism to activate cell death in a UPR dependent manner remains unclear. This pathway, however, appears to be conserved since the induction of ER-stress mediated apoptosis was also reported for *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. In *S. pombe* the ER-membrane resident chaperone calnexin (Cnx1, Cne1 in bakers yeast) was identified to promote the apoptotic process involving other key players of the UPR system like Ire1 or BiP (Kar2 in bakers yeast) (Guérin et al., 2008). This was in line with data from mammalian cell lines that were devoid of calnexin and displayed a decline in ER-stress dependent apoptosis underlining the conservation of this mechanism between lower and higher eukaryotic systems (Zuppini et al., 2002; Groenendyk et al., 2006). Moreover, in *S. cerevisiae* constant ER stress and consequently programmed cell death was induced by perturbing the metabolism of sphingolipids, highly conserved and important components of eukaryotic membranes which led to dimorphic endoplasmic reticulum and mitochondria (van Meer et al., 2008; Kajiwara et al., 2012). Expectedly, the unfolded protein response was also activated by the ER stress and could therefore presumably play a role in mediating apoptotic signals in the yeast system (Kajiwara et al., 2012). Based on these findings, it could be assumed that the here studied tRNA modification mutants may accumulate cytoplasmic (maybe also ER-localised) protein aggregates and may thereby be jeopardised for elevated UPR mediated apoptosis induction. Thus, these mutants might also suppress the activation of the unfolded protein response, even among TM treatment (**Publication III**, **Figure** A2 **A-C**), to prevent the programmed cell death similar to the human cell lines in the studies of Shcherbakov et al., (2019). This would in sum indicate an adaptation mechanism of the cells to the absence of cooperating tRNA modifications and the perturbance of translation and proteostasis, probably in different cell compartments including the ER that might enforce an apoptotic program. Nonetheless, it must be elucidated in future studies if any of the different

options, alone or in combination may explain the complicated relationship between tRNA modification defects and the consequently compromised UPR system.

7.3 Transcriptional adaption in response to translational defects

The transcriptome, proteome and metabolome represent an interconnected regulome controlled by various signalling cascades and master regulators which means that influencing one or more parts of this network provokes an adaptive reaction of the other compartments (see also chapter **4.4** and following). The absence of cooperating tRNA modifications in this regard was demonstrated to severely impact the translation process (**Publication I** and **Publication** II) and perturbs the unfolded protein response (**Publication III**). The latter and many other studies, however, implies that translational incapabilities mediated by modification defects are not only affecting protein biosynthesis but also other signalling cascades. This was supported by investigations on singular tRNA modification defects (e.g. mcm^5/s^2U, t^6A) demonstrating modulative effects on the transcriptome and metabolome (Zinshteyn and Gilbert, 2013; Nedialkova and Leidel, 2015; Pollo-Oliveira and de Crécy-Lagard, 2019; Laxman et al., 2013; Damon et al., 2014; Scheidt et al., 2014; Karlsborn et al., 2016; Thiaville et al., 2016; Chou et al., 2017; Gupta et al., 2019). Thus, the experiments reported in **Publication IV** aimed to analyse transcriptional aberrations in the tRNA modification double mutants, relate these events to possible adaptive responses in metabolism and signaling and identify possible causes for these effects. The results are discussed in the following chapters.

7.3.1 Loss of tRNA modifications induces starvation responses

As described in the previous chapters, the four double mutants *urm1 tcd1*, *elp3 tcd1*, *urm1 deg1* and *elp3 deg1* suffer from two different malfunctional tRNAs: $tRNA^{Lys}_{UUU}$ (*tcd1* combinations) and $tRNA^{Gln}_{UUG}$ (*deg1* combinations). Despite this fact, all mutants show similar phenotypes concerning morphological defects, sensitivity against environmental stressors and dysfunctionality of the UPR system (**Publication I** and **Publication** III). This, however, implies a similar, potentially adaptive effect on translational processivity that was exemplified by the accumulation of protein aggregates or MoTTs identification (**Publication I** and **Publication** IV). Accordingly, to examine the cause of the related defects, the transcriptome of all four double mutants was analysed for possible common alterations that could account for the common phenotypes. Interestingly, analysis of the transcriptome of all four double mutants revealed drastic common changes regarding the regulation of a variety of pathways. The changes involved up to 1780 induced and 1769 suppressed genes in transcript abundance of which 878 (401 induced, 477 suppressed) were commonly deregulated in these mutants (**Publication IV**). Gene ontology (GO) analysis of the overlaps revealed (i) a general induction of various starvation pathways and (ii) a suppression of transcriptional and translational effectors alongside with diauxie responsive genes, which are normally either activated (i) or

silenced (ii) upon entrance into the stationary phase (de Virgilio, 2012; Gasch et al., 2000; Thevelein et al., 2000; Smets et al., 2010). This was surprising, since the total RNA for the transcriptome analysis was extracted from mutant cells cultivated until exponential growth phase that should not promote any stationary phase programs (**Publication IV**). Nonetheless, further inspection of the significantly suppressed genes indicated a transcript decline on participants of the transcriptional regulation (e.g. *RRN9*, *RBA50*, *RPC53*, *RPC82*, *POL5*), translational machinery (e.g. *ZUO1*, *SSZ1*, *ANB1*, *RPG1*) and responsive to stationary growth phase (e.g. *HXK2*, *YAP6*, *ATF2*, *COG1*) (**Publication IV**, **Figure S2**, **Table S8**).

However, the overlapping activated pathways clearly pointed to an induction of genes controlled by the nitrogen catabolite repression (NCR, e.g. *MEP2, MEP3, DAL80, GAT1*) and general amino acid controlled pathway (GAAC) or are responsive to glucose repression (e.g. *TSL1*, *GSY2*, *GLC3*) as well as the general stationary phase (Gasch et al., 2000) (**Publication IV**, **Figure S2**, **Table S7**). Additionally, upregulation of respiratory genes (*COX3*, *COX4*, *COX5A*, *COX5B*, *COX13*, *COX20*, *ATP3*, *ATP4*, *ATP7*, *ATP16*, *ATP17*) known to be induced in the stationary phase by yeast were also demonstrated for the four double mutants underlining the alteration of the transcriptional program (**Publication IV**, **Table S6** and **S12-S14**). To support these findings, qRT-PCR was performed to quantify selected marker genes indicative for the NCR (*MEP2*) (Hofman-Bang, 1999), common stationary phase induction (*HSP12*) (Praekelt and Meacock, 1990) and glucose repression responses (*HXK1*) (Lobo and Maitra, 1977; Herrero et al., 1995). This approach confirmed the induction of the tested pathways in the exponential phase and a minor further increase in stationary phase as exemplified for the severest mutant *elp3 deg1*. Moreover, the transcriptional activation of all three marker genes was reversible via overexpression of the specific malfunctional tRNA (**Publication IV**). Thus, the transcriptional aberrations detected in all four double mutants indicate an induction of the stationary phase program already in the exponential growth phase which is caused by the ASL modification defects and the resulting translational incompetence. Similar responses were also found for tRNA modification mutants individually defective for the synthesis of mcm^5/s^2U, t^6A, $\Psi_{38/39}$ or other ASL resident modifications which induced the GAAC, NCR, respiration and carbohydrate metabolism (Zinshteyn and Gilbert, 2013; Nedialkova and Leidel, 2015; Scheidt et al., 2014; Thiaville et al., 2016; Chou et al., 2017). Investigations regarding the induction of the GAAC involving *elp*, KEOPS- and Urm1-pathway related mutants indicated an activation of the pathway independent of Gcn2 (see also chapter **4.4.2**) (Zinshteyn and Gilbert, 2013; Thiaville and de Crécy-Lagard, 2015). These findings added on to a line of evidence that implies various mechanisms independent of the kinase or tRNA charging status to promote GAAC activation by either diminishing the tRNA pool (in yeast) or ribosomal stalling due to absence of a specific tRNA (in mice) (McCormick

et al., 2015; Ishimura et al., 2016). However, comparing these previous findings with the transcriptome analysis of the tRNA modification double mutants illustrated a different picture. The data set indicated a more diverse expression pattern of GAAC controlled genes exemplified on two different amino acid biosynthesis pathways for lysine and arginine (**Publication IV**, **Figure S4**). The genes *ARG1-8*, *CPA1* and *CPA2* represent well known targets of Gcn4 (Natarajan et al., 2001) and seemed to be ambivalently expressed in the mutant set which held also true for the 8 target genes of the lysine pathway (**Publication IV**, **Figure S4**). Thus, a general induction of the GAAC appears to be highly improbable in these tRNA modification mutants and might rather point to a general loss of regulatory control of this pathway.

Since other stationary phase pathways are induced besides the GAAC in the modification mutants and various interconnections between these signal cascades have been reported in the past, other possibilities concerning the inappropriate activation of the starvation responses can be assumed. Alongside to the increase of NCR, respiratory metabolism and glucose repression assigned genes, RNAseq data (**Publication IV**, **Figure S2**) together with different supportive experiments confirmed that another stationary phase responsive pathway, namely (macro-) autophagy, was strongly induced in the *tcd1* and *deg1* combinations as well as *elp6 ncs2*. Interestingly, all these pathways are in *S. cerevisiae* canonically regulated by TORC1, Snf1 complex (Snf1C) and protein kinase A (PKA) (see chapter **4.4.1**) (de Virgilio, 2012; Gasch et al., 2000; Thevelein et al., 2000; Smets et al., 2008; Conrad et al., 2014). The Tor1 complex for example controls the nitrogen catabolite repression via extensive phosphorylation of Gln3 blocking nucleus entrance of the transcription factor and also inhibits autophagy activation by hyperphosphorylation of Atg13 upon adequate nutrient supply (Kamada, 2010; Coschigano and Magasanik, 1991; Noda and Ohsumi, 1998; Beck and Hall, 1999; Blinder et al., 1996; Kamada et al., 2000, 2010; Cheong et al., 2005; Kabeya et al., 2005, 2007; Feller et al., 2013). The complex additionally participates via the action of Sch9 in cooperation with PKA in the general stress response and again autophagy to (i) prevent the nuclear localization of Msn2, Msn4 and Rim15 and (ii) negatively regulate autophagy, respectively (Papinski and Kraft, 2016; Budovskaya et al., 2005; Urban et al., 2007; Yorimitsu et al., 2007; Wanke et al., 2008; Stephan et al., 2009; Smets et al., 2010; Conrad et al., 2014). TORC1 seems to indirectly influence the GAAC due to the action of the Sit4 phosphatase on Gcn2 and impacts glucose dependent repression and respiration induction via the Snf1C (Hinnebusch, 2005; de Virgilio, 2012; Cherkasova and Hinnebusch, 2003; Hedbacker and Carlson, 2009; Kubota et al., 2003; Smets et al., 2008; Usaite et al., 2009; Conrad et al., 2014). Snf1C on the other hand is itself involved in glucose dependent regulation of respiratory and carbohydrate metabolism as well as the GAAC (via Gcn2 and Gcn4), the NCR (via Gln3) and autophagy (de Virgilio, 2012; Hedbacker and Carlson, 2009; Wang et al., 2001; Bertram et al., 2002; Shirra et al., 2008;

Usaite et al., 2009; Cherkasova et al., 2010; Smets et al., 2010; Conrad et al., 2014). Hence, it is possible, that the activation of different starvation pathways detected in the tRNA modification double mutants is somehow controlled by inactivation of TORC1 affecting a plethora of downstream transcriptional programs, maybe in response to issues with the nutrient uptake or signalling. Supporting evidence was provided by the examination of the autophagy induction which seemed to depend on the presence or dephosphorylation of the canonical TORC1 clients Atg1 and Atg13, respectively (**Publication IV**) (Kamada, 2010; Kamada et al., 2000, 2010; Cheong et al., 2005; Kabeya et al., 2005;). Deletion of *TOR1* or treatment of WT cells with L-methionine sulfoximine (MSX), a glutamine depleting agent and consequently inactivating TORC1, lead to an expected induction of *MEP2* but had no effect on the *HXK1* expression and accordingly challenged this hypothesis (**Publication IV**, **Figure S6**). Thus, the inactivation of the Tor1 complex did not promote expression of the glucose repressed *HXK1* gene suggesting that induction of starvation responsive pathways in the tRNA modification mutants may not occur by TOR1 inactivation alone.

Interestingly, examination of the RNAseq dataset revealed not only increased expression of glucose repressed genes (e.g. *HXK1, TSL1*, *GSY2* and *GLC3*) but also the suppression of transcription factors or other effectors necessary for the control of this pathway. Among these were *CYC8*, *YAP6* and *NRG1* which are either responsible for the association (Yap6, Nrg1) or part of the Cyc8-Tup1 corepressor complex that negatively modulates glucose repressed genes (Treitel and Carlson, 1995; Smith and Johnson, 2000). Moreover, the major effector *HXK2* clearly declined in the transcript level and encodes a hexokinase involved in the suppression of glucose controlled genes like *HXK1* or *GLK1* (**Publication IV**, **Table S11**) (Gancedo, 1998; Rodríguez et al., 2001). This was remarkable, since the glucose repression signalling cascade is canonically regulated by the Snf1 complex (Snf1C) and induced upon glucose decline (de Virgilio, 2012; Gasch et al., 2000; Thevelein et al., 2000; Smets et al., 2008; Conrad et al., 2014).

Hence, the demonstrated transcriptional alterations may not only be attributed to the action of major regulatory kinase complexes like TORC1 or Snf1C but also to the decline of important transcriptional repressors or effector enzymes. The transcriptome analysis of the four tRNA modification mutants might support this view (as described above) whereas the cause for such an event is not clear until now. Thus, reprogramming of the transcriptome appears to be a common incident presumably provoked at least in part by a general translational incompetence and subsequent protein aggregation, which will be discussed in the following chapters.

7.3.2 Ambiguous promotion of protein aggregation in tRNA modification mutants?

Protein aggregation appears to be a common symptom of different tRNA modification defects and therefore result from translational incompetence. These proteostasis deficiencies were predominantly linked to the absence of modifications which are normally installed in the ASL

of tRNAs and therefore turned out to be critical for the translation process. Thus, the loss of $mcm^5s^2U_{34}$, (c)t^6A_{37}, $\Psi_{38/39}$ and combinations thereof impeded frame maintenance, biosynthesis of MoTTs and, potentially, translational slow-down of the ribosome at specific codons. So far, however, ribosomal pausing has been confirmed only for the *wobble* uridine modification defects and was suggested to mechanistically account for protein aggregation in U34 modification deficient mutants. A similar protein aggregation phenomenon in additional mutants including those studied in this thesis may point to a similar effect of the modification defects on ribosomal pausing (**Publication I**, **II** and **IV**; Nedialkova and Leidel, 2015; Pollo-Oliveira and de Crécy-Lagard, 2019; Tükenmez et al., 2015; Thiaville et al., 2016; Pollo-Oliveira et al., 2020). Nedialkova and Leidel (2015) were the first to find indications for a decline of protein homeostasis in consequence of perturbed translation due to the loss of $mcm^5s^2U_{34}$. Surprisingly, analysis of the protein aggregates demonstrated that the polypeptides were not enriched for lysine, glutamine and glutamate which would be encoded by the corresponding modification dependent A-ending codons (Nedialkova and Leidel, 2015). The results are inconsistent with an involvement of modification tuneable transcripts in the aggregation process that might be assumed based on previous reports (**Publication I** and **IV**; Gu et al., 2014; Patil et al., 2012) and argued for a more general disturbance of protein folding during the translation process (Nedialkova and Leidel, 2015). Similar occasions might be supposed for proteostasis defects demonstrated for *elp3 tcd1* and *elp3 deg1*. Both mutants display different patterns of modification defects which can be only found in combination on the $tRNA^{Lys}_{UUU}$ (mcm^5/s^2U_{34} and ct^6A_{37}) for the *tcd1* combinations or the $tRNA^{Gln}_{UUG}$ (mcm^5/s^2U_{34} and $\Psi_{38/39}$) for the *deg1* combinations (**Publication I**). Accordingly, the malfunctional tRNAs were found responsible for the translational deficiencies indicated by the decline of Gln and Lys rich MoTTs and promotion of protein aggregation in the double mutants. This was confirmed by rescue experiments employing overexpression of both hypomodified tRNAs improving the translation of lysine or glutamine rich proteins, respectively, and diminishing protein aggregates (**Publication I** and **Publication** IV).

Nonetheless, protein aggregation was initialized in both mutants resulting in similar aggregation patterns of *elp3 tcd1*, *elp3 deg1* and even *elp6 ncs2* despite the different malfunctional tRNAs (**Publication IV**, **Figure S7**). Thus, according to the comparable outcome of tRNA modification losses concerning cytological and morphological phenotypes in the different mutants (**Publication I** and **Publication** IV), disturbed proteostasis (i.e. protein aggregation) may represent a common driver of those defects. Protein aggregation therefore might be promoted similarly by distinct modification defects. Presumably, the identity of the weakly decoded codon is irrelevant as long as it perturbs the translational process alongside with the general protein folding (**Publication IV**). Nascent polypeptide folding is achieved in a co-translational manner depending on the translational speed and can be modulated by the

mRNA-codon composition, -secondary structure, tRNA abundance and -content (Zama, 1995; Makhoul and Trifonov, 2002; Saunders and Deane, 2010; Novoa and Ribas de Pouplana, 2012; Cortazzo et al., 2002; Torrent et al., 2018). In dependence on these effectors, the ribosomal speed is adjusted during translation to allow the proper folding of the arising polypeptide which is also thought to play a regulatory role on proteostasis (Zhang et al., 2009; Yanagitani et al., 2011). Artificially enforced ribosomal pausing caused by tRNA modification loss, possibly together with frame shifting events, may therefore impede global protein folding. Therefore, this mechanism might represent a key promoter of protein aggregation and could be also induced by other occasions (e.g. tRNA degradation). For instance, the hypomodified $tRNA^{Val}_{AAC}$ of *trm8 ncl1* is subjected to rapid tRNA decay (RTD) due to the lack of m^7G_{46} and m^5C_{49} at semi-permissive temperatures (37°C) (Alexandrov et al., 2006; Chernyakov et al., 2008). Degradation of this specific tRNA over time could also promote ribosomal stalling at the GUU codon leading to perturbation of translational speed and protein folding. Indeed, the heat-stressed double mutant displayed an increase of protein aggregation (**Figure A3 A**) and at the same time lost substantial amounts of the $tRNA^{Val}_{AAC}$ (**Figure A3 B**). In line with translation defects, other tRNA modification mutants like *trm5* (**Figure A1 A**) or *pus1 urm1* and *pus1 elp3* (**Figure A1 B**) displayed a similar accumulation of protein clusters, further supporting the hypothesis described above (Khonsari and Klassen, 2020; Lee et al., 2007).

Another explanation for the protein aggregation of the studied double mutants can be extracted from the transcriptome analysis of **Publication IV**. The categories ‘de novo’ protein folding and protein folding were found among the ten GO terms with the highest significance for supressed genes. This included various chaperones (e.g. *SSA1*, *SSA2*, *SSA4*, *SSZ1*, *SSE1*, *ZUO1*) and heat shock proteins (*HSP82*, *HSP42*, *HSP104*, *HSP60*) which are either supporting protein folding during the translation process or are strongly upregulated due to proteotoxic stress caused by environmental triggers (**Publication IV**, **Table S7** and **S15**) (Estruch, 2000; Gasch et al., 2000). These findings were unexpected since the protein aggregation of the *tcd1* and *deg1* combinations suggested an induction of the different protein folding enzymes to resolve protein aggregation as previously described for the *elp6 ncs2* mutant (Nedialkova and Leidel, 2015). Nonetheless, the same transcriptional analysis revealed that the heat shock proteins Hsp12 and Hsp26 were strongly induced in all mutant combinations (**Publication IV**) presumably indicating the loss of regulatory control of stress responses. The expression of various chaperones and HSPs is regulated by the activity of different transcription factors which either specifically respond to environmental triggers or to more general stresses (Estruch, 2000). As described in the previous section, various transcriptional regulators and factors are transcriptionally repressed, among which are different effectors playing important roles in various stress responsive pathways (e.g. *SMP1*, *CST6*, *CRZ1*) (**Publication IV**, **Table S11**). Thus, it could be assumed that maybe other major stress triggered transcription factors like

Msn2 and Msn4 (Estruch and Carlson, 1993; Martínez-Pastor et al., 1996) are also transcriptionally suppressed in response to the translational incapabilities of the tRNA modification mutants adding on to the protein aggregation by blocking the expression of HSPs and protein folding enzymes. Unfortunately, mRNAs for both genes escaped the RNAseq analysis.

Interestingly, besides the expressional decrease of chaperones and heat shock proteins, macro-autophagy and mitophagy appeared to be activated in all four double mutants (**Publication IV**, **Figure S2**, **Table S3**). Autophagy is canonically controlled by TORC1 accompanied by PKA and Snf1C to be induced upon starvation and/or in the stationary phase (as described above) (Noda and Ohsumi, 1998; Suzuki and Ohsumi, 2007; Papinski and Kraft, 2016; Wang et al., 2001; Budovskaya et al., 2005; Yorimitsu et al., 2007; Stephan et al., 2009;). Nevertheless, autophagic processes are also triggered to degrade proteotoxic clusters alongside with the UPS which is a conserved mechanism between lower and higher eukaryotes (Dikic, 2017; Zaffagnini and Martens, 2016). Interestingly, different reports also demonstrated an involvement of the mitochondria and mitophagy in the disposal of protein aggregates. According to these findings, mistranslation induced protein aggregates are transported into the mitochondria and are subsequently degraded via mitophagy. Moreover, translational disturbances and with that protein aggregates seem to affect the functionality of the mitochondria which the cell presumably tries to compensate via the upregulation of the organelle biogenesis (Ruan et al., 2017; Shcherbakov et al., 2019). Since the transcriptome analysis of *urm1 tcd1*, *elp3 tcd1*, *urm1 deg1* and *elp3 deg1* also indicates an induction of mitophagy- as well as mitochondrial respiratory genes, it can be suggested that similar processes might take place in the aggregation prone tRNA modification mutants (**Publication** IV, **Figure S2**, **Table S10** and **S13**).

Conclusively, protein aggregation might be triggered by a mix of different events. This could involve the decline in translational speed and protein folding as well as negatively influencing the transcriptional regulation of HSPs and chaperones all adding up to the accumulation of protein clusters. Nevertheless, induction of autophagy and probably also mitophagy may represent a way of the tRNA modification mutants to compensate the proteostasis defect.

7.3.3 Translational incapability affects transcriptome regulation(?)

The previous sections detailed alterations of translation, transcription and other consequences in the *urm1 tcd1*, *elp3 tcd1*, *urm1 deg1* and *elp3 deg1* mutants due to the combined lack of functionally cooperating tRNA modifications. Nonetheless, all described deficiencies could be tracked back to the two malfunctional $tRNA^{Lys}_{UUU}$ and $tRNA^{Gln}_{UUG}$, since overexpression of each rescued the observed defects in the specific mutants (**Publication I-Publication** IV). Since the physiological, transcriptional and in part translational symptoms of the different double mutants resemble each other, it may be assumed that there is a common cause responsible

for those events. Thus, it is likely that the corruption of the translation process promotes the transcriptional and other downstream aberrations, regardless of the modification defect that caused it. The presented and discussed findings imply that there might be different mechanisms compromised in response to the translation defects. Hence, the promoted deficiencies can be triggered on two occasions: either by (i) the uncontrolled accumulation of misfolded protein clusters and/or during (ii) the translational process monitored by co-translational effectors and pathways.

Protein aggregation in this regard appears to represent an comprehensible option to provoke downstream events in tRNA modification mutants since it appears to be a common consequence in response to translational incapability in various eukaryotic systems (**Publication I** and **IV**, **Figure A1**, **A3** and **A4**; Nedialkova and Leidel, 2015; Rojas-Benítez et al., 2013; Laguesse et al., 2015; Thiaville et al., 2016; Freeman et al., 2019; Pollo-Oliveira et al., 2020). In line with this hypothesis, a report investigating the influence of continuative mistranslation of the leucine CUG-codon by an engineered $tRNA^{Ser}_{CAG}$ revealed an impact not only on the proteome but also on the transcriptional regulation (Paredes et al., 2012). The consequential transcriptome alterations resembled those found in the four tRNA modification double mutants in regard of the modulation of signal cascades normally induced or suppressed upon starvation or stationary phase entry. This included the induction of GAAC, glucose repressed carbohydrate metabolism and oxidative stress response while the gene expression of translation relevant factors globally declined (**Publication IV**; Paredes et al., 2012). The artificial mistranslation however also triggered the activation of the UPR, of stress responsive heat shock proteins and chaperones presumably to cope with the proteotoxic stress (Paredes et al., 2012). This contrasted the findings for the four double mutants since both feedbacks appeared to be either functionally impeded or transcriptionally suppressed (**Publication III** and **Publication** IV). Hence, translational complications either caused by tRNA modification loss or environmentally/artificially induced mistranslation events might differ in some way. For instance, mistranslation and subsequent proteotoxic stress impede in human cell lines the induction of the UPR to prevent ER stress mediated apoptosis (see also chapter **7.2**) and moreover lead to an import of protein aggregates into the mitochondria for mitophagy dependent degradation (Shcherbakov et al., 2019). A similar process has been described for yeast in response to heat shock induced protein aggregation (Ruan et al., 2017). Hence, a comparable mechanism can be anticipated for the studied tRNA modification mutants (see also chapter **7.3.2**).

Overall, protein aggregation seems to bear the capacity to modulate the transcriptome, probably by forcing the cell to react to the continuative proteotoxicity resulting in the loss of regulatory control. Clearance of single misfolded proteins and clusters is among others achieved by the ubiquitin proteasome system (UPS) which critically depends on the 26S

proteasome to degrade ubiquitin-marked (ubiquitinylated) proteins (Finley et al., 2012). Hence, it is highly likely that this pathway is also involved in the degradation of protein aggregates in tRNA modification mutants. For instance, the transcription factor gene *RPN4* known to promote expression of proteasomal genes was disrupted in the *elp6 ncs2* background which led to an aggravation of the growth phenotype indicating the importance of a functional UPS in the mutant background (Nedialkova and Leidel, 2015). Alongside to this important task, the 26S proteasome is responsible for the eukaryotic transcription control via the regulation and degradation of transcription factors and transcriptional (co-)activators (Muratani and Tansey, 2003; Geng et al., 2012). The ubiquitination status of those factors/activators controls their activity, localisation and stability, monitored by the proteasome complex probably to prevent undesired induction of transcription (Geng et al., 2012). Therefore, transcriptional regulation as well as degradation/clearance of damaged proteins or protein aggregates, respectively, lies in the responsibility of the 26S proteasome. Based on this link, it can be assumed that an ongoing supply of protein aggregation might overwhelm the complex and in consequence perturb or block other functions of it. In human cells the expression of aggregation prone proteins like huntingtin or a folding mutant of the cystic fibrosis transmembrane conductance regulator was capable to inhibit the UPS resulting in an arrests of the cell cycle (Bence et al., 2001). A similar approach by overexpressing the same huntingtin variant in different yeast cells (WT or *deg1*) promoted comparable cytological and morphological defects that moreover were comparable to the documented deficiencies of *urm1 tcd1*, *elp3 tcd1*, *urm1 deg1* and *elp3 deg1* (**Publication I**). Hence, it can be assumed that the efficient protein aggregation dependent UPS inhibition might also influence the half-life of transcription factors and compromise the transcriptional regulation in those mutants enforcing the transcriptome aberrations described above. Nevertheless, the RNAseq analysis of the *tcd1* and *deg1* combinations pointed also to a decline of the transcription machinery alongside with transcription factors and effectors (**Publication IV**, **Figure S2**, **Table S7** and **S11**). Possibly both the transcriptional and proteasomal modulation of transcription relevant factors are cooperatively causing the deregulation of the transcriptome.

Interestingly, co-translational monitoring of the protein biogenesis as well as various translational quality control pathways are linked to the 26S proteasome activity. Three major pathways are known to act during translational issues and react to specific obstacles concerning translation elongation or termination. The nonsense-mediated decay (NMD) is activated upon premature termination of the translation process by premature stop codons (PTC) on the mRNA (Losson and Lacroute, 1979; Popp and Maquat, 2013; Lykke-Andersen and Bennett, 2014; Maquat et al., 1981). On the other hand, translational elongation can be affected by mRNA aberrations e.g. by forming secondary structures blocking the ribosome or by a lack of a stop codon leading to no translation termination. The first issue activates the no-

go decay (NGD) while the latter induces the non-stop decay (NSD) (Doma and Parker, 2006; Harigaya and Parker; Frischmeyer et al., 2002; van Hoof et al., 2002). Irrespective which pathway is triggered by corrupted translation, the cell tries to recycle the ribosome and tRNA whereas the faulty nascent polypeptide as well as the mRNA must be degraded, whereas the latter is achieved by different specific nucleases employed by the three pathways. Hence, subsequential to the NGD or NSD the ribosome-associated quality-control (RQC) is enforced to split the 40S and 60S ribosomal subunits by Dom34 and Hbs1 (Doma and Parker, 2006; Lykke-Andersen and Bennett, 2014; Passos et al., 2009; Tsuboi et al., 2012). After this step, the RQC involves the association of Cdc48 by Rqc1 to the 60S to extract the nascent peptide chain and to afterwards mark it for proteasomal degradation by the ubiquitin ligase (E3) Ltn1 and Rqc2 (Bengtson and Joazeiro, 2010; Brandman and Hegde, 2016; Brandman et al., 2012; Defenouillère et al., 2013). Since both NGD and NSD appear to be intimately connected to the RQC it may be suggested that the NMD also relies on this quality control pathway (Lykke-Andersen and Bennett, 2014; Tsuboi et al., 2012; Shao et al., 2013; Matsuda et al., 2014). Nevertheless, only Cdc48 has been found to promote probably in concert with the key factor Upf1 (and Upf3) tRNA-bound nascent peptide chain degradation by the proteasome during the nonsense-mediated decay (Lykke-Andersen and Bennett, 2014; Takahashi et al., 2008; Kuroha et al., 2009, 2013; Verma et al., 2013). However, since different findings point to frame shifting and ribosome pausing events due to the combined or individual loss of mcm^5/s^2U_{34}, $(c)t^6A_{37}$ and/or $\Psi_{38/39}$ it is possible that one or more of the described pathways may be activated in the corresponding mutants (**Publication II**; Zinshteyn and Gilbert, 2013; Nedialkova and Leidel, 2015; Ranjan and Rodnina, 2017; Klassen and Schaffrath, 2018; Lecointe et al., 2002; Rezgui et al., 2013; Tükenmez et al., 2015; Thiaville et al., 2016; Joshi et al., 2018). For instance, disruption of the NMD key factor *UPF1* in an *elp3* background appeared to improve growth of the mutant on Ade- media presumably by stabilisation of the PTC harbouring *ADE2* mRNA (Klassen and Schaffrath, 2018). Additionally, NMD (at least in mammalian organisms) appears to be involved in the negative regulation of different stress responsive pathways like the UPR or autophagy which might indicate a similar role in bakers yeast (Gardner, 2008; Goetz and Wilkinson, 2017; Mendell et al., 2004; Wengrod et al., 2013;). Hence, the translational defects in tRNA modification mutants may engage the NMD, NGD and/or NSD and subsequentially RQC to protect protein homeostasis but conversely may also promote the presumed defects of the 26S proteasome leading to the demonstrated transcriptional aberrations. A first attempt, however, to test the RQC participation in proteostasis protection was done in the *elp6 ncs2* background. Disruption of the RQC participants *DOM34* or *HBS1* in the tRNA modification mutant resulted only in mild aggravation of translational and phenotypic defects probably contradicting an involvement of the ribosome recycling system in coping with protein aggregation stress (Nedialkova and Leidel, 2015). Nevertheless, blockage

of any of the co-translational quality-control pathways may also account to protein aggregation and transcriptional abnormalities. For instance, deletion of RQC factors like *LTN1* or *RQC2* induces the Hsf1 dependent heat shock response. This occurs most likely to clear aggregation-prone polypeptide chains and might resemble stress responses detected in some tRNA modification mutants (Brandman and Hegde, 2016; Satyal et al., 2000; Brandman et al., 2012; Alings et al., 2014; Damon et al., 2014; Shen et al., 2015).

Additionally, tRNA modification double mutants displayed decreased transcript levels of chaperones known to be also involved in the support of co-translational protein folding, potentially adding on to the detected stress responses. This applied to both components of the ribosome-associated complex (RAC) namely *ZUO1* and *SSZ1* (**Publication IV**, **Table S7** and **S15**). Both are directly attached to the translation machinery and are supporting the protein folding process in cooperation with the chaperones Ssb1/Ssb2 (Yan et al., 1998; Gautschi et al., 2001). Hence, a decline or abolishment of one or both RAC components is a well-known trigger for protein aggregation, which was also confirmed in **Publication IV** (Gamerdinger, 2016; Willmund et al., 2013). Moreover, disruption of *ZUO1* triggered the expression of the stress marker genes *HSP12* and *MEP2,* indicating a link between deficiencies in the co-translational protein folding support, protein aggregation and starvation stress/stationary phase response (**Publication IV**). In sum, protein aggregation could be a major trigger of transcriptional abnormalities in tRNA modification mutants by influencing various regulatory pathways (**Publication IV**, **Figure 7**). Nevertheless, it remains to be investigated in future studies if this holds true, what proteins and mechanisms are contributing to aggregation or resolving of them and which of the described possibilities are responsible for the loss of regulatory control of the transcriptome.

8. References

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9. Authors‘ Contribution to Publications

University Kassel, Faculty 10 Mathematics und Nature Science

Declaration of the cumulative dissertation in the doctoral subject Biology

The authors‘ contributions for each publication will be stated clearly according to the “Allgemeine Bestimmungen für Promotionen an der Universität Kassel (AB-PromO) § 7 vom 18.05.16” and “Neufassung der Besonderen Bestimmungen des Fachbereichs Mathematik und Naturwissenschaften der Universität Kassel zu den Allgemeinen Bestimmungen für Promotionen an der Universität Kassel (AB-PromO) § 7 vom 15.11.2017“.

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Topic of the Dissertation:

“**Investigation of the functional synergy and involvement of different tRNA modifications in translational control”**

2. Numbered list of publications and detailed explanation of authors’ contribution:

I. Klassen, R., Ciftci, A., Funk, J., **Bruch**, **A**., Butter, F., & Schaffrath, R. (2016). tRNA anticodon loop modifications ensure protein homeostasis and cell morphogenesis in yeast. *Nucleic acids research*, *44*(22), 10946-10959.

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II. Klassen, R., **Bruch**, **A**., & Schaffrath, R. (2017). Independent suppression of ribosomal+ 1 frameshifts by different tRNA anticodon loop modifications. *RNA biology*, *14*(9), 1252-1259.

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III. **Bruch**, **A**., Klassen, R., & Schaffrath, R. (2018). Unfolded protein response suppression in yeast by loss of tRNA modifications. *Genes*, *9*(11), 516.

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IV. **Bruch**, **A**., Laguna, T., Butter, F., Schaffrath, R., & Klassen, R. (2020). Misactivation of multiple starvation responses in yeast by loss of tRNA modifications. *Nucleic Acids Research*.

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4.2 Dr. Roland Klassen signature:

4.3 Dr. Falk Butter signature:

4.4 Dr. Teresa Laguna signature:

4.5 Johanna Funk signature:

4.6 Akif Ciftci signature:

10. Erklärung gemäß § 8 Abs. 1 Satz 2 lit. D AB-PromO

Hiermit versichere ich, dass ich die vorliegende Dissertation selbständig, ohne unerlaubte Hilfe Dritter angefertigt und andere als die in der Dissertation angegebenen Hilfsmittel nicht benutzt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten oder unveröffentlichten Schriften entnommen sind, habe ich als solche kenntlich gemacht. Dritte waren an der inhaltlichen Erstellung der Dissertation nicht beteiligt; insbesondere habe ich nicht die Hilfe eines kommerziellen Promotionsberaters in Anspruch genommen. Kein Teil dieser Arbeit ist in einem anderen Promotions- oder Habilitationsverfahren durch mich verwendet worden.

Gudensberg, der _____

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11. Acknowledgements

I would like to thank both my supervisors Prof. Dr. Raffael Schaffrath and Dr. Roland Klassen who encouraged me to work on this fascinating project in the framework of the SPP1784 “Chemical Biology of native Nucleic Acid Modifications” and their support during this time. I highly appreciate the assistance, discussions, advices and ideas which helped me to develop on a scientific and personal level. This involved not only the conceptualisation of the experiments and the resulting papers but also the attendance to the various SPP1784 conferences and the EMF-program.

I also want to thank all co-authors of the different publications imbedded in this thesis, especially Dr. Falk Butter and Dr. Teresa Laguna who supervised and worked on RNA sequencing and corresponding bioinformatics of our mutant set.

A special thanks goes to Dr. Roland Klassen for the daily help in the lab in regard of data interpretation, discussions and advices concerning every aspect of the scientific work. Moreover, he and Leonie Vogt were untiringly providing helpful suggestions regarding this work, which I am appreciating very much.

Nonetheless, without the great work atmosphere in the department of microbiology laboratory and in the “SPP office” this work would have never come to a successful end. Thus, I would like to thank Roland, Koray, Harmen, Alex, Cindy, Bahar, Maria, Karsten, Uschi, Lars, David, Leonie, Meike, Özge, Valentina, Anna and all other staffs/students/PhD-students who were working in our lab for contributing to this great four years either by fruitful discussion, many(!) coffee breaks or just helpfulness and kindness. Additionally, a special thanks goes to Birgit Jäger for assisting on every administrative issue, her kindness and general support.

I thank all professors who agreed to review this thesis and/or to attend to the disputation.

Finally, I thank my family and friends for their patience and understanding, when I decided to stay in lab for one “last” experiment instead of meeting up, and their support during this time. This work is dedicated to my parents.

12. Appendix

The following presents experimental results aiming to extend the data of the **publications I-IV**. The figures are assigned to the respective publication by roman numerals. The accompanied Material & Methods section summarizes the utilized *S. cerevisiae* strains, primers and experimental approaches which were used for the results in case they are not already detailed in the publications imbedded in this dissertation.

12.1 Material & Methods

Table A1. *S. cerevisiae* strains used throughout the different experiments in **Fig. A1** and **Fig. A3**.

Strain	Genotype	Reference/source
BY4741	*MAT***a**, *his3Δ, leu2Δ, met15Δ, ura3Δ*	Euroscarf, Frankfurt
RK420	BY4741 *trm8Δ::KanMX4 ncl1Δ:: SpHIS5*	Roland Klassen
RK480	BY4741 *trm5Δ::SpHIS5*	Roland Klassen
RK495	BY4741 *pus1Δ::SpHIS5 elp3Δ::KlLEU2*	Roland Klassen
RK496	BY4741 *pus1Δ::SpHIS5 urm1Δ::KlLEU2*	Roland Klassen

Table A2. Oligonucleotides used for the experiment in **Fig. A3B**.

Oligonucleotide	Sequence (5´ - 3´)	Target	Purpose
tRNAValAACfw	GGTTTCGTGGTCTAGTCGGTTA	*tV(AAC)*	RT-PCR detection of $tRNA^{Val}_{AAC}$
tRNAValAACrv	GATTTCGCCCAGGATCGAACTG	*tV(AAC)*	RT-PCR detection of $tRNA^{Val}_{AAC}$
tRNA_Gly_Fw	GCGCAAGTGGTTTAGTGGT	*tG(GCC)*	RT-PCR detection of $tRNA^{Gly}_{GCC}$
tRNA_Gly_Rv	TGCGCAAGCCCGGAATCGAAC	*tG(GCC)*	RT-PCR detection of $tRNA^{Gly}_{GCC}$

12.1.1 Total RNA extraction and RT-PCR

Total RNA was isolated from strains (**Table A1**) cultivated until OD_{600}=1.0 at 30°C in YPD as described in **Publication IV**. A portion of the same cultures was additionally stressed at 37°C for 4 hours, harvested and subjected to RNA extraction. The RNA samples were further processed and afterwards used to perform RT-PCR according to (Khonsari and Klassen, 2020). The primer-pairs tRNAValAACfw/tRNAValAACrv and tRNA_Gly_Fw/tRNA_Gly_Rv (**Table A2**) were utilized to amplify the RTD-target $tRNA^{Val}_{AAC}$ or the $tRNA^{Gly}_{GCC}$ as a control, respectively, and the resulting PCR-products were separated on a 2% agarose gel.

12.2 Results of interest for publication I

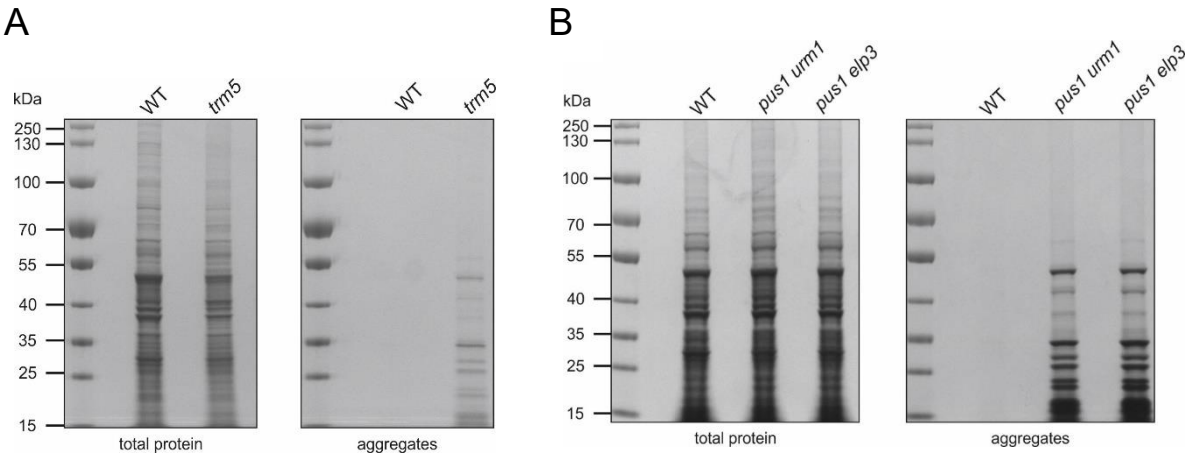

Figure A1 Loss of additional tRNA modifications, individually or in combination, induces protein aggregation. The indicated tRNA modification single (**A**) or double (**B**) mutants were incubated in YPD media until early log phase (OD_{600}=1) and 50 OD_{600} units were subjected to protein aggregate isolation as previously described (Koplin et al., 2010). Equal amounts of protein extracts of the different indicated strains (left panels) were used for protein aggregate preperation (right panel). Detection was achieved via SDS-PAGE and Coomassie staining.

12.3 Results of interest for publication III

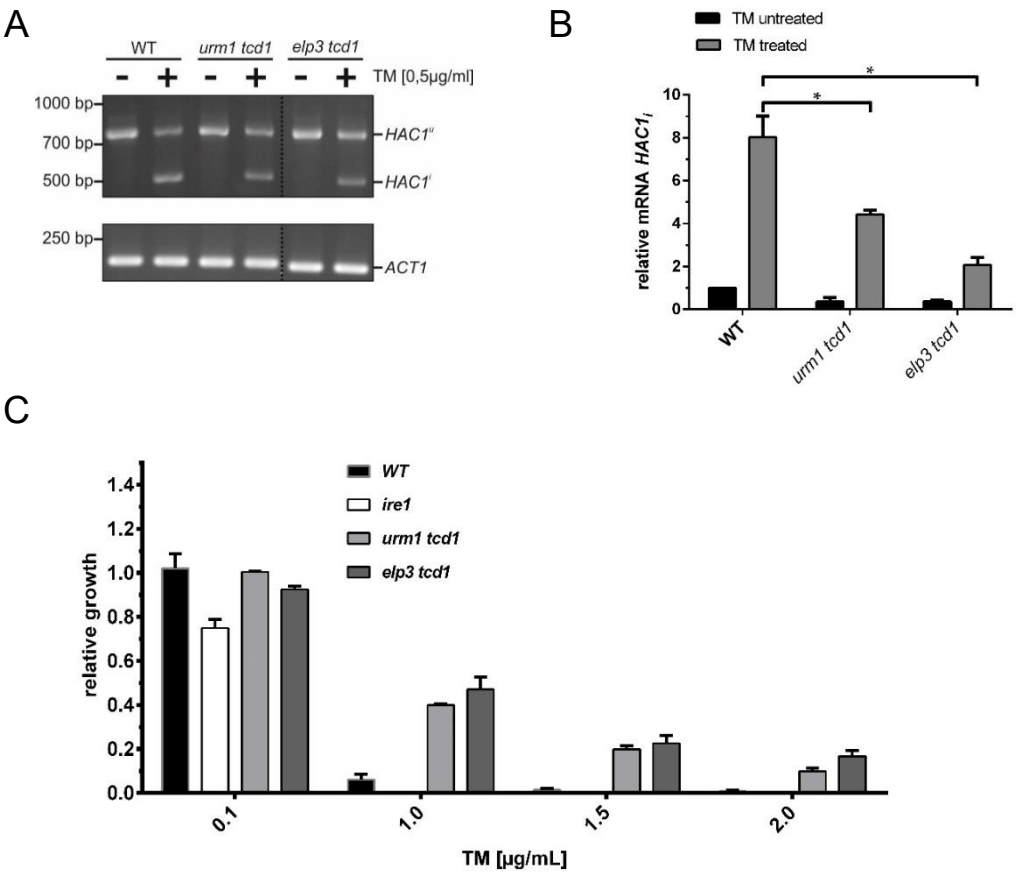

Figure A2 Examination of TM induced phenotypes and *HAC1* splicing of the *urm1 tcd1* and *elp3 tcd1* mutants. The indicated strains were incubated in YPD until early-log phase (OD_{600}=1) and subjected to total RNA isolation for RT-PCR (**A**) or qRT-PCR (**B**) to measure the *HAC1* transcript splicing in the WT and both double mutants according to (De-Souza et al., 2014) and **Publication III**, respectively. Additionally, all three strains were treated with 0.5 µg/mL tunicamycin (TM) for 3h as a control (**+**). The unprocessed transcript is represented by $HAC1^u$ whereas the spliced variant is indicated by $HAC1^i$. In both approaches, the *ACT1* mRNA level was utilized as a loading control (**A**) or for normalisation of the quantified $HAC1^i$ mRNA (**B**) according to (Pfaffl, 2001). For the latter experiment, three biological replicates and technical triplicates were used for qRT-PCR and statistical significance was calculated conducting the two-tailed *t*-test and indicated with asterisks (* $p < 0.05$). (**C**) The WT, i*re1*, *urm1 tcd1* and *elp3 tcd1* were each cultivated in YPD medium for 24 h treated with the indicated concentrations of TM. The standard deviations indicated on the bars were derived from experimental approaches involving three biological replicates.

12.4 Results of interest for publication IV

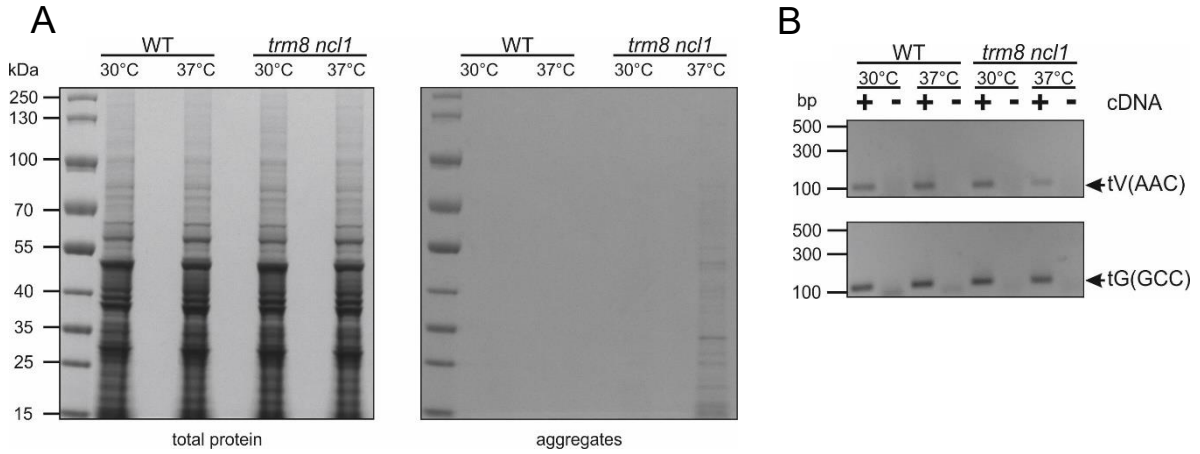

Figure A3 Heat stress induced rapid tRNA decay (RTD) promotes protein aggregation in *trm8 ncl1.* The indicated strains were cultivated in YPD at 30°C until they reached the exponential growth phase (OD_{600}=1). A portion of the cultures were harvested (50 OD_{600} units) and the rest was further incubated at semi-permissive temperature (37°C) for 4 hours. After this step, similar OD_{600} units of the cells were again harvested and all obtained yeast pellets were utilised for protein aggregate (**A**) and total RNA (**B**) extraction (Koplin et al., 2010; **Publication IV**). Preparation and detection procedures for protein aggregate isolation were followed as described in **Figure A1**. Total RNA isolates (1 µg) of the indicated strains were used for revere transcription PCR (RT-PCR) with specific primers for the known RTD-target $tRNA^{Val}_{AAC}$ (tV(AAC)) (Alexandrov et al., 2006) and the non-target control $tRNA^{Gly}_{GCC}$ (tG(GCC)) as described in (Khonsari and Klassen, 2020). The application (**+**) or the lack (**-**) of cDNA for the RT-PCR experiments are indicated.

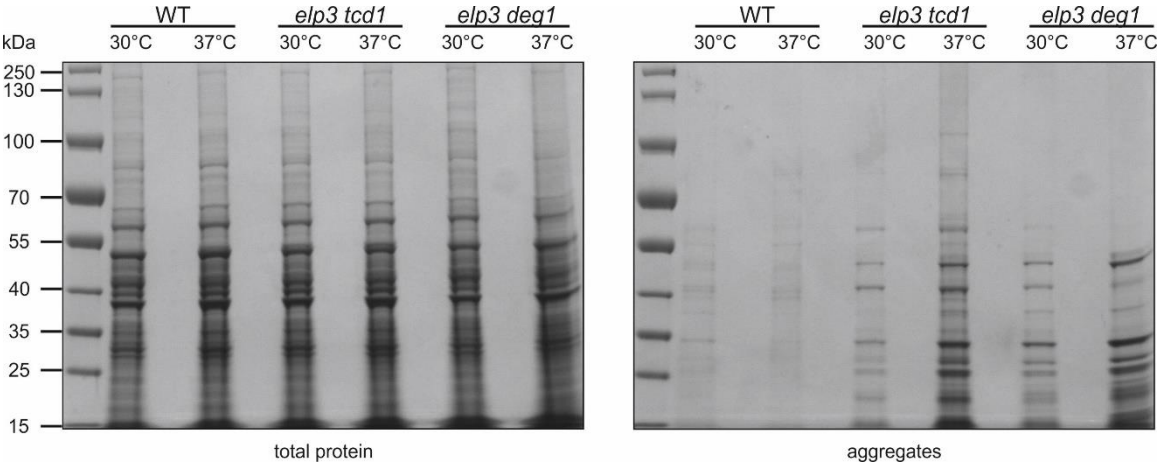

Figure A4 Effect of heat stress on protein aggregation in tRNA modificaion mutants. Incubation of the WT, *elp3 tcd1* and *elp3 deg1* was performed at permissive temperature (30°C) until mid log phase (OD_{600}=1). After harvesting 50 OD_{600} units, the remaining culture was shifted to semi-permissive temperatures (37°C) for 4 hours. The following harvesting (50 OD_{600} units) and application of the differentially treated cells to protein aggregate isolation was executed as detailed in **Figure A1** and **Figure A3**.