The Importance of tRNA Modification and *SSD1* Status for Protein Homeostasis and Cell Viability

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“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.”

Marie Curie

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Publications

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List of Abbreviations

A site	Aminoacyl site
AMP	Adenosine monophosphate
AMPK	5´AMP-Activated protein kinase
AS	Acceptor steam
ASL	Anticodon steam and loop
ATG*X*	Autophagy related
ATP	Adenosine triphosphate
CAMP	Cyclic adenosine monophosphate
Cbf5	Centromere binding factor 5
CBK1	Cell wall biosynthesis kinase 1
CCA	Cytosine-cytosine-adenine tail
CDC19	Cell division cycle 19
CFU	Colony forming unit
CFW	Calcofluor-white
CJD	Creutzfeldt Jacob disease
CLS	Chronological life span
CWI	Cell wall integrity
DEG1	Depressed growth rate 1
DL	Dihydrouridine loop
DNA	Deoxyribonucleic acid
DR	Dietary restriction
DTT	Dithiothreitol

E site	Exit site
EDTA	Ethylenediaminetetraacetic acid
eIF2	Eukaryotic initiation factor 2
ELP*X*	Elongator Protein
G1 phase	Growth 1 phase
GFP	Green fluorescent protein
HAc	Hydrogen acetate
HCl	Hydrogen chloride
HSP*X*	Heat shock protein
IPOD	Insoluble protein deposit
JUNQ	Juxtra nuclear quality control compartment
KCl	Potassium chloride
LB	Lysogeny broth
mcm^5	5-methoxycarbonylmethyl
Met	Methionine
MET22	Methionine requiring 22
$MgCl_2$	Magnesium chloride
ml	Milliliter
mM	Millimolar
mRNA	Messenger ribonucleic acid
mTOR	Mechanistic target of rapamycin
NCL1	Nuclear protein
ncm^5	5-carbamoylmethyl
NCR	Nitrogen catabolic repression

NCS*X*	Needs Cla4 to survive
nM	Nanomolar
OD	Optical density
P site	Peptidyl site
pAp	adenosine 3´,5´ bisphosphate
PAS	Pre-autophagosomal structure
P-bodies	Processing bodies
PBs	P-bodies
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PH	Potential of hydrogen
PKA	Protein kinase A
PLD	Prion like domain
PMSF	Phenylmethylsulfonyl fluoride
PPase	Pyrophosphatase
PUS*X*	Pseudouridine synthase
PVDF	Polyvinylidene fluoride
RAC	Ribosome Associated Complex
rcf	Relative centrifugal force
RLS	Replicative life span
RNA	Ribonucleic acid
RNP	RNA guided ribo nucleo protein
RNQ1	Rich in asparagine (N) and glutamine (Q) 1
rpm	revolutions per minute

Rpn4	Regulatory Particle Non-ATPase
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
RTD	Rapid tRNA decay
S (phase)	Sythesis phase
SDS	Sodium dodecyl sulfate polyacrylamide
SIT*X*	Suppressor of initiation of transcription
SNF1	Sucrose nonfermenting 1
snRNA	Small nuclear ribonucleic acid
SSD1	Suppressor of SIT4 deletion
Ssd1-d	Suppressor of SIT4 deletion -dead
SSD1-v	Suppressor of SIT4 deletion -viable
TL	Thymidine loop
TORC*X*	Target of rapamycin complex
TOR*X*	Target of rapamycin
TRM*X*	tRNA methyltransferase
tRNA	Transfer ribonucleic acid
U	Ubiquitin
UBA4	Ubiquitin activating
UPS	Ubiquitination proteasome system
URE2	UREidosuccinate transport 2
URM1	Ubiquitin related modifier 1
V	Volt
v/v	volume/volume

VL	Variable loop
w/v	weight/volume
WT	Wild type
YNB	Yeast nitrogen broth
YPD	Yeast extract peptone dextrose
ZUO1	Zuotin
Ψ	Pseudouridine
°C	Degree celsius
µg	Microgram
µl	Microliter
5-FOA	5-Fluoroorotic acid
5-FU	5-fluorouracil

1. Abstract

Chemical modifications of anticodon loop are required to support tRNA function in mRNA decoding and to prevent protein aggregation in the eukaryotic model system *Saccharomyces cerevisiae*. This work investigates the effects of mutation in the gene encoding the Deg1 pseudouridine synthase. It is known that the mutation of the human *DEG1* orthologue *PUS3* causes severe neurological and developmental defects, emphasizing the general importance of the modification. However, the exact cellular consequences of *deg1*/*pus3* mutation remain to be resolved. This thesis shows that in yeast, the absence of a functional Deg1 synthase induces slow growth, heat and drug sensitivities and protein homeostasis defects. In addition, depending on the specific selection of the yeast strain background, a shortened chronological lifespan could also be observed, and strong negative genetic interactions of *DEG1* with other genes involved in anticodon loop modification were detected. Strain background influence on phenotypic variation is partly due to allelic variation of the gene encoding the RNA binding protein Ssd1. A common laboratory yeast strain used in this work contains the *ssd1-d* allele encoding a truncated non- functional Ssd1 variant. This allele is linked to phenotypes that partially overlap with those of *deg1*. Thus, enhancing *deg1* phenotypes by *ssd1-d* may result from the additivity of negative phenotypes and may not necessarily involve shared molecular mechanisms. Since protein homeostasis defects could be functionally involved in aging phenotypes, life spans and protein aggregation in *deg1* mutants and strains lacking a ribosome-associated chaperone involved in co-translational protein folding is compared. While both strains accumulated protein aggregates, only the *deg1* mutant exhibited a reduced life span. Hence, other cellular consequences than protein aggregation may cause the *deg1* aging phenotype. Since yeast's lifespan is also known to be affected by the TOR (target of rapamycin) kinase activity, the rapamycin sensitivities and autophagy (a process under the direct control of TOR) were analyzed. The absence of *DEG1* is found to induce rapamycin sensitivity and increased autophagy. Both effects are enhanced in the absence of functional Ssd1, pointing to a variation of TOR activity in the different yeast strain backgrounds. Direct translational effects of the *deg1* mutation were studied using a glutamine-rich Rnq1 protein. The results suggest that the combined absence of functional *SSD1* and *DEG1* aggravates the expression defect of the protein, suggesting that translation is negatively affected by mutation in both genes.

2. Abstract in German/ Zusammenfassung

Chemische Modifikationen der Anticodon-Schleife sind erforderlich, um die tRNA-Funktion bei der mRNA-Decodierung zu unterstützen und die Proteinaggregation im eukaryotischen Modellsystem *Saccharomyces cerevisiae* zu verhindern. Diese Arbeit untersucht die Auswirkungen von Mutationen im *DEG1* Gen, das eine Pseudouridin-Synthase kodiert. Es ist bekannt, dass die Mutation des humanen *DEG1*-Orthologs *PUS3* schwere neurologische und Entwicklungsstörungen verursacht, was die allgemeine Bedeutung der Modifikation unterstreicht. Die genauen zellulären Folgen müssen jedoch noch geklärt werden. Diese Arbeit zeigt, dass in der Hefe das Fehlen einer funktionellen Deg1-Synthase zu langsamem Wachstum und Empfindlichkeit gegenüber Hitze und verschiedenen chemischen Agenzien führt sowie Defekte in der Proteinhomöostase auslöst. Darüber hinaus konnte je nach spezifischer Selektion des Hefestammhintergrunds auch eine verkürzte chronologische Lebensdauer beobachtet werden, und es wurden starke negative genetische Wechselwirkungen von *DEG1* mit anderen an der Anticodon Loop-Modifikation beteiligten Genen nachgewiesen. Der Einfluss des Stammhintergrunds auf die phänotypische Variation ist teilweise auf allelische Variation des Gens zurückzuführen, das das RNA-bindende Protein Ssd1 codiert. Ein üblicher Laborhefestamm, der in dieser Arbeit verwendet wird, enthält das *ssd1-d*-Allel, das für eine verkürzte, nicht funktionale Ssd1-Variante kodiert. Dieses Allel ist mit Phänotypen verbunden, die teilweise mit denen von *deg1* überlappen. Daher kann die Verstärkung von *deg1*-Phänotypen durch *ssd1-d* aus der Additivität negativer Phänotypen resultieren und muss nicht notwendigerweise gemeinsame molekulare Mechanismen beinhalten. Da Defekte der Proteinhomöostase funktionell an alternden Phänotypen beteiligt sein könnten, werden Lebensdauern und Proteinaggregation in *deg1*-Mutanten und Stämmen, denen ein Ribosomen-assoziiertes Chaperon fehlt, das an der co-translationalen Proteinfaltung beteiligt ist, verglichen. Während beide Stämme Proteinaggregate akkumulierten, wies nur die *deg1*-Mutante eine reduzierte Lebensdauer auf. Daher können andere zelluläre Folgen als die Proteinaggregation den *deg1*-Alterungsphänotyp verursachen. Da bekannt ist, dass die Lebensdauer von Hefen auch von der TOR-Kinaseaktivität (Target of Rapamycin) beeinflusst wird, wurden die Rapamycin-Empfindlichkeit und die Autophagie (ein Prozess unter direkter Kontrolle von TOR) analysiert. Es wurde festgestellt, dass das Fehlen von *DEG1* eine Rapamycin-Empfindlichkeit und eine erhöhte Autophagie induziert. Beide Effekte werden in Abwesenheit von funktionellem Ssd1 verstärkt, was auf eine Variation der TOR-Aktivität in den unterschiedlichen Hefestämmen hindeutet. Direkte Translationseffekte der *deg1*- Mutation wurden unter Verwendung eines glutaminreichen Rnq1-Proteins untersucht. Die Ergebnisse legen nahe, dass das kombinierte Fehlen von funktionellem *SSD1* und *DEG1* den Expressionsdefekt des Proteins verschlimmert, was darauf hindeutet, dass die Translation durch Mutationen in beiden Genen negativ beeinflusst wird.

3. Introduction

3.1. Transcription and translation

In all living organisms, transcription is a process of making RNA from DNA templates in the nucleus. Transcription begins with the attachment of transcription factors to the TATA box, and RNA polymerase is then capable of attaching to the antisense DNA strand and starts synthesizing the RNA template, and the newly formed RNA strand is released to be post-processed at the end (Levine & Tjian, 2003; Spitz & Furlong, 2012).

There are various types of RNA, and the most prominent ones are messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA), which exist in all living entities. One of the transcriptional products in eukaryotic cells is a pre-mRNA that undergoes post-transcriptional modification. At this point, modified guanine will be added to the 5´end of the mRNA, and a poly-A tail (containing lots of adenosine monophosphates) will be added to the 3´end of the mRNA to stabilize and prepare it for the translation process. In some cases, parts of the mRNA sequence are introns that are not coding for protein get sliced out with splicing mechanism. The exons, which are coding sequences, stay in the mRNA and result in a mature mRNA (Proudfoot et al., 2002).

Synthesizing protein occurs during the translation process which employs mature mRNA as the template and takes place in the cell's cytoplasm. Translation can be divided into three stages, starting with initiation, followed by elongation, and ending with termination. The eukaryotic mRNA has a 3´end, a part containing the codons encoding specific amino acids, and a methylated cap at the 5´end. Translation initiation begins when the small subunit (40S) of the ribosome attaches to the cap site of the mRNA strand and moves to the translation initiation site. As explained, mRNA contains codons that code for specific amino acids. At this stage, tRNA molecules containing the complementary anticodon for mRNA codons bind to the mRNA. The first mRNA codon is typically the AUG codon which is complementary with the CAU anticodon in a tRNA molecule. At 3´end (the acceptor stem of the tRNA), the corresponding amino acid is attached, which is methionine (Met) for the tRNA responsible for translation initiation, and it decodes for the AUG codon in the mRNA. Eukaryotic initiation factor (eIF2) mediates the binding of $tRNA_i^{Met}$ to the 40S subunit

of the ribosome (Shin et al., 2011). The large subunit of the ribosome (60S) is then binding to create the peptidyl (P) and the aminoacyl (A) sites. The first tRNA occupies the P site, and the second tRNA with the complementary anticodon for the mRNA codon is placed in the A site. The methionine carried by the first tRNA is then transferred to the A sites amino acid, and the first tRNA moves to the exit (E) site and leaves the ribosome, and both ribosome subunits move along the mRNA, and the next tRNA enters. These are the basic steps of elongation. As elongation continues, the growing peptide continually transfers to the A site tRNA, and the ribosome moves along the mRNA, and new tRNAs keep entering the A site until a stop codon of the mRNA appears in the A site. In the end the release factor protein binds to the stop codon and initiates the translation termination. At this point, the ribosome (80S) dissociates and the protein gets released (Dever et al., 2016).

3.2. tRNA modification in *Saccharomyces cerevisiae*

The chemical structure of a specific tRNA molecule was first determined for yeast $tRNA^{Ala}$, including several modified nucleosides (Holley et al., 1965). It was shown that tRNA has a cloverleaf-like secondary structure, and the anticodon codon was predicted as ICG where the inosine (I) is a modified form of adenosine. This finding helped Francis Crick to propose the wobble hypothesis in 1966. It implies that wobble pairing occurs when a single tRNA species decodes multiple codons differing in the third nucleotide of the mRNA codon. This involves non-Watson-Crick base pairings between the anticodon position 34 of the tRNA and the third codon base and was referred to as “Wobble position”. Wobble decoding often involves a modified base at the tRNA position 34, which helps the tRNA to decode multiple codons, coding for the same amino acid (Agris et al., 2007). In addition to their occurrence at the Wobble position and functional involvement in expanding the decoding ability, tRNA modifications occur at various other positions (Figure 1) and were analyzed in different organisms in further studies. tRNA modifications are important for efficient mRNA translation, and some of them are also required for efficient aminoacylation, which is the process of charging tRNA species with the correct amino acid (Hayashi et al., 1994; Senger et al., 1997).

For effective translation, tRNAs need to maintain their cloverleaf shaped structure. The structural stabilization of the tRNA is usually related to modifications in the main body of the tRNA, like pseudouridines which are rigidifying the tRNA structure by

driving the sugar component of the nucleobase into C3´-endo (El Yacoubi et al., 2012; Duechler et al., 2016; Lorenz et al., 2017). tRNA modifications in the yeast *Saccharomyces cerevisiae* have been extensively studied, and recently the importance of tRNA modification in higher eukaryotes, like humans, is becoming more apparent by finding deregulated tRNA modifications and modification enzymes in neurological diseases, cancer or mitochondrial linked disorders (Begley et al., 2013; Bykhovskaya et al., 2004; Delaunay et al., 2016; Kopajtich et al., 2014; Shimada et al., 2009).

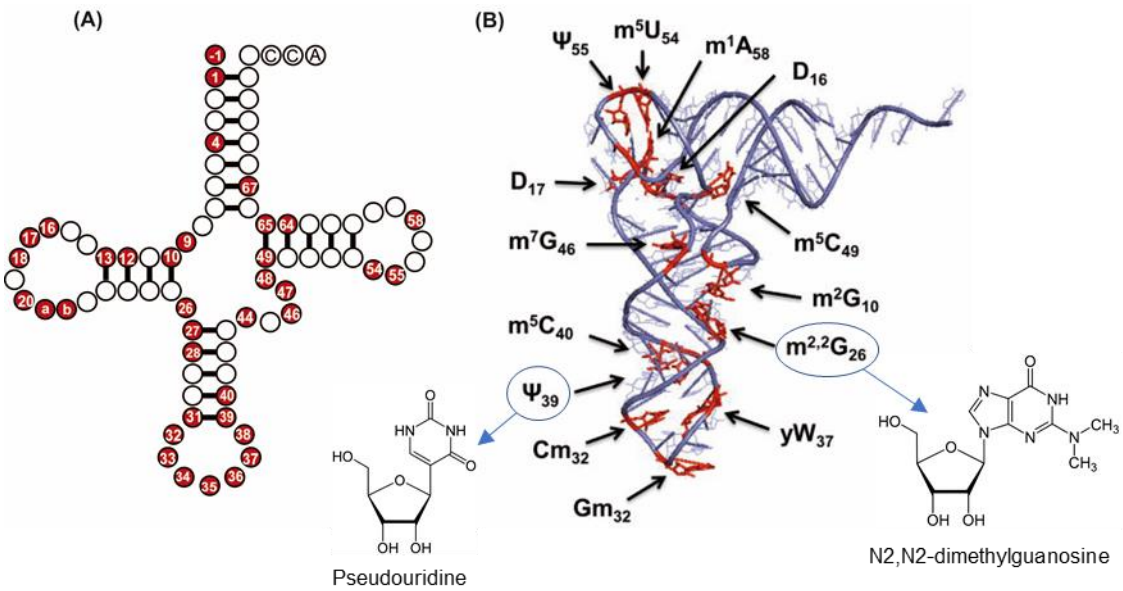

Figure 1. tRNA structure and the positions of modified nucleotides. (A) The two-dimensional structure of a tRNA molecule in *Saccharomyces cerevisiae*. Each ball represents a single nucleotide. Modified nucleotides are represented in red, and the highest density of these modifications is observed in the anticodon stem loop position. (B) Three-dimensional structure of tRNA. This structure belongs to $tRNA^{Phe}GAA$, and modified nucleotides are highlighted in red. The position of two of the modifications in yeast are shown with blue circles, and the chemical structure of these modifications (Ψ_{39} and $m^{2,2}G_{26}$) are shown next to their modification sites. The image is modified from (Jackman & Alfonzo, 2013).

3.2.1. Pseudouridylation sites in the tRNA molecule

One of the most frequent tRNA modifications is pseudouridine (Ψ) which was first discovered in 1957, and it was thought to be a fifth core nucleotide (Davis & Allen, 1957). It was named "pseudouridine" when more characterizations showed that it is an isomer of the nucleoside uridine (Decatur & Fournier, 2002). Pseudouridine can appear in the

acceptor stem, D-stem, anticodon stem loop, and T-loop of the tRNA (Spenkuch et al., 2014; Rintala-Dempsey & Kothe, 2017; Motorin & Helm, 2019), see Figure 2A.

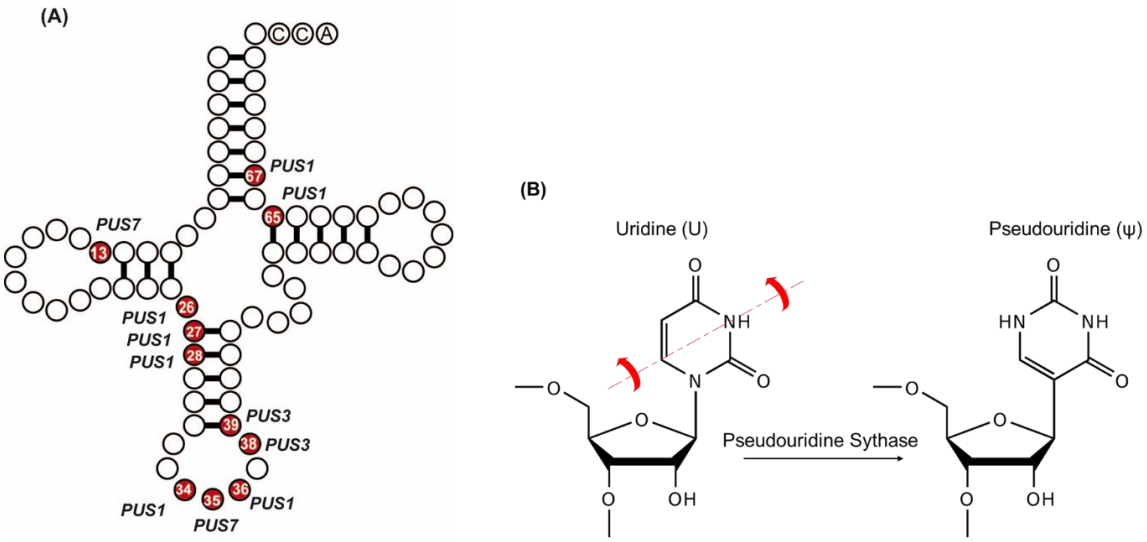

Figure 2. Positions of pseudouridylation in tRNA. (A) Pseudouridine synthase (PUS) dependent Ψ modification sites are present in different regions of the tRNA molecule (acceptor stem, D-stem, anticodon stem loop, and T-loop) (Wu et al., 2011; Lovejoy et al., 2014; Shaheen et al., 2016). (B) Chemical structure of uridine and pseudouridine. Ψ synthases break the N-glycosidic bond in uridine and catalyze the rotation of the uracil base and reattachment to the sugar via a C-glycosidic bond. The figure is modified from (Pseudouridylation.svg| Wikimedia Commons contributors).

In pseudouridine, uracil attaches by a carbon-carbon bond instead of the usual nitrogen-carbon glycosidic bond in its isomer uridine (Spenkuch et al., 2014) and Figure2B. Pseudouridine synthases catalyze the isomerization of uridine to pseudouridine (Figure 2B). In eukaryotes, whenever pseudouridine synthases (Pus1 through Pus9), are not a part of a complex and exist individually, they are characterized by different modification sites. When these enzymes are a part of the catalytic domain of RNA-guided-ribonucleoprotein (RNP) complexes, they are known as Cbf5 complex in yeast, and can be found in the nucleus, mitochondria, and in the cytoplasm. There is still not so much information available about these enzymes in humans. There is a high rate of homology between yeast and human PUSes, but pseudouridine synthases modify human tRNAs in a different way compared to the way they get modified in yeast (Rintala-Dempsey & Kothe, 2017). 1.4% of the total rRNA nucleotides in eukaryotes are pseudouridines (Ge & Yu, 2014), and the presence of Ψ is also reported in small nuclear RNAs (snRNA) (Reddy et al., 1972; Melaron et al., 1991; Patton,

1991). Pseudouridylation is more abundant in rRNA and tRNAs, and with the help of transcriptome-wide mapping methods, it is possible to recognize the presence of pseudouridine also in mRNA. The pseudouridine (Ψ) ratio to uridine (U) is about 0.2 to 0.6% in the mRNA which is much lower than in other RNAs (Carlile et al., 2014; Lovejoy et al., 2014; X. Li et al., 2015).

3.2.2. What does pseudouridylation do?

An improvement of the structure of the tRNA molecule is reported for most of the modifications as their specific role (Spenkuch et al., 2014), and because pseudouridine exists in different locations of the tRNA, it is assumed to play a role in maintaining the tRNA's structure. There are multiple Ψ synthases responsible for modifying specific positions (Figure 2), and defects of some of them are related to human diseases (Hawer et al., 2019; McKenney et al., 2017; Rintala-Dempsey & Kothe, 2017). The growth rate, amino acid levels, lipid content, and global transcription of amino acid biosynthesis genes can be influenced by a lack of specific pseudouridine synthases in yeast cells (Bozaquel-Morais et al., 2018; Chou et al., 2017; Lecointe et al., 1998; Mülleder et al., 2016). In yeast, pseudouridylation at positions 38 and 39 of the tRNA can lead to frameshifting by destabilizing the tRNA interaction in the E site of the ribosome (Bekaert & Rousset, 2005). On the other hand, the absence of pseudouridine modification in humans is related to intellectual disability (Shaheen et al., 2016). In human and yeast, snRNA Ψs can affect functional splicing (Dönmez et al., 2004; Wu et al., 2011; Karijolich et al., 2015), and in rRNA sequences, the distribution of pseudouridines and their location reveal an essential role of pseudouridylation in ribosome assembly with correct structure for a proper occurrence of the protein synthesis (King et al., 2003; X. hai Liang et al., 2007; Piekna-Przybylska et al., 2008; X. H. Liang et al., 2009). There is little known about pseudouridylation's role in mRNA. Based on in vitro studies, this modification seems to affect translation, and might enhance the mRNA translational capability (Karikó et al., 2008). Pseudouridylation may as well suppress the termination of translation by turning a nonsense codon like UGA to a sense codon like ΨGA which codes for phenylalanine and tyrosine (Karijolich & Yu, 2011; Parisien et al., 2012).

Pseudouridine modification is linked to human diseases, and it was first reported in a study in 1973 in urinary metabolites in cancer patients (Waalkes et al., 1973).

Pseudouridine can't get recycled, and is removed from the body by urine, like all other modified nucleosides. The amount of Ψ is related to RNA turnover and the glomerular filtration rate, which can be the reason for a higher level of Ψ in cancer patients, which suggests that the evaluation of pseudouridine level can be used as a tumor marker (Gehrke & Kuo, 1979; Seidel et al., 2006). Followed up by other researches in this area, increased levels of Ψ was observed in blood or the tissues of patients with different types of cancer, e.g., breast (Zheng et al., 2005), colorectal (Feng et al., 2005), esophageal (Masuda et al., 1993), gallbladder (Jiao et al., 2014), ovarian (J. Chen et al., 2012), prostate (Stockert et al., 2021) and small cell lung cancer (Tamura et al., 1987). In general, there are many methods for diagnosing the mentioned types of cancer, for example using mass spectrometry or ELISA, and each of them has its benefits and limits; and quantification of Ψ amount is one of the fastest and financially affordable methods available. Additive to cancer, some other diseases are as well reported as the result of disturbed pseudouridylation, like sideroblastic anemia (MLASA) (Bykhovskaya et al., 2004), growth delay, secondary microcephaly, and intellectual disability (Casas & Fischel-Ghodsian, 2004; Zeharia et al., 2005; Metodiev et al., 2014; M. Cao et al., 2016).

3.2.3. Elongator related tRNA modifications mediation

Eukaryotic elongator complex is built up of two copies of its six subunits, named Elp1 through Elp6, and was first identified in yeast, combined with RNA polymerase II, and its primary function is at the wobble uridine site of the tRNA and modifying uridines (Otero et al., 1999; Wittschieben et al., 1999; Esberg et al., 2006). Elongator is responsible for introducing 5-methoxycarbonylmethyl (mcm^5) and 5-carbamoylmethyl (ncm^5) groups to U_{34} at the wobble position of several eukaryotic tRNAs and stabilizing the interaction between the codon and anticodon (Huang et al., 2008; Johansson et al., 2008; Vendeix et al., 2012). Several phenotypes related to the role of elongator in cellular functions in yeast can get rescued by over-expression of $tRNA^{Gln}UUG$ and $tRNA^{Lys}UUU$, whose modifications are both elongator dependent (Esberg et al., 2006). Among the elongator's six subunits, Elp3 can be found in all three domains of life (Selvadurai et al., 2014). Elp3 is the elongator's catalytic subunit; however, the translation rate of proteins containing lots of codons decoded by elongator-dependent modified tRNAs will get influenced in case of loss of any of the six elongator subunits (Bauer et al., 2012; Huang et al., 2005). Some severe human diseases such as breast cancer,

intellectual disability, or amyotrophic lateral sclerosis are derived from deficiencies or mutations of elongator subunits (Najmabadi et al., 2011; Torres et al., 2014; Bento-Abreu et al., 2018; Hawer et al., 2019).

5 methoxycarbonylmethyl 2 thiouridine (mcm^5s^2U) can be found at the wobble position of $tRNA^{Gln}UUG$, $tRNA^{Lys}UUU$ and $tRNA^{Glu}UUC$. There are two different pathways responsible for mcm^5s^2U formation, the elongator introduces the mcm^5 and ncm^5 side chain at C5 of the U_{34}, and the Urm1 pathway, which is responsible for thiolation (sulfur transfer) at the C2 site of U_{34} (Huang et al., 2005, 2008; Johansson et al., 2008; Leidel et al., 2009). Some identified Urm1 pathway members, involved in tRNA thiolation, are Urm1, Uba4, Ncs2 and Ncs6 (Huang et al., 2008; Nakai et al., 2008; Leidel et al., 2009).

3.2.4. Negative interaction between tRNA modification genes

Different modifications can be present in different positions of the same tRNA and sometimes at the same nucleotide. The presence of more than one modification in a tRNA molecule opens up the possibility of interaction between the modifications. The inactivation of modifications involved in a functional cross-talk, influences in vivo phenotypes or synthetic effects, and an individual modification defect may show a more negligible effect compared to a combined modification defect. In several cases, severe phenotypes are specifically observed in the combined absence of two distinct tRNA modification genes, a condition referred to a negative genetic interaction (Esberg et al., 2006; Björk et al., 2007; C. Chen et al., 2009; Klassen et al., 2015; Nedialkova & Leidel, 2015).

It is known that in some cases specific tRNA species are functionally impaired in the combined absence of different modifications. Two good examples for this are $tRNA^{Gln}UUG$ and $tRNA^{Pro}UGG$, which cause temperature sensitive growth phenotypes in absence of mcm^5/ncm^5U_{34} and $\Psi_{38/39}$. A strong translational defect of the mRNA as well occurs when both mcm^5s^2U and $\Psi_{38/39}$ are absent. These two modifications can be found in the anticodon stem and loop of the tRNA and the negative genetic interaction of the respective genes is possibly due to the folding defects of the tRNA (Figure 3). It is also observed that lack of two specific modification genes, may increase the level of protein aggregation, which will be explained in the following chapters, and it might indicate the correlation of

previously described phenotypes with protein aggregation (Nedialkova & Leidel, 2015; Klassen et al., 2016).

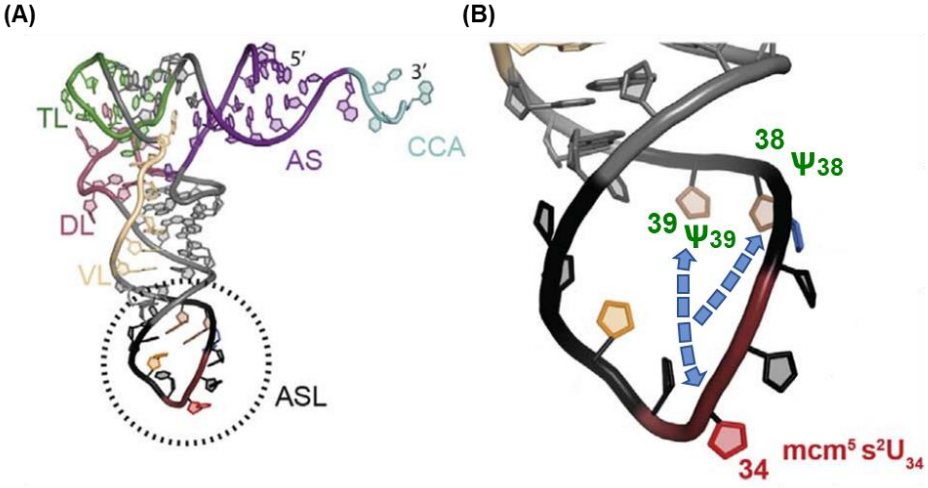

Figure 3. Negative interaction of tRNA modifications. (A) Tertiary structure of the tRNA molecule and its different regions. The Acceptor Stem (AS; violet), Thymidine Loop (TL; green), Variable Loop (VL; yellow), Anticodon Stem and Loop (ASL; black), and Dihydrouridine Loop (DL; pink). The cytosine-cytosine-adenine tail is as well visible (CCA; light blue). (B) The ASL site of the tRNA and the position of mcm^5s^2U34 and $\Psi_{38/39}$ modifications. The blue arrows represent the assumed functional collaboration between the shown modifications. The figure is modified from (Sokołowski et al., 2018).

3.3. Protein aggregation in yeast cells

The amino acids chains formed during the translation, need to fold appropriately to produce a functional protein. Protein aggregates appear when misfolding of proteins happens. As a result of protein misfolding, so-called “Protein conformational diseases” can appear (Valastyan & Lindquist, 2014; Tao & Conn, 2018). These include neurodegenerative disorders such as Parkinson’s disease, Alzheimer’s disease, Huntington’s disease, and Cystic fibrosis (Merlini et al., 2001). In yeast, the accumulation of protein aggregates in two types of inclusion bodies, known as JUxta Nuclear Quality Control Compartment (JUNQ) and Insoluble PrOtein Deposit (IPOD), have been reported (Spokoini et al., 2012; Kumar et al., 2016). There is a high concentration of proteasome in JUNQ, which provides the possibility of getting rid of soluble ubiquitinated aggregates; the non-ubiquitinated aggregates will get transferred to IPOD and removed from the cell by autophagy later on (Kaganovich et al., 2008), see Figure 4.

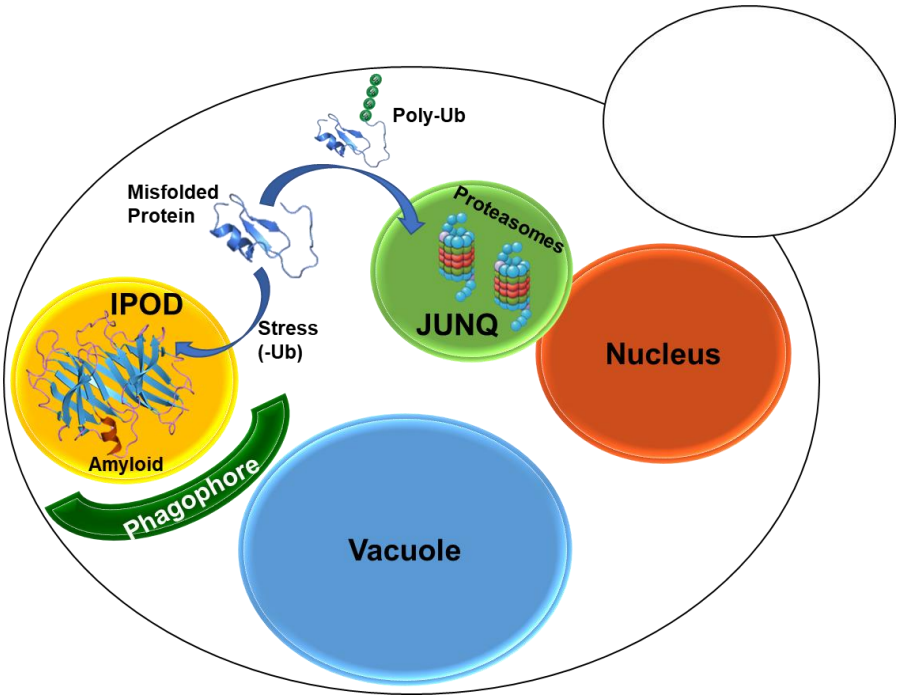

Figure 4. Accumulation of protein aggregates in JUNQ and IPOD. The misfolded proteins can enter into two types of inclusion bodies, the JUNQ and IPOD. The soluble ubiquitinated misfolded proteins will get accumulated in JUNQ, which has a high concentration of proteasomes. In contrast, other aggregated proteins which are not ubiquitinated and form amyloids will get transferred to IPOD, which will get removed from the cell by autophagy due to the presence of IPOD next to Phagophore assembly site (PAS). The figure is modified from (Rothe et al., 2018; Spokoini et al., 2012).

3.3.1. tRNA modification and protein aggregation

In yeast, several modification defects removing base modification from the anticodon loops of specific tRNAs were shown to result in an increased protein aggregate formation (Klassen et al., 2016; Schaffrath & Klassen, 2017; Pollo-Oliveira et al., 2020). There are two models suggested for protein aggregate formation in response to combined defects in tRNA modifications. In 2015, Nedialkova and Leidel showed that optimal codon translational rates require the presence of modified tRNAs. When the tRNA anticodon modification mcm^5s^2U is lacking, a codon-specific ribosome pausing during translation occurs because of the established decoding defects on CAA (Gln) and AAA (Lys) codons. Due to this slowdown, the nascent polypeptide emerging from the ribosome protein will have an increased tendency to misfold, resulting in protein aggregation (Nedialkova & Leidel, 2015). The second model suggests that the formation of protein aggregates might be related to increased mistranslation. The decoding inefficiency of U_{34}

hypomodified tRNA may result in other non-cognate tRNAs to have an increasing chance to erroneously decode CAA, AAA or other codons normally decoded by elongator dependent tRNAs. Due to this model, the resulting amino acid chain would contain an elevated number of wrong amino acids, interrupting protein folding and causing aggregate formation (Prokhorova et al., 2017).

There are studies about the influence of negative interaction between tRNA modifications on protein aggregation. It is shown that the absence of U_{34} modifications, mcm^5U and s^2U or the lack of ncm^5/mcm^5U and $\Psi_{38/39}$ in the same tRNA will enhance the formation of protein aggregation, and as observed in *ncs2 elp6*, *urm1 deg1* or *elp3 deg1* double mutants, the amount of accumulated aggregates in the absence of two tRNA modifications, is much more than the amount of formed aggregates in a single modification mutant (Nedialkova & Leidel, 2015; Klassen et al., 2016).

3.3.2. Rnq1 and protein aggregation

Two types of protein aggregates can be distinguished: ordered and disordered aggregates. Ordered protein aggregation can form amyloid fibrils which are β-sheet rich protein structures and are resistant to proteolytic degradation, whereas disordered aggregation produces amorphous protein aggregates (Uversky, 2003; Dobson, 2004; Chiti & Dobson, 2006). Ordered protein aggregation causes human diseases such as Creutzfeldt-Jakob disease (CJD). An example for ordered protein aggregates in yeast is [*PIN*$^+$], which is a prion form of the Rnq1 protein. (Sondheimer & Lindquist, 2000; Derkatch et al., 2004).

Prions are pathogenic misfolded proteins capable of triggering typical forms of the same protein and making them fold abnormally. In yeast, the non-mendelian trait [*PIN*$^+$] is needed for the de novo appearance of [*PSI*$^+$] as well as [*URE3*] (Derkatch et al., 1998; Wickner et al., 2000). *SUP35* and *URE2* are two genes respectively responsible for the induction of de novo appearance of [*PSI*$^+$] and [*URE3*], and the overexpression of prion domains of the Sup35 and Ure2 proteins was shown to be sufficient for prion appearance, which was how this domain was identified. Furthermore, Rnq1 as a potential yeast prion protein was identified due to the similarity with Sup35 and Ure2 prion domains and its high Q and N content (Masison & Wickner, 1995; Derkatch et al., 1996; Mick F. Tuite, 2000).

There are no phenotypic changes observed as the result of inactivation of Rnq1, and to show that this protein is capable of prion formation, overproduction of its prion domain was used. Rnq1 shows different behaviors in different yeast backgrounds (Hines et al., 2011); it can be soluble in one yeast strain while aggregated in another. The aggregated form of Rnq1 is transferred by cytoplasmic mixing, and this transmission needs to happen in the presence of Hsp104 chaperone, which is responsible for refolding and remodeling the previously aggregated and denatured proteins (Parsell et al., 1994; Sondheimer & Lindquist, 2000). The [*PIN*$^{+}$] prion can be cured by a low concentration of the protein denaturant guanidine hydrochloride (GdnHCl) or by deletion of the Hsp104 chaperone (Sondheimer & Lindquist, 2000).

It is shown that the lack of one and specially two tRNA modifications in the yeast cells leads to a clear downregulation of Rnq1 protein levels in the cells. The severe downregulation of this protein in double mutants is related to a significant translational inefficiency, and the translational defect induced by these strains is related to the decreased function of tRNA^{Gln}UUG which causes a wrong decoding of CAA codons (Klassen et al., 2016; Schaffrath & Klassen, 2017).

3.4. Aging in *Saccharomyces cerevisiae*

Understanding aging is important for treating or preventing age-related diseases like different types of cancer or heart disease. Aging is defined as a time-dependent decline in physiological function influenced by genetic or external factors, and it leads to increased mortality rates and decreased reproductive rates (Goldsmith, 2015).

In yeast, aging is studied using two different approaches; replicative and chronological assays. Replicative life span (RLS) is measured by counting the number of daughter cells produced by a single mother cell until the mother cell is no longer capable of division; the chronological aging is based on measuring the time in which cells stay viable in the stationary phase of growth (Barton, 1950; Fabrizio & Longo, 2003) and Figure 5.

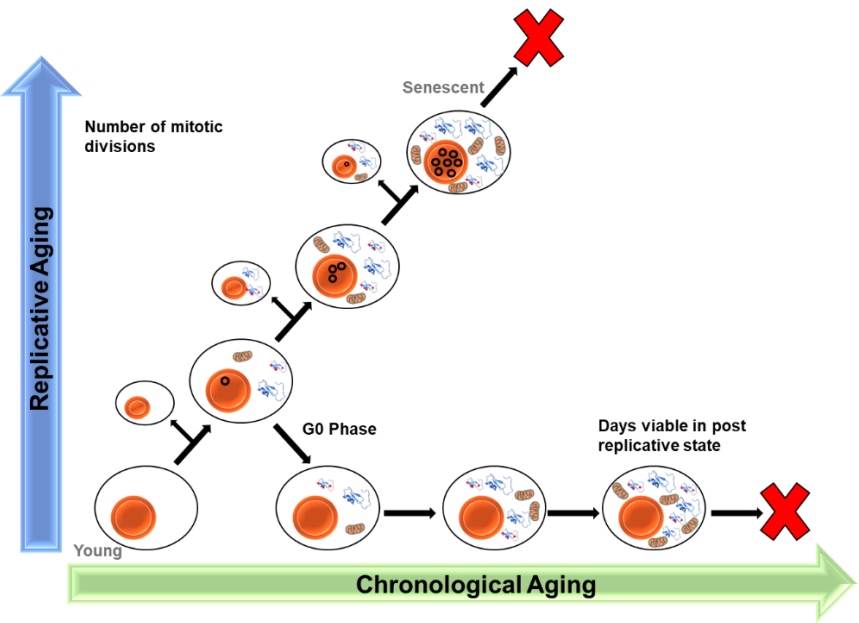

Figure 5. Replicative and chronological aging in *Saccharomyces cerevisiae.* Replicative aging can be measured by the times a mother cell divides and produces daughter cells until it becomes senescent. Chronological aging is measured by the time cells can stay viable in the stationary phase. The figure is modified from (Oliveira et al., 2017).

3.4.1. Dietary restriction as an established aging factor in yeast

Environmental nutrients play a significant role in yeast's life span. Different studies show that either reduction in amino acid levels or glucose concentration in the growth medium will increase both replicative and chronological life span (CLS) (Reverter-Branchat et al., 2004; Powers et al., 2006). This nutrient-induced life span extension in yeast involves changes in the activity of the target of rapamycin (TOR) kinase, extensively introduced in the next chapter, and the Ras-cAMP-PKA pathway which controls cell functions in response to the changes of the nutrition level in the environment (Smith et al., 2008; Kaeberlein et al., 2013).

In dietary restriction (DR) conditions, several cell processes are regulated by nutrient signaling pathways like AMP-activated protein kinase (AMPK). In mammals, the general role of AMPK is to coordinate growth and metabolism. Analysis of AMPK orthologues in other eukaryotes shows that this coordination is usually the result of low energy conditions (Thelander et al., 2004; Baena-González et al., 2007; Bokko et al., 2007; Hedbacker & Carlson, 2008; Narbonne & Roy, 2009; Johnson et al., 2010). Snf1 (sucrose non-fermenting 1) is the analogous AMPK complex in the yeast *Saccharomyces cerevisiae*. It is shown that Snf1 activity is

necessary to optimize the CLS in dietary restriction conditions (Woods et al., 1996; Wierman et al., 2017). Dietary restriction can enhance many other processes similar to Snf1 signaling during diauxic shift (transition of the log phase to stationary phase), like mitochondrial respiration and improvement of the arrest in the G1 phase in the cell cycle, which can help the cell to undergo repair mechanisms or go to the apoptotic pathway in the stationary phase (D. L. Smith et al., 2007; Weinberger et al., 2007; Choi & Lee, 2013; Tahara et al., 2013; Murad et al., 2016).

3.4.2. Target of rapamycin (TOR) pathway and aging

The TOR kinase is responsible for regulating growth, cell division, and metabolism in eukaryotes in response to nutrients and hormonal cues. The mechanistic target of rapamycin (mTOR) was first identified in studies about rapamycin as an antifungal drug and its growth inhibitory features (Heitman et al., 1991). Investigations in the budding yeast *Saccharomyces cerevisiae* show that yeast has two paralogs of TOR, *TOR1* and *TOR2* (Martin & Hall, 2005; De Virgilio & Loewith, 2006).

TOR kinase can be found in the form of two different complexes; TORC1 and TORC2. TOR2 can specifically be a part of the TORC2 complex, responsible for actin organization. TORC1 complex in response to nutrients or stress or presence of rapamycin can control different functions of cells like autophagy, translation stress-responsive transcription, nutrient import, and ribosome biogenesis (Wullschleger et al., 2006) and Figure 6.

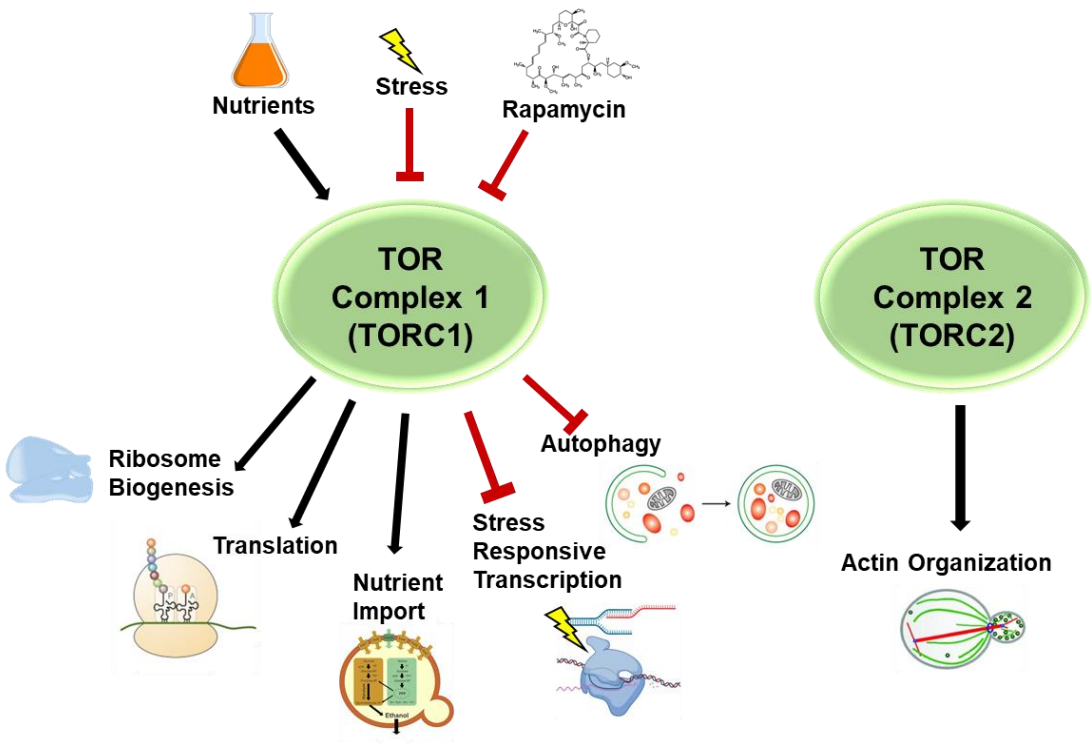

Figure 6. TORC1 and TORC2 in the yeast *Saccharomyces cerevisiae*. TOR complex 2 is responsible for actin organization, and TOR complex 1 controls ribosome biogenesis, translation, nutrient import, stress-responsive transcription, and autophagy under the influence of presence or absence of nutrients, stress factors, or rapamycin. The image is modified from (Biomol GmbH | Rapamycin | CAS 53123-88-9 | AdipoGen Life Sciences; Research – Institute of Biochemistry | ETH Zurich; Microbe Notes | Transcription vs. Translation; Microbe Notes | Protein Synthesis in Eukaryotes;(Wullschleger et al., 2006; D. Li et al., 2013; Mishra et al., 2014; Nijland et al., 2014).

It is established that TOR signaling in yeast influences both replicative and chronological life span, and deletion of *TOR1* or inhibition of TORC1 can increase both life spans. The replicative life span can be as well increased as a result of mutations in downstream targets of TOR, which are involved in the synthesis of proteins (Fabrizio et al., 2001; Kaeberlein et al., 2005; R. W. Powers et al., 2006; Medvedik et al., 2007; Steffen et al., 2008). Other eukaryotes have only one TOR (mTOR in mammals) instead of both *TOR1* and *TOR2* in yeast, and it is shown that TOR activity also influences the aging in mammals (Hasty, 2010). TOR has different functions in different tissues of mammals, and its inhibition can be beneficial or harmful for the organism. Thus, studying TOR's inhibition for aging-related diseases like cancer or Huntington's disease might ease the understanding of the TOR signaling pathway's influence on aging in mammals (Wullschleger et al., 2006).

3.4.3. The role of tRNA modification in aging

tRNAs play an important role in aging and can influence the life span while undergoing modifications (Zhou et al., 2021). As explained previously, tRNA modifications can affect the stability of tRNA, codon recognition, or/and aminoacylation. Regarding the "Protein aggregation in yeast cells" and "tRNA modification and protein aggregation" chapters, the relation between the lack of tRNA modification and protein aggregation seems to cause the age-related diseases, which might lead us to this general hypothesis that these modifications have a negative impact on aging; however, different studies in yeast replicative and chronological life span shows that the absence of some of these modifications can cause accelerated aging; there are as well cases in which the lack of specific modifications is followed up by the extension of life span (Table 1; based on *Saccharomyces* Genome Database (SGD)), this gives a particular value to further studying the effect of tRNA modification on aging.

Table 1. Replicative and chronological lifespan, under the control of responsible genes for tRNA modification in yeast background S288C

Modification	Gene Name	RLS	CLS	References
$mcm^5\underline{s}^2U$	*URM1*	No Data	Decreased	(Marek & Korona, 2013)
$mcm^5\underline{s}^2U$	*NCS2*	Increased	Decreased	(McCormick et al., 2015), (Campos et al., 2018)
$mcm^5\underline{s}^2U$	*NCS6*	Decreased	Increased	(Yu et al., 2021), (Garay et al., 2014)
$\underline{mcm}^5s^2U$, $\underline{ncm}^5s^2U$	*ELP3*	Decreased	Decreased/ Increased	(Yu et al., 2021), (Campos et al., 2018; Eisenberg et al., 2009)
$\underline{mcm}^5s^2U$, $\underline{ncm}^5s^2U$	*ELP4*	Increased/ Decreased	Decreased	(McCormick et al., 2015; Yu et al., 2021), (Marek & Korona, 2013)

3.4.4. Protein aggregation and aging

During replicative aging in *Saccharomyces cerevisiae*, the level of oxidatively damaged and carbonylated proteins increase. These changes will lead to modification of proteins and can cause protein aggregates formation. The carbonylated proteins and the oxidized proteins can maintain in the parental cell during the cell division (Stadtman, 1992; Bota & Davies, 2002).

The cell division in yeast cells is asymmetrical, and it is related to the individual aging of the mother cell (Kennedy et al., 1994). The mother cell undergoes age-related changes like increasing generation time, reducing mating ability, and increasing cell size (Sinclair & Guarente, 1997). The mother cell will die after 20-30 times division (Mortimer & Johnston, 1959; Kaeberlein et al., 1999, 2001), which could be due to the accumulation of oxidized or carbonylated proteins. The influence of protein aggregation on chronological aging is not yet well studied. However, the influence of trehalose on protein hemostasis was studied, and it was shown that in the early stages of chronological aging, trehalose plays an essential role in cell's life span by binding to the newly synthesized proteins and helping them fold properly and preventing the formation of aggregates. Trehalose also protects the proteins from oxidative carbonylation and protects the cell against oxidatively damaged proteins and aggregates formation in chronologically young cells. However, in the stationary phase of growth, trehalose plays a role in shortening the life span by inhibiting the chaperone from refolding the misfolded proteins (Arlia-Ciommo et al., 2014). In general, it seems that protein aggregation plays a vital role in cells' aging, and an acceleration of aging will follow the formation of more protein aggregates. However, this effect has not yet been proven.

3.5. Autophagy in yeast

Autophagy is the degradative pathway in eukaryotic cells, responsible for delivering cytoplasmic proteins and sometimes organelles into the lysosomes in mammals and vacuoles in yeasts. The vacuole has different roles in yeast, among others, it is responsible for the regulation of intracellular pH and osmosis, degradation of proteins, storage of amino acids and small ions, and polyphosphates (Li & Kane, 2009; Wiederhold et al., 2009). However, an essential function of autophagy is cargo sequestration by autophagosomes. Autophagosomes are double membrane-

bound structures resulting from phagophore formation from pre-autophagosomal structure (PAS) (Hollenstein & Kraft, 2020). Different conditions can induce autophagy in eukaryotes such as pathogen infection, hormones, growth factors, or an insufficient amount of nutrients which is the leading cause of autophagy activation in the yeast (Abeliovich & Klionsky, 2001; Quan & Lee, 2013; Kim et al., 2019).

Autophagy can be selective or non-selective (bulk). Non-selective autophagy can result from starvation, whereas selective autophagy can occur in the presence of specific substrates in cells that are no longer needed (Baba et al., 1997; Dunn et al., 2005; Kanki & Klionsky, 2008).

Studies in yeast revealed that autophagy induction is mainly a result of nutrient starvation, and there is a group of proteins responsible for autophagy in yeast, and they are called autophagy related (ATG) proteins (Suzuki et al., 2013). As explained before, the TOR pathway responds to environmental nutrients and can induce autophagy, under starvation conditions; the TOR pathway as well induces autophagy in the presence of rapamycin due to its starvation mimicking effect. The inactivation of TORC1 leads to the formation of autophagosomes with the help of Atg1 kinase and some of its regulatory ATG proteins (Figure 7). Other ATG members take part in lipid kinase complex formation, or as a component of two ubiquitin-like systems. Atg8 and Atg12 are the two ubiquitin-like proteins involved in conjugation reactions, and due to their presence in autophagy-related membranes, it is suggested that they are taking part in building these membranes. Additive to Atg8's participation role in the formation of autophagosomal membranes, this protein is also responsible for the proper adhesion of cargoes into autophagosomes during selective autophagy (Nakatogawa et al., 2009). In general, autophagy has many roles in physiological as well as pathological conditions, and it has diverse impacts on the cells.

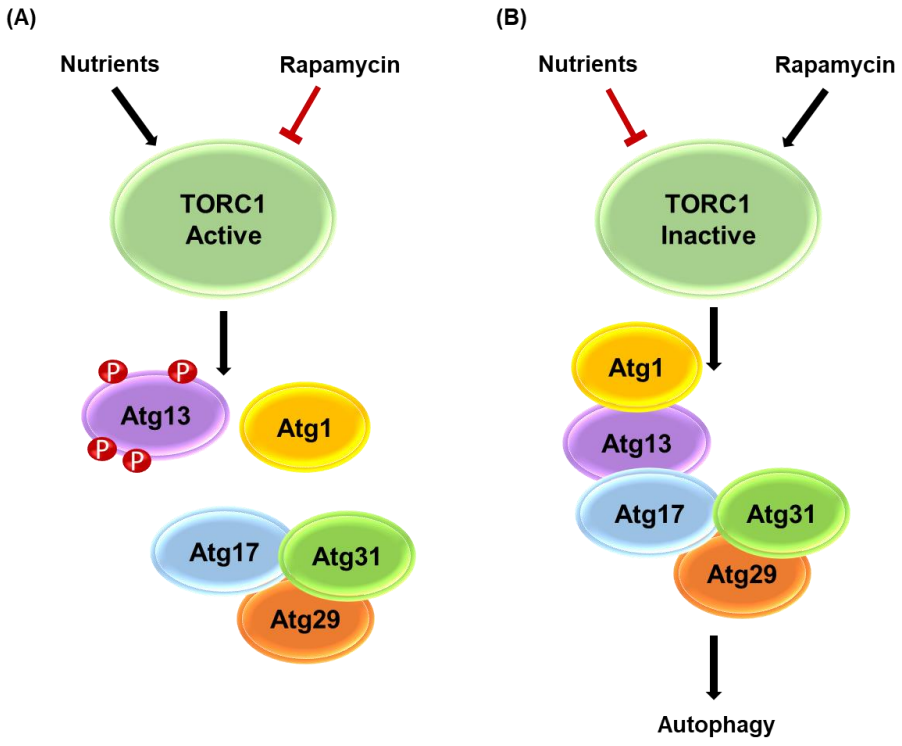

Figure 7. Activation and inactivation of TORC1. (A) In the presence of nutrients and/ or absence of rapamycin, the TOR complex 1 is activated, and the Atg13 stays phosphorylated, and there is no autophagy as a result. (B) In the starvation situation and/ or presence of rapamycin, Atg13 is dephosphorylated and Atg1 and some of its regulatory ATG proteins (Atg17, Atg29 and Atg31) will bind together and the autophagy gets activated. The figure is modified from (Schalk, 2011).

3.5.1. Autophagy and tRNA modification

In *Saccharomyces cerevisiae*, in the middle of the exponential phase of growth, the intake of nutrient can switch from fermentation to cellular respiration, and it's done by diauxic shift. The diauxic shift helps cells switch the substrate for growth from glucose to ethanol and use both resources to reach the stationary phase (Galdieri et al., 2010). In the stationary phase, due to the low amounts of nutrient, cells activate the starvation transcriptional programs and undergo autophagy (Thevelein et al., 2000; Smets et al., 2010). This change is needed for cells to deal with the starvation situation by reducing translation and expressing essential proteins for using alternative nutrient sources (Cardenas et al., 1999; Rohde et al., 2008; Smets et al., 2010).

In a recent study (Bruch et al., 2020), the quantification of mRNA levels of genes involved in stationary phase responses, nitrogen catabolic repression (NCR), and glucose starvation was done in both exponential and stationary phases. This

experiment was performed for a wild type (WT), and a mutant strain with mcm^5/ncm^5U_{34} and $\Psi_{38/39}$ modifications defect in its tRNA. The moderate difference between mRNA levels in the double mutant's stages of growth shows the importance of tRNA modification to prevent cells from starving and undergoing autophagy in the early stages of their growth phase. It was shown that in tRNA modification mutants the autophagy was activated during the exponential phase of growth which is normally getting activated in the stationary phase. This was shown to be due to the expression of starvation marker genes during the exponential phase in tRNA modification mutants that are usually expressed only in the stationary phase of growth (Bruch et al., 2020). However, the relation between autophagy and tRNA modification is still not fully understood.

3.5.2. Role of autophagy in yeast aging

There are different ways that yeast cells can be influenced by autophagy. Independent studies show that the inactivation of TOR can extend both RLS and CLS, but the mechanism of this phenomenon is not yet known (Kaeberlein et al., 2005; Wei et al., 2009). However, there are some pieces of evidence on the involvement of autophagy induction, which mainly shows that autophagy is beneficial for the cell and will help the organism to live longer (Paglin et al., 2001; Rich et al., 2003; Papandreou et al., 2008; Eisenberg et al., 2009; Ruckenstuhl et al., 2014). The autophagy induction has a tight bond to TOR signaling reduction (Noda & Ohsumi, 1998). The Sch9 kinase is needed for TORC1 mediated regulation in the biogenesis of the ribosome and initiation of translation and can function dependently or independently of TOR. A deletion of *SCH9* was found to extend both RLS and CLS (Fabrizio et al., 2001; Kaeberlein et al., 2005; Yorimitsu et al., 2007; Smets et al., 2008; Wei et al., 2009); on the other hand, this deletion has a slight impact on autophagic starvation induction. It is shown that caloric restriction regulates the vacuolar acidity by nutrient-sensing pathways (Hughes & Gottschling, 2012), and Sch9 is one of the nutrient-sensing kinases suggested for the cell´s function under caloric restriction (Kaeberlein et al., 2005). One possible reason for the induction of autophagic starvation is the Sch9 role in maintaining the vacuolar acidification, which is reduced during aging and decreases the capability of vacuoles in neutral amino acids storage (Ruckenstuhl et al., 2014; Wilms et al., 2017).

In a study on Parkinson's disease, α-synuclein, which is an essential component of protein aggregates, known as Lewy bodies, was ectopically expressed in yeast (Spillantini et al., 1998). It was observed that limiting nutrients or inhibition of TOR both can reduce the toxicity of α-synuclein and can rescue the accelerated chronological aging caused by the expression of α-synuclein; this expression leads to an increased rate of autophagy which was suppressible by TOR inhibition and/or dietary restriction. However, the limitation of CLS reduction and normal aging after lowering the rate of autophagy revealed that too much autophagy activity is as well not beneficial for aging, and autophagy of different ranges can influence cells differently (Stefanis et al., 2001; Xilouri et al., 2009; Choubey et al., 2011; Sampaio-Marques et al., 2012). Additive to TOR/Sch9, there are some other ways in which autophagy can influence aging, and they are mainly through nutrient-sensing pathways like the Ras-cAMP-PKA pathway or the AMPK/Snf1 pathway (Broach, 2012); however, it is still unknown if autophagy is sufficient for extending the lifespan.

3.6. Ssd1 in *Saccharomyces cerevisiae*

Ssd1 (Suppressor of Sit4 Deletion) is a translational repressor and RNA binding protein which can promote cell wall integrity. It was shown to travel between nucleus and cytoplasm of the cell regularly and binds to cell wall encoding mRNAs which aids in transferring them to polarized cell growth sites in the cell (Kurischko & Broach, 2017). Ssd1 usually interacts with the mRNA by its N terminal prion-like domain (PLD), and in stress situations and/or during the stationary phase, Ssd1 transfers the mRNAs to processing bodies (P- bodies)/(PBs) where mRNAs are either stored, repressed, or degraded. Cbk1 (Cell wall Biosynthesis Kinase 1) is a serine/threonine protein kinase that plays a role in polarized growth, and it's known that it is a highly conserved tumor suppressor. Cbk1 is responsible for phosphorylation of Ssd1 in the cytoplasm. In the phosphorylated state, Ssd1 plays a role in localized translation of the cell wall protein encoding mRNAs and regions of polarized growth. In the non-phosphorylated state, it rather associates with P-bodies and thus represses the expression of its bound mRNAs (Kurischko & Broach, 2017) and Figure 8.

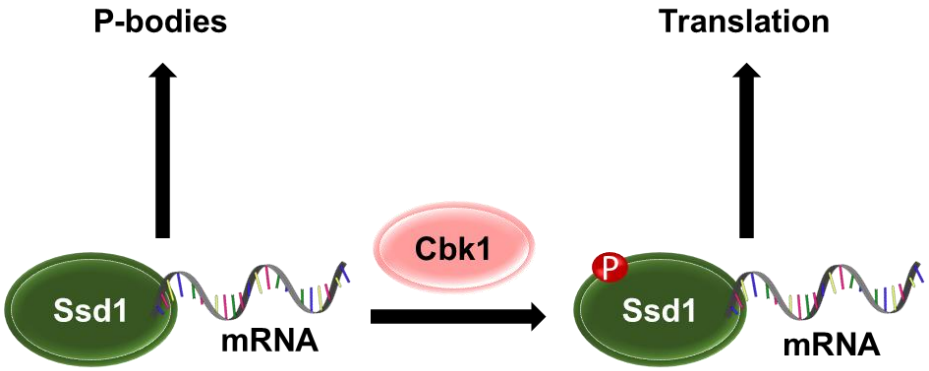

Figure 8. Phosphorylation of Ssd1 by Cbk1 kinase. In the cytoplasm, when Ssd1 binds to mRNA and is not phosphorylated due to stress conditions, the Ssd1, mRNA complex will be transferred to P-bodies. In the presence of Cbk1, the Ssd1 gets phosphorylated and translation happens.

3.6.1. Known and unknowns about Ssd1 functions

The important function of Ssd1 in yeast is explained in the previous chapter; however, more functions are described. During early studies on Ssd1, as it's involved in its name, scientists found the connection between Ssd1 and Sit4 (Suppressor of Initiation of Transcription). The Sit4 Pyrophosphatase (PPase) is needed in the late G1 phase for progression into the S phase of the cell cycle. In the mentioned research, three different models are suggested for the relation between Ssd1 and Sit4. In one model, Ssd1 is considered as a Sit4 substrate that can function without its phosphorylation regulation state with Sit4's help. In the other model, Ssd1 can partially dephosphorylate the Sit4 substrate even though it has nothing in common with other protein phosphatases. In the final model, Ssd1 can provide some functions in a parallel pathway to the Sit4 pathway. All of these models were suggested for the *SSD1-v* morphotype of *SSD1*. Based on this study, *SSD1* has two morphotypes, *SSD1-v* (v for deletion for *SIT4* viable) and a truncated version of it called *ssd1-d* (d for deletion for *SIT4* dead) (Sutton et al., 1991), see Figure 9.

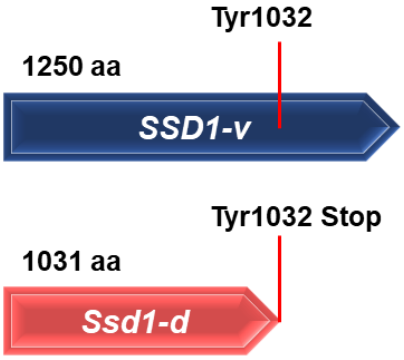

Figure 9. *SSD1*'s different morphotypes. There are two different allelomorphs for the *SSD1* gene, *SSD1-v*, which can be found in the BY4741 strain in this study which consists of 1250 amino acids, and *ssd1-d*, with 1031 amino acids, which is the truncated form of *SSD1-v* due to its premature stop codon at Tyr1032 position.

SSD1-v can act as a mutation suppressor for genes involved in cAMP responses (Sutton et al., 1991; Yukifumi et al., 1994), or the genes coding for RNA polymerase III or splicing factors (Stettler et al., 1993; Luukkonen & Séraphin, 1999). It is also shown that the Ssd1 function is vital for the cells to enter into quiescence in case of limitation of nutrition and growth factors, and it seems that its function is much more crucial in diploid than haploid cells. In a common laboratory strain, W303-1B, which contains the *ssd1-d* allele of *SSD1*, the quiescence pathway is very much restricted to the haploids (Miles et al., 2019). In a recent study, the replacement of *ssd1-d* with *SSD1-v* allele doubled the formation of quiescent (Q cells) in haploids, and the cells were able to live longer in a stationary phase (Li et al., 2009). In the same strain as a diploid, the identical replacement increased the formation of Q cells as well as decreased sporulation by ten folds, and this means that *SSD1-v* introduction to this strain shifts the sporulation to cellular quiescence, and the mechanism behind it is yet unknown (Miles et al., 2019).

A relation between Ssd1 with TORC1 and TORC2 is as well reported (Reinke et al., 2004; Cardon et al., 2012), and recently due to the rescue of TOR complex mutants´ rapamycin sensitivity, by Ssd1 overexpression in the mentioned mutant strains, it is suggested that Ssd1 has a functional role in the TORC1 pathway; however, its mechanism is not yet fully understood (Chang et al., 2021).

3.6.2. tRNA modification and Ssd1

As explained previously, *SSD1* has two different morphotypes; *SSD1-v* and *ssd1-d*. *ssd1-d* is the truncated form of *SSD1-v*, and it is also the nonsense allele compared to *SSD1-v*, which encodes the full-length functional protein in the cell (Sutton et al., 1991; Jorgensen et al., 2002).

In a recent study about *SSD1*, the influence of this gene on the elongator-dependent tRNA modifications in yeast is shown. It was observed that the lack of mcm^5/ncm^5 modification at U_{34} in different *SSD1* backgrounds causes an enhanced temperature sensitivity in a *ssd1-d* background strain; in the same strain, a reduced growth phenotype was also observed in the presence of other stress factors, all of which were suppressible by *SSD1-v* expression in the *ssd1-d* background (Xu et al., 2019, 2020). It was demonstrated that the *ssd1-d* allele does neither affect the formation of mcm^5/ncm^5 nor tRNA levels (Xu et al., 2019). The allelic variation of *SSD1* also affects the negative genetic interaction between mcm^5/ncm^5 and s^2 modification genes *elp3* and *ncs2*. An *elp3 ncs2* double mutant displays a synthetic sick and slow-growth phenotype in an *SSD1-v* background. In *ssd1-d* in contrast, the negative genetic interaction of *elp3* and *ncs2* results in synthetic lethality. It was speculated that this enhanced phenotype of *elp3* and *ncs2* could be caused by elevated protein aggregation (Xu et al., 2019) (see below).

3.6.3. Role of Ssd1 in protein aggregation

Protein aggregation can result from excessive heat, oxidative stress, aging, mutation, and a low availability of chaperones. In yeast cells, normal tolerance against higher temperatures requires a functional heat shock protein Hsp104. This protein acts as a protein disaggregase and also plays a role in prion maintenance, as explained in the “RNQ1 and tRNA modification mutants” chapter, and it also impacts yeast cells’ longevity. Hsp104 disaggregates large protein aggregates into smaller pieces and thereby fulfills an important and essential role in the inheritance of yeast prions (Shorter & Lindquist, 2005). It was shown that Ssd1 is needed for protein disaggregation by Hsp104, and it influences the ability of Hsp104 to interact with the Sti1 (stress Inducible 1) cochaperone to bind protein aggregates. This functional relation between Ssd1 and the Hsp104 chaperone lowers the amount of protein aggregates in the cell and improves survival under

stress conditions (Mir et al., 2009). It remains unknown, however, whether the phenotypic variation of U_{34} tRNA modification mutants by the *SSD1* status indeed occurs because of changes in protein aggregates levels.

3.6.4. Influence of Ssd1 on autophagy and aging

Autophagy is a recycling and degradative pathway in the cell, and as mentioned before, it is mainly induced due to starvation. However, in a recent study, it is suggested that both enhanced mRNA transport to p-bodies via Ssd1 and induction of autophagy will cause a longer lifespan in yeast strains (Hu et al., 2018).

It has been shown that six autophagy related genes are up-regulated in *SSD1-v* background, and among them are *ATG1*, *ATG13* and *ATG17*, which are parts of a kinase complex that is responsible for autophagy activation in starvation conditions (Díaz-Troya et al., 2008). Based on this result and what is explained in the "Autophagy in yeast" chapter, it can be assumed that the *SSD1-v* strain has a higher capacity to activate the autophagy under stress conditions, and the presence of this allele can thus be beneficial for cells (Li et al., 2009).

SSD1-v is not only required for high levels of stress resistance, normal autophagy and prevention of protein aggregation but also positively affects replicative and chronological lifespan in yeast (Kaeberlein & Guarente, 2002; Fabrizio & Longo, 2003; Kaeberlein et al., 2004). A reduced chronological life span of a *ssd1-d* strain was observed compared to a *SSD1-v* wild type strain, but no difference was observed between the CLS of *ssd1* mutant strain and *ssd1-d* WT. This indicates a complete loss of function of truncated Ssd1 protein expressed from the *ssd1-d* allele (Li et al., 2009). Furthermore, in a study on elongator mutants in both background strains, it has been shown that the *ssd1-d* allele is required for histone acetylation and telomeric gene silencing defects in the mentioned mutants. Thus, the *ssd1-d* allele increases the sensitivity of yeast cells lacking mcm^5/ncm^5 modifications in U_{34} position of their tRNA molecules. It is also observed that the cells lacking the interaction between mcm^5/ncm^5 and s^2 modifications can be viable, although slow-growing in *SSD1-v*, but lethal in *ssd1-d* background strain (Xu et al., 2019).

4. Aim of the Study

This thesis analyzes the role of the RNA binding protein Ssd1 in the phenotypic variation of a mutation in the pseudouridine synthase gene *DEG1* in baker's yeast. Previous work established a role of Ssd1 in the phenotypes of yeast mutants lacking a functional Elongator complex involved in wobble uridine modification (Xu et al., 2019). Since it remained unknown whether this Ssd1 effect extends to other modification genes, *DEG1* was selected for further analysis, which shares phenotypes with Elongator mutants. Elongator genes also display negative genetic interaction with *DEG1*, and in this work, it is determined whether the synthetic phenotypes of combined mutants are affected by the loss of Ssd1 function. These approaches rely on using two commonly used yeast strain backgrounds that are known to differ in their allelic variant of *SSD1*, of which a truncated non-functional version is present in one strain. To check whether the observed differences in phenotypes occurs because of the mentioned allelic variation, plasmid-based complementation with a full-length *SSD1* gene is performed. Specific tRNA modification mutants cause protein aggregation, possibly because of ribosomal slow down at codons requiring modified tRNA for efficient decoding. This thesis aims to determine to which extent such effect on protein homeostasis is involved in the pleiotropic phenotypes of the *deg1* mutant, and whether this is modulated by Ssd1. Since protein homeostasis declines during cellular aging and is thought to represent an important aging factor, the effect of *deg1* mutation alone and in conjunction with *ssd1* mutation or lack of other tRNA modification genes on yeast’s life span is determined. Recent studies indicate a role of certain tRNA modifications in the signaling of nutrient availability, possibly via effects on the target of rapamycin (TOR) kinase complex. To test whether *DEG1* and *SSD1* modulate this effect, the effect of the mutation in either gene on autophagy, a process directly controlled by nutrient availability and communicated via TOR signaling, is analyzed.

5. Results

5.1. Influence of yeast strain backgrounds on *deg1* mutant phenotypes

The in vivo consequences of Deg1 dependent Ψ modification at positions 38 and 39 of the tRNA molecule are studied in this work, in two different common laboratory yeast strain backgrounds. BY4741 and W303-1B. BY4741 is directly derived from the first completely sequenced yeast strain, S288C, from which it differs only in terms of auxotrophic markers, while W303-1B was created by Rodney Rothstein and has a complex genealogy involving S288C (Thomas & Rothstein, 1989) but also various other laboratory strains (termed D311-3A, D190-9C, Σ1278B and SK1) (Ralser et al., 2012). The assessment of the phenotypic variation of a *DEG1* deletion by yeast strain backgrounds was motivated by the recent demonstration of such variation for mutants lacking the tRNA modification gene *ELP3* (Xu et al., 2019, 2020). There are some fundamental differences between these two strains, like the red colony color for W303-1B due to a mutation in the adenine biosynthetic pathway (Dorfman, 1969) or the higher thermotolerance in the BY4741 strain. In addition, these strains have a polymorphic *SSD1* locus. The BY4741 strain contains the *SSD1-v* allele while, W303-1B contains the *ssd1-d* allele with a premature termination codon (Sutton et al., 1991; Xu et al., 2019). It was shown that the phenotypic variation of *elp3* mutants in the two strain backgrounds is due to the two different *SSD1* alleles present in the strains (Xu et al., 2020). To determine whether the *SSD1* allelic variant also influences other tRNA modification mutant phenotypes, the focus is on *deg1* and determined temperature and drug sensitivities, genetic interaction with other tRNA modification genes, and other cellular processes directly linked to tRNA modification loss in both strain backgrounds. The results are partially published (Khonsari et al., 2021).

5.1.1. Comparison of *elp3* and *deg1* mutant phenotypes in *ssd1-d* and *SSD1-v* strains

To analyze whether the variable alleles of *SSD1* influence the induced yeast phenotypes in the absence of Deg1 pseudouridine synthase, *deg1* mutants were generated in both *SSD1-v* (BY4741) and *ssd1-d* (W303-1B) strain backgrounds. As a comparison, *ELP3* was also deleted in both backgrounds. The first

phenotype to be analyzed was the growth capability at different temperatures. In both strain backgrounds, the modification defects caused a growth defect at elevated temperatures (Figure 10A) (Khonsari et al., 2021).

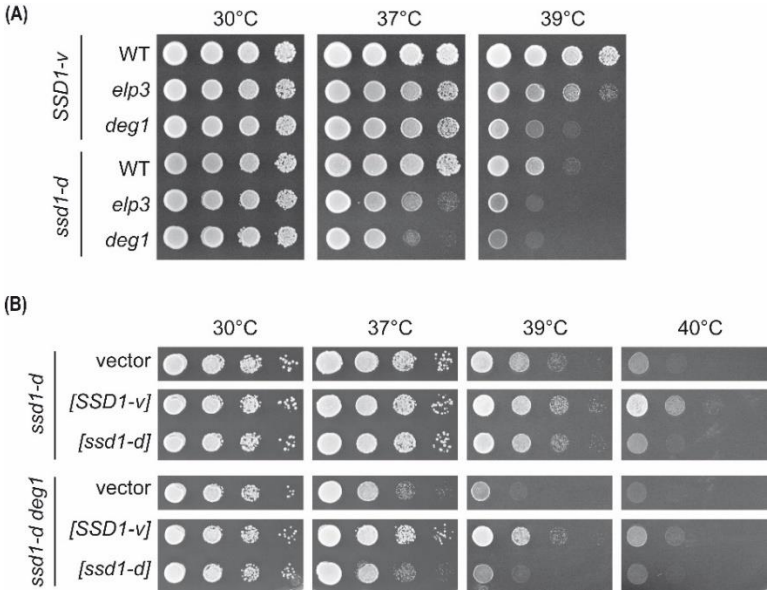

Figure 10. *elp3* and *deg1* mutants´ temperature sensitivity in the *SSD1-v* and *ssd1-d* strain backgrounds. *SSD1-v* refers to BY4741, while *ssd1-d* refers to W303-1B. (A) Wild type (WT) and mutant strains were serially diluted and spotted on YPD plates. All plates were incubated at the indicated temperature for 48 h. (B) Both WT and *deg1* mutant in the *ssd1-d* background (W303-1B) with the empty vector, *SSD1-v* or *ssd1-d* containing plasmids were serially diluted and spotted on YPD plates. All plates were incubated at the specified temperature for 48 h.

The *deg1* mutant is more temperature sensitive in comparison to *elp3*, and the difference is observable in both *ssd1-d* and *SSD1-v* strains (Figure 10A). However, the observed growth defect at 37°C for both tRNA modification mutants is more severe in the *ssd1-d* background as compared to the *SSD1-v* strain. At 37°C, only the *ssd1-d elp3* and *deg1* mutants, but not the similar mutants in *SSD1-v*, display a significant growth defect. However, at 39°C, both, *ssd1-d* and *SSD1-v* derived *elp3* and *deg1* mutants show reduced growth as compared to the appropriate wild type control and the effect if more pronounced for *deg1* as compared to *elp3* (Figure 10A). This assay also revealed an elevated temperature sensitivity of the *ssd1-d* strain W303-1B as compared to BY4741 at 39°C. The *ssd1-d* allele was previously shown to cause growth defects at higher temperatures (Kaeberlein et al., 2004).

To investigate whether the growth phenotype differences are indeed because of the allelic variation of the *SSD1* gene and not because of other genetic differences between W303-1B and BY4741, the *SSD1-v* allele was expressed in the *ssd1-d* strains and tested for phenotypic complementation. Both *ssd1-d* and *ssd1-d deg1* strains were transformed with an empty vector or a plasmid carrying *SSD1-v* [*SSD1-v*]. As a control, a plasmid containing *ssd1-d* [*ssd1-d*] was included in this experiment. As shown in Figure 10B, the growth defect of the *ssd1-d* wild type and the *deg1* mutant at higher temperatures can be suppressed in the presence of *SSD1-v*. Growth for both *ssd1-d* and *ssd1-d deg1* strains was improved at elevated temperature with the help of *SSD1-v* [*SSD1-v*] expression, but it was not improved upon expression of either *ssd1-d* [*ssd1-d*] or empty vector controls. Hence, the enhanced temperature sensitivity of *ssd1-d* strains is caused by the *ssd1-d* allele, and *SSD1-v* itself positively affects thermoresistance in the wild type strain and the tRNA modification mutant. Based on this finding, the enhancement of *elp3* and *deg1* phenotypes under the influence of the *ssd1-d* allele could be due to an additive effect caused by two independent mechanisms increasing thermosensitivity.

5.1.2. Comparison of rapamycin sensitivity in *SSD1-v* and *ssd1-d* backgrounds

In addition to temperature sensitivity, the effect of different alleles of *SSD1* was tested on the *elp3* and *deg1* mutants' rapamycin phenotypes. Different tRNA modification mutants, including *elp3* and *deg1*, were previously shown to exhibit elevated sensitivity against the TOR inhibitor drug rapamycin. Consistently, *deg1* and *elp3* mutants showed enhanced rapamycin sensitivity in comparison to the respective wild type control (Klassen et al., 2017; Xu et al., 2019) in both strains (Figure 11A). The sensitivity to rapamycin was generally increased in the *ssd1-d* background. However, distinct from the temperature phenotypes, *elp3* showed stronger rapamycin sensitivity as compared to *deg1*. Notably, the *ssd1-d* wild type strain also displays aggravated rapamycin sensitivity compared to the *SSD1-v* wild type strain (Figure 11A). To check whether the observed difference in rapamycin sensitivity between the *ssd1-d* and *SSD1-v* strains is because of the dissimilar status of *SSD1* in the two backgrounds, the rapamycin phenotype of an *ssd1* deletion strain was analyzed. The *ssd1-d* phenotype complementation by the ectopic expression of *SSD1-v* was as well tested. *ssd1* deletion in the *SSD1-v* strain causes an enhanced

drug sensitivity, and *SSD1-v* expression in the *ssd1-d* strain suppresses the phenotype (Figure 11B). Based on these observations, rapamycin resistance is influenced by the *SSD1* status, independent of any tRNA modification defect. Hence, the increased rapamycin sensitivity in *ssd1-d* tRNA modification mutants could result from an additive effect caused by independent consequences of the lack of tRNA modification and the defect of Ssd1 (Khonsari et al., 2021).

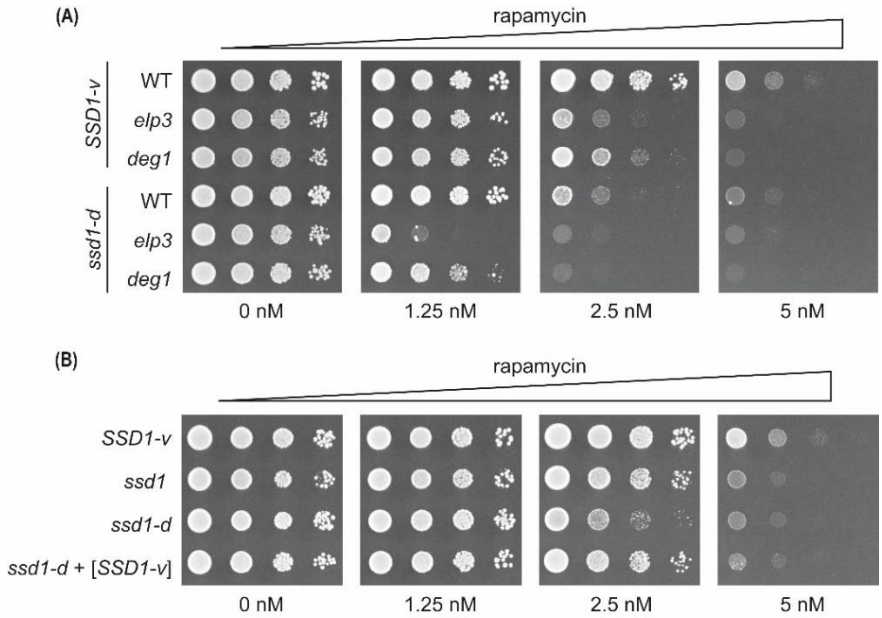

Figure 11. Rapamycin sensitivity of *deg1* and *elp3* mutants is influenced by *SSD1*. (A) Comparison of rapamycin sensitivity between the tRNA modification mutants in both *SSD1-v* and *ssd1-d* backgrounds. WT, *elp3*, and *deg1* mutants of both *SSD1* related strains were serially diluted and spotted on YPD plates containing the demonstrated amounts of rapamycin. All plates were incubated for 48 h at 30°C. (B) Rapamycin sensitivity in the presence, absence, defect, and expression of *SSD1*. All yeast strain backgrounds for this experiment: *SSD1-v* (BY4741); *ssd1* (BY4741); *ssd1-d* (W303-1B); *ssd1-d* + [*SSD1-v*] (W303-1B with *SSD1-v* plasmid pPL092). Strains were spotted on YPD plates with the shown concentrations of rapamycin and were incubated at 30°C for 48 h.

5.1.3. Genetic interaction of *DEG1* with mcm^5s^2U relevant genes

Because *ssd1-d* can modulate the growth phenotypes of *elp3* and *deg1* tRNA modification mutants individually, the effect of different alleles of *SSD1* on the negative genetic interaction between U_{34} and $U_{38/39}$ modifiers was also tested. A substantial interdependent growth defect results from the combination of lack of U_{34} modification caused by mutations of either *URM1* or *ELP3* and deletion

of *DEG1* (Klassen et al., 2016). The *elp3 deg1* double mutant is viable in the *SSD1-v* background but shows a very severe growth defect at 30°C (Klassen et al., 2016). The *urm1 deg1* double mutant displays almost a normal growth at 30°C but grows extremely slow at 37°C (Klassen et al., 2016).

The plasmid shuffling method, which was previously used to generate the *SSD1-v* double mutant strains, was utilized to check for the phenotypes of both *elp3 deg1* and *urm1 deg1* double mutants in the *ssd1-d* background. First, *elp3* and *urm1* mutations were each stabilized with a suitable 5 fluoroorotic acid (5-FOA) counter selectable plasmid providing either *ELP3* or *URM1*, respectively. Afterwards, the *DEG1* gene was deleted, and the loss of *urm1*- and *elp3*-complementing plasmids was tested by checking growth on 5-FOA supplemented media (Figure 12A, B). Both double mutants, (*elp3 deg1* and *urm1 deg1*) in the *ssd1-d* background did not show growth on 5-FOA media, which was in contrast to the control strains lacking the additional *deg1* defect (Figure 12C, D). Therefore, both mentioned double mutants are inviable in the *ssd1-d* strain background. Thus, the previously observed synthetic sick genetic interaction between *urm1/elp3* and *deg1* in *SSD1-v* is further enhanced in the *ssd1-d* strain, in which a synthetic inviable interaction has occurred (Figure 12C, D) (Khonsari et al., 2021).

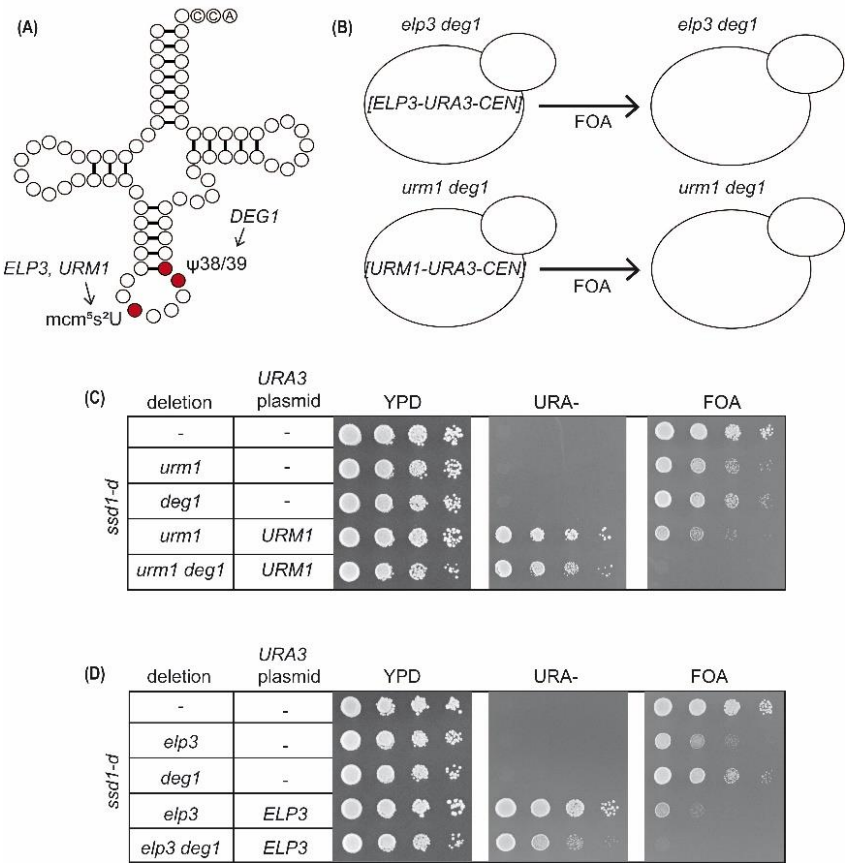

Figure 12. Plasmid shuffle assay in the *ssd1-d* strain to characterize genetic interaction between *DEG1* and *ELP3 or URM1*. (A) The $mcm^5s^2U_{34}$ and $\Psi_{38/39}$ modification sites and genes involved are indicated on a tRNA molecule. (B) The concept of plasmid shuffle assay in *elp3 deg1* or *urm1 deg1* double mutants carrying *URA3-CEN* plasmids that introduce the *ELP3* or *URM1* wild type gene functions, respectively. 5-FOA (FOA) medium is counter selective for the *URA3* based plasmids. Therefore, growth or non-growth on 5-FOA media reveals the double mutant phenotype. (C) Result of plasmid shuffle assay in the *deg1 urm1* double mutant. (D) Result of plasmid shuffle assay for the *deg1 elp3* strain. WT and indicated mutant strains with and without *URA3* based plasmids. All strains were serially diluted and spotted on YPD, URA- and FOA medium. YPD and URA- plates were incubated at 30°C for 48 h, the FOA plates were incubated for 72 h at the same temperature.

5.1.4. Suppression of synthetic lethal genetic interactions of *DEG1* by $tRNA^{Gln}UUG$

Negative growth phenotypes of *deg1 urm1* and *deg1 elp3* double mutants in *SSD1-v* background strain were shown to be partially rescuable with the overexpression of $tRNA^{Gln}UUG$ (Klassen et al., 2016). A defect of the mentioned tRNA is thought to be responsible for the double mutants' negative phenotypes, and higher amounts of the hypomodified tRNA can make up the functional impairment. To check if the synthetic inviable interaction in the *ssd1-d* background between *elp3/urm1* and *deg1* can also be

suppressed, the plasmid shuffle technique shown in Figure 12 was repeated in the presence or absence of elevated tRNA^{Gln}UUG levels. The latter was achieved by including either a multicopy plasmid containing the *tQ(UUG)* gene or the appropriate empty vector in the plasmid shuffle approach. As shown in Figure 13, both *ssd1-d elp3 deg1* and *urm1 deg1* strains display growth improvement on 5-FOA media upon overexpression of tRNA^{Gln}UUG construct, but not with the empty vector control. However, both double mutant strains needed substantially prolonged incubation times for colony formation on 5-FOA media. Hence, growth is significantly delayed compared to the wild type or the respective single tRNA modification mutants. Thus, tRNA^{Gln}UUG overexpression causes a rescue of the synthetic lethal interactions in *ssd1-d* strain between *ELP3/URM1* and *DEG1* (Figure 13), but growth is severely impaired in comparison to either single mutant or the wild type.

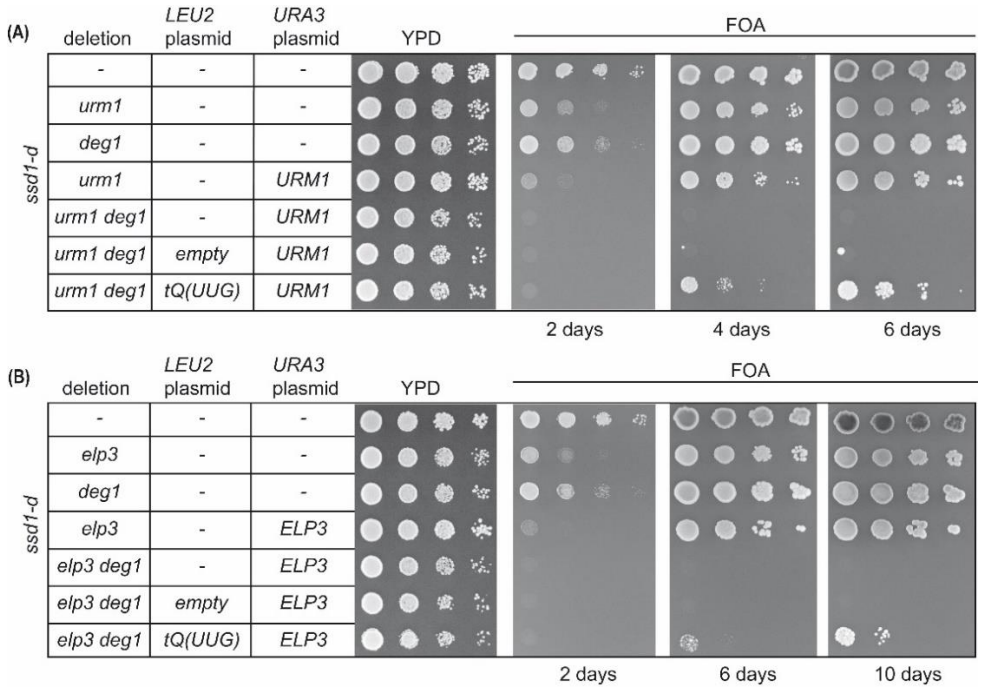

Figure 13. Suppression of the synthetic lethality of combined tRNA modification mutants by tRNA overexpression. All strains are generated in the W303-1B background and contain represented gene deletions, and additionally either no plasmid (-), empty *LEU2* vector pRS425 (empty), *LEU2*-tRNA^{Gln}UUG high copy (h.c.) vector pRK55 (*tQ(UUG)*) or single copy (s.c.) *URA3* vectors introducing *ELP3* and *URM1*, respectively. (A) Rescue of *urm1 deg1* by tRNA overexpression. (B) Rescue of *elp3 deg1* by tRNA overexpression. All trains were serially diluted and spotted on YPD and FOA plates. YPD plates were incubated at 30°C for 48 h, and the FOA plates were incubated at 30°C as represented.

5.1.5. Role of *SSD1* in the negative genetic interaction of *DEG1* with U_{34} tRNA modification genes

As outlined above, the negative genetic interaction of *DEG1* with U_{34} modification genes is aggravated in the *ssd1-d* strain W303-1B as compared to the *SSD1-v* strain BY4741. However, it remained unclear, whether this difference is due to the allelic variation of *SSD1* in the two strains or might be caused by other genetic differences. To address this, it was investigated whether the synthetic lethal interaction between *deg1* and *elp3/urm1* in the *ssd1-d* background is suppressible by the presence of *SSD1-v*. If the enhanced negative genetic interaction is exclusively due to the *ssd1-d* allele present in the W303-1B strain, ectopic expression of *SSD1-v* should suppress the observed synthetic inviability of *deg1 urm1* and/or *deg1 elp3*. To analyze this possibility, the plasmid shuffle approach shown in Figure 12 was conducted in the presence or absence of an *SSD1-v* containing single copy vector. As shown in Figure 14, the *ssd1-d elp3 deg1* and *urm1 deg1* double mutants remain inviable in the presence of the *SSD1-v* expression construct. All strains were also spotted on URA- as well as LEU- plates, which was used as a control to confirm the presence of *ELP3*/*URM1* complementing *URA3* plasmids and the *SSD1-v* containing *LEU2* plasmid. In contrast to the observation made with $tRNA^{Gln}UUG$ overexpression (Figure 13), even the extension of the incubation time of 5-FOA plates for both *elp3 deg1* and *urm1 deg1* mutant strains did not enable visible growth of these strains (Figure 35). Thus, *SSD1-v* expression does not rescue the synthetic lethal interactions between *ELP3/URM1* and *DEG1* in the *ssd1-d* strain, suggesting that additional genetic differences between the strains do influence the strength of the combined mutant phenotype.

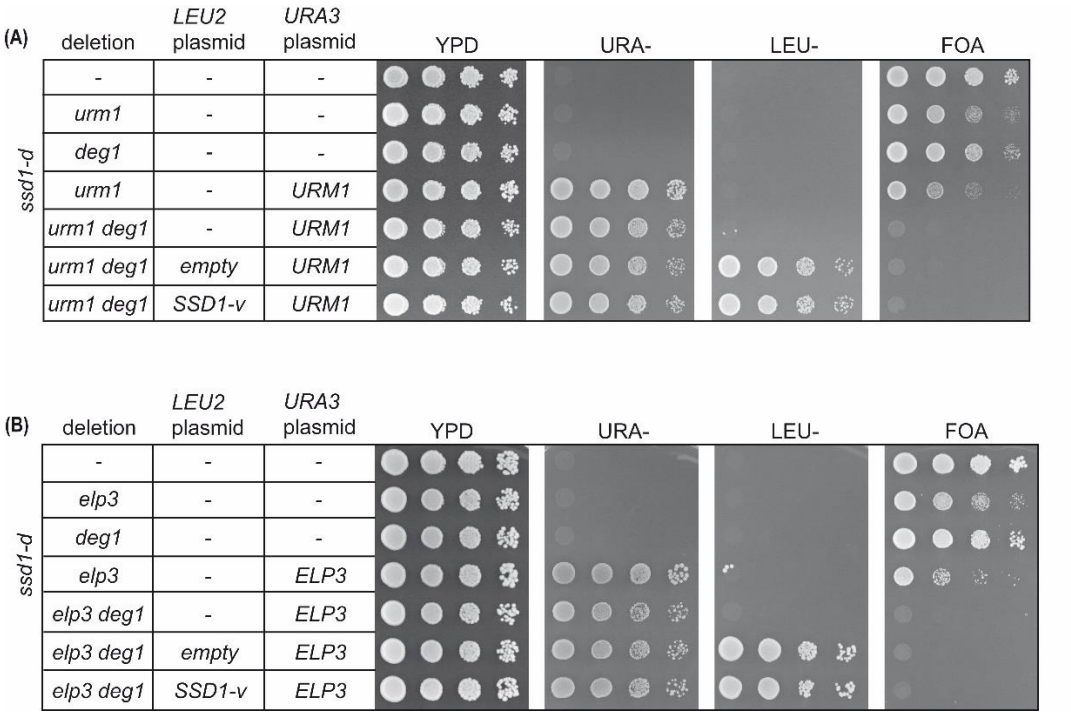

Figure 14. The synthetic lethality of *deg1 urm1* and *deg1 elp3* strains in W303-1B (*ssd1-d*) is not rescued by *SSD1-v*. All strains are in the W303-1B background with indicated gene deletions and either no plasmid (-), empty *LEU2* vector YCplac111 (empty), *LEU2-SSD1-v* plasmid pPL091 or single copy *URA3* vectors introducing *ELP3* and *URM1*, respectively. (A) *SSD1-v* expression is not rescuing the *urm1 deg1* double mutant. (B) *SSD1-v* expression is not rescuing the *elp3 deg1* double mutant. All strains were serially diluted and spotted on YPD, URA-, LEU- and FOA plates. The plates were incubated at 30°C for 48 h.

5.2. Phenotypic variation of other tRNA modification defects by SSD1

In this part of the results section, the phenotype of other tRNA modification mutants, including another pseudouridine modification mutants are tested in both *SSD1-v* and *ssd1-d* strain backgrounds. The mutants used in this part are *pus1*, *trm1*, *trm8* and *ncl1*. The Pus1 protein is responsible for pseudouridylation at position 1, 26, 27, 28, 34, 36, 65, and 67 (Simos et al., 1996; Motorin et al., 1998; Behm-Ansmant et al., 2003, 2006). Trm1 is responsible for $m^2{}_2G$ (N_2, N_2 dimethylguanosine) modification at position 26 (Edqvist et al., 1994); Trm8 in charge of m^7G (7-methylguanosine) modification at position 46 (Alexandrov et al., 2002) and the Ncl1 protein is accountable for m^5C (5-methyl cytidine) modification at position 48 (Wu et al., 1998). These strains are chosen based on their high temperature sensitivity, which was also observed in the *deg1* mutant. In addition, this chapter intends to show if all tRNA modification mutants in the *ssd1-d* background exhibit similarly elevated temperature sensitivity compared

to the same mutations in the *SSD1-v* background. The results of this part are partially published in (Khonsari et al., 2021).

5.2.1. Other thermosensitive tRNA modification mutants

Loss of either Pus1, Trm1, Trm8, and Ncl1 enzymes are known to induce a thermosensitivity phenotype. Still, it remains unknown whether this effect is enhanced in the absence of a functional Ssd1 protein as observed for *deg1* and *elp3* (Figure 10A). The mutant phenotypes of the strains carrying complete deletions of *PUS1*, *TRM1* or *NCL1* were compared in *ssd1-d* and *SSD1-v* backgrounds. All three mutants (*pus1*, *trm1*, *ncl1*) in the *SSD1-v* background presented strong temperature sensitivity in comparison to the *SSD1-v* wild type (Figure 15A), which was a conformation for earlier reports (Gustavsson & Ronne, 2008; Ruiz-Roig et al., 2010; Khonsari & Klassen, 2020). However, the *ssd1-d* strain again showed increased temperature sensitivity compared to the *SSD1-v* strain, and only a slight further enhancement was seen for *ssd1-d pus1* compared to *ssd1-d* wild type. Unexpectedly, the *ncl1* and *trm1* mutants in the *ssd1-d* background were not notably more thermosensitive than the *ssd1-d* control (Figure 15A). Hence, an enhancement of tRNA modification mutant phenotypes in the *ssd1-d* strain is not generally present and appears to be rather specific for *elp3*, *urm1* and *deg1*. To obtain additional information about genetic interaction strength of tRNA modification genes in both *SSD1* backgrounds, the *TRM8* methyltransferase gene was deleted in wild type and *ncl1* mutants (Alexandrov et al., 2005). In *ncl1 trm8* mutants, a robust negative genetic interaction is well described and mechanistically related to rapid tRNA decay of $tRNA^{Val}AAC$ (Alexandrov et al., 2006). Such decay was shown to be triggered at elevated temperatures (37°C) and results in a complete lack of growth of an *SSD1-v ncl1 trm8* double mutant at the mentioned temperature. However, all genetic investigations of RTD in absence of *NCL1* and *TRM8* were carried out in the *SSD1-v* (BY4741/4742) background and it remained unknown whether it is influenced by strain background and/or *SSD1* variation. Hence, the synthetic temperature sensitivities of *ncl1 trm8* in both, *ssd1-d* and *SSD1-v* strains was compared. As shown in (Figure 15B), an intense synthetic temperature sensitivity of *ncl1 trm8* double mutants is observable in both strain backgrounds. However, in *ssd1-d ncl1 trm8* compared to the *SSD1-v* counterpart, the defect is

dampened rather than enhanced (Figure 15B). Hence, also negative genetic interactions between distinct tRNA modification genes is not generally enhanced in the *ssd1-d* strain, and thus, might be specific for the studied cases involving *deg1*, *elp3* and *urm1* or functionally equivalent mutants (e.g. *elp1,2,4,5,6* and other tRNA thiolation deficient mutants) (Khonsari et al., 2021).

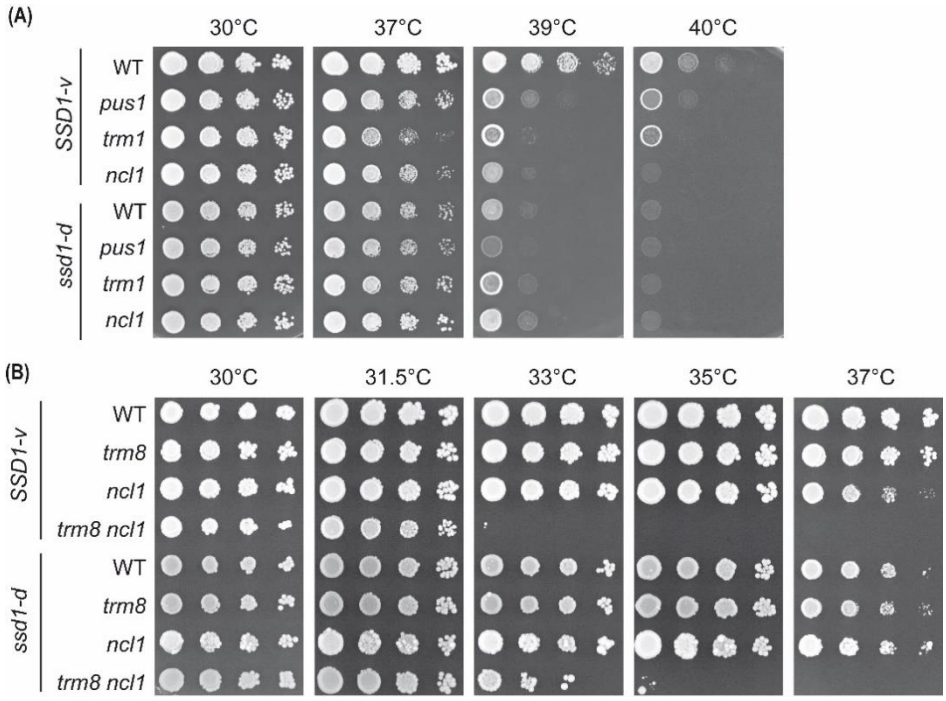

Figure 15. Comparison of thermosensitivity in both *SSD1* backgrounds of other tRNA modification mutants. (A) The wild type and *pus1, trm1,* and *ncl1* mutant strains in *SSD1-v* and *ssd1-d* backgrounds were serially diluted, spotted on YPD plates. The plates were then incubated for 48 h at elevated temperatures. (B) Wild type and *trm8, ncl1,* and *trm8 ncl1* double mutants in both *SSD1* backgrounds were serially diluted and spotted on YPD plates, and incubated for 48 h at elevated temperatures.

5.2.2. 5-fluorouracil sensitive tRNA modification mutants

Another described phenotype for *trm1* and *pus1* mutants in the *SSD1-v* background is an aggravated sensitivity to the anticancer drug 5-fluorouracil (5-FU) (Gustavsson & Ronne, 2008). The efficiency of the drug was highly increased by including slight thermal stress during the incubation time, and this effect is thought to result from a destabilizing effect of the drug on the hypomodified tRNAs. Because of the unanticipated lack of strong temperature sensitive phenotypes for *trm1* and *pus1* mutants in the *ssd1-d* background, the 5-FU phenotype in both *SSD1* backgrounds was analyzed. As compared to the

SSD1-v wild type, *trm1* and *pus1* strains containing the same *SSD1* allele demonstrate enhanced sensitivity to 10 mg/ml 5-FU at 30°C, but this effect was not observed in the corresponding *ssd1-d* strains (Figure 16). Anyhow, by applying the drug at 37°C, both *trm1* and *pus1* mutants in *ssd1-d* and *SSD1-v* backgrounds became severely sensitized compared to the wild type controls, confirming the expected phenotype in single mutants. Interestingly, the *pus1* and *trm1* mutants in the *ssd1-d* background appeared a little more resistant against this effect in comparison to the same strains in the *SSD1-v* background (Figure 16). Thus, 5-FU phenotypes of both *trm1* and *pus1* mutants in the *ssd1-d* strain are generally confirmed but are improved compared to the *SSD1-v* background. This result provides another example of dampened rather than enhanced tRNA modification mutant phenotypes in the *ssd1-d* strain and underscores our assumption that phenotypic enhancement by *ssd1-d* is rather specific for U_{34} modification defects and the *deg1* mutation.

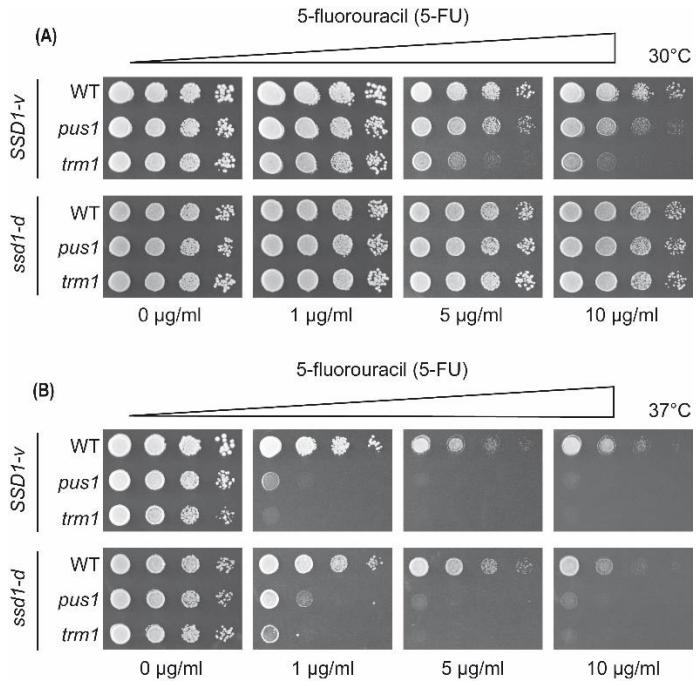

Figure 16. 5-fluorouracil (5-FU) phenotype of wild type and tRNA modification mutants in both *SSD1* backgrounds. (A) WT, *pus1* and *trm1* mutants in *ssd1-d* and *SSD1-v* backgrounds were spotted on YPD plates with the displayed amounts of 5-FU and incubated for 48 h at 30°C. (B) Identical cell suspensions as in (A) were spotted on YPD plates containing the demonstrated amounts of 5-FU and incubated for 48 h at 37°C.

5.3. Effect of Ssd1 on the expression of Rnq1 in the absence of $\Psi_{38/39}$

In the yeast *Saccharomyces cerevisiae*, the presence of both mcm^5s^2U and $\Psi_{38/39}$ in $tRNA^{Gln}UUG$ was shown to be required for efficient translation of mRNA encoding the Q and N rich Rnq1 protein (Klassen et al., 2016). In the same study, it was also observed that in the *SSD1-v* background, there is a downregulation of the Rnq1 protein level in the absence of *DEG1*, which was due to post-transcriptional effects, presumably reflecting a reduced translational efficiency of $tRNA^{Gln}UUG$ already in the absence of Deg1 dependent Pseudouridine alone (Klassen et al., 2016). It remains unknown, however, whether such translational defect (an in-turn phenotypes associated with it) are influenced by the *SSD1* allelic variation present in the yeast strains BY4741 and W303-1B. To clarify this, the influence of the *SSD1* locus on the Rnq1 protein level in both WT and *deg1* mutants of both yeast strain backgrounds was tested in comparison. Since Rnq1 is a well-studied yeast prion and both strain backgrounds are known to carry the prion version of Rnq1 ([*PIN+*]) (Derkatch et al., 2004; Nakayashiki et al., 2005; Wickner et al., 2007), it was also asked whether the translational defect of $tRNA^{Gln}UUG$ in *deg1* mutants influences the aggregation propensity of Rnq1. Therefore, protein aggregates were analyzed, and the level of Rnq1 protein in the total protein aggregates content of the cell was compared between the WT and the *deg1* mutant in both *SSD1-v* and *ssd1-d* backgrounds. Fluorescence microscopy using Rnq1-GFP fusion proteins was additionally used to obtain more evidence on aggregation formation. Since yeast prions can easily be eliminated using GdnHCl treatment (Eaglestone et al., 2000), this agent was used to cure the [*PIN+*] prion in WT and *deg1* mutants of both strain backgrounds, and to compare the effects of *deg1* mutation on the cellular levels of prion and non-prion forms of Rnq1.

5.3.1. Expression of the glutamine-rich prion protein Rnq1 in *deg1* mutants

It is known that the efficiency in decoding $tRNA^{Gln}UUG$ is crucially dependent on pseudouridine at position 38 (Han et al., 2015; Sokołowski et al., 2018; Borchardt et al., 2020). Thus, enhanced phenotypes of *deg1* in the *ssd1-d* background compared to the same mutation in *SSD1-v* could be due to a further increased decoding defect of $tRNA^{Gln}UUG$ in the *ssd1-d* strain. For a comparison between the two *SSD1* background strains regarding the expression defect related to $tRNA^{Gln}UUG$ in the absence of pseudouridylation at position 38 and 39, Rnq1 was

expressed as a GFP fusion protein (Sondheimer & Lindquist, 2000) in WT and *deg1* mutants of both *SSD1* strains. As mentioned before, a significant downregulation in the amounts of Rnq1-GFP protein was observed in the *SSD1-v deg1* mutant compared to the *SSD1-v* wild type control. Surprisingly, however, the Rnq1-GFP levels were similar in the *ssd1-d* wild type and *ssd1-d deg1* mutant strains (Figure 17). Hence, expression of the Gln-rich Rnq1 appears to be improved rather than weakened in the *ssd1-d deg1* mutant in comparison to the *SSD1-v* strain. Thus, based on the study of Rnq1-GFP levels in [*PIN*$^+$] strain, no general intensified defect in the translation of the Ψ_{38} deficient tRNA is detectable (Khonsari et al., 2021).

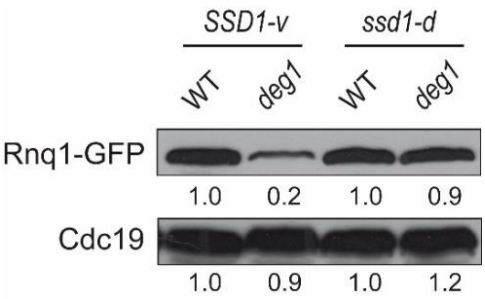

Figure 17. Comparison of the Gln-rich Rnq1-GFP prion protein levels in wild type (WT) and *deg1* mutants in the *SSD1-v* and *ssd1-d* strains BY4741 and W303-1B, respectively. Total protein from wild type and *deg1* mutants in both strain backgrounds expressing Rnq1-GFP was used for Western analysis by anti GFP and anti Cdc19 antibodies. The intensity of GFP and Cdc19 signals are normalized to the respective wild type intensity.

5.3.2. Effect of Deg1 and Ssd1 on the level of Rnq1-GFP in protein aggregates

A downregulation of Rnq1-GFP was observed in total protein extracted from *SSD1-v deg1* compared to *SSD1-v* wild type strain but not between *ssd1-d deg1* strain and *ssd1-d*. Since both yeast strains are carriers of the [*PIN+*] prion, which is the amyloid aggregate form of Rnq1 (Derkatch et al., 2001) it appeared possible that the modification defect has distinct effects on the prion properties of Rnq1 in both strain backgrounds. Since the protein extraction method routinely used for yeast relies on the cell breakage by glass beads and subsequent removal of cell debris by centrifugation (see methods section 6.14.), it appeared possible that distinct Rnq1 aggregate sizes or other properties might influence the detection of the Rnq1-fusion protein in total protein extracts as analyzed in Figure 17. Thus, a protocol to isolate the total protein aggregate content of cells was used (see methods section 6.18.) in order to analyze the effect of the *deg1*

mutation specifically on the aggregated form of Rnq1. Therefore, protein aggregates were extracted from all GFP-tagged yeasts and GFP signals detected by Western analysis. In contrast to the results obtained for total protein extracts, a clear downregulation of the Rnq1-GFP signal was observed in the aggregate fraction from both *SSD1-v deg1* and *ssd1-d deg1* mutants in comparison to their respective WT (Figure 18A), and this downregulation was comparable between the two strains. The Western analysis shows that the *deg1* mutation decreases the amount of Rnq1 protein aggregates in both backgrounds almost equally, even though it has a different influence on the signal in total protein in the two *SSD1* backgrounds. It seems possible that the GFP signal in the total protein (after removal of cell debris and large aggregates) represents the soluble (non-aggregated) fraction of Rnq1-GFP and possibly smaller aggregates that do not pellet using the low speed centrifugation applied in this step. Thus, strain differences in Rnq1 aggregate sizes might indeed influence the apparent Rnq1 protein levels in total protein preparations. When fluorescence microscopy was applied to detect Rnq1-GFP, the sizes of foci known to represent Rnq1-GFP aggregates are indeed smaller in the *ssd1-d* strains (Figure 18B), which might support the assumption that a higher fraction of small aggregate forms of Rnq1 result in elevated detection of the GFP signal in *ssd1-d deg1* relative to *SSD1-v deg1* in total protein preparations.

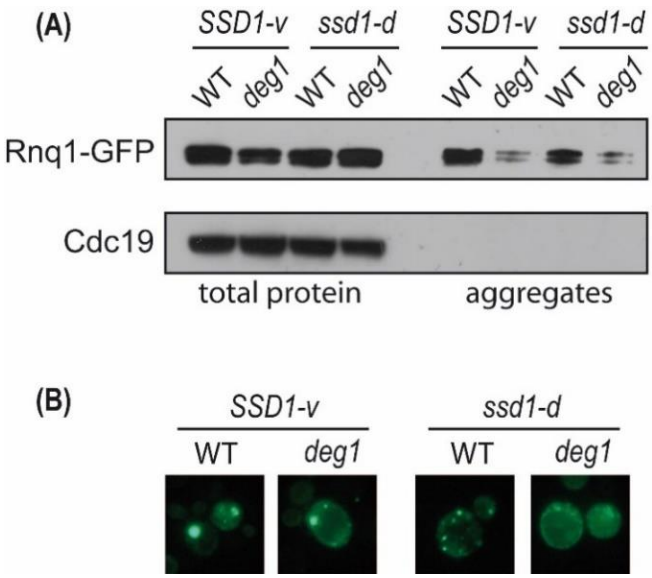

Figure 18. Comparison of total protein and protein aggregate levels of Rnq1-GFP in *SSD1-v* and *ssd1-d* backgrounds. (A) Total protein and protein aggregates were extracted from WT and *deg1* mutants of *SSD1-v* and *ssd1-d* yeast strains and were used for Western analysis with anti GFP and anti Cdc19 antibodies. (B) The Rnq1-GFP fluorescence detected by microscopy of the indicated strains.

5.3.3. Effect of *deg1* mutation on the expression of Rnq1 after curing of the prion form

It is known that the growth of yeast strains carrying the prion versions of Sup35 ([*PSI*$^+$]) or Rnq1 ([*PIN*$^+$]) in the presence of 1-5 mM of GdnHCl leads to the elimination of the prion forms due to inhibition of prion propagation (Tuite et al., 1981; Eaglestone et al., 2000). After few generations of growth in the presence of GdnHCl, only the fully soluble from of Sup35 and Rnq1 are present in cells. Therefore, GdnHCl is frequently used to eliminate prions ("curing") in yeast studies (Tuite et al., 1981; Derkatch et al., 1996, 2004; Eaglestone et al., 2000). The efficiency of prion curing can be tracked using the expression of GFP fusions of Sup35 or Rnq1, which form visible foci when prion conversion takes place and display homogeneous signal distribution in the cells when the prion is eliminated. In the context of the *deg1* effect on Rnq1 expression and prion properties, the GdnHCl was applied to eliminate the [*PIN*+] prion. Here, the purpose of this approach was to investigate whether loss of *DEG1* has a differential effect on Rnq1-GFP levels when the protein exists in the non-amyloid soluble form in yeast cells. To control for prion curing, fluorescence microscopy was used before and after the treatment for all strains used for this investigation (Figure 19A). WT and *deg1* mutants of both strain backgrounds expressing Rnq1-GFP were grown twice on YPD containing 3 mM GdnHCl. To ensure all strains retained the *URA3* vector carrying the *RNQ1-GFP* fusion protein, all strains were spotted on URA- plates; a temperature sensitivity test was also performed for this set of strains to track and validate the temperature sensitivity phenotype linked to *DEG1* deletion and to conclude about possible phenotypic effects of the presence or absence of the prion (Figure 19B). A small improvement for *SSD1-v deg1* and *ssd1-d* at 39°C and for *ssd1-d deg1* at 37°C was observed after elimination of [*PIN*+] (Figure 19B), suggesting some negative effects of the prion on thermotolerance. To compare the expression defect of Rnq1 caused by the absence of Deg1 in [*pin*-] and [*PIN*+] strains, total protein was extracted, before and after the treatment with GdnHCl. The GFP signal in these protein samples was compared by Western analysis. As observed before, an apparent downregulation of Rnq1-GFP prion protein level in the *SSD1-v deg1* mutant compared to the *SSD1-v* wild type was detected in the [*PIN*+] background (Figure 19C). This effect was comparable in the [*pin*-] variants of the same strains. Interestingly, however, a severe downregulation of

the Rnq1-GFP signal was observed in the [*pin*-] *ssd1-d deg1* mutant in comparison to the [*pin*-] *ssd1-d* strain. This result is in striking contrast to the effect observed for the [*PIN*+] versions of the same strains, where *deg1* mutation had no effect on Rnq1-GFP protein levels (figure 19C). Another marked difference is the appearance of a number of smaller signals detected with the anti-GFP antibody in all [*pin*-] but none of the [*PIN*+] strains, which could reflect protein degradation products of the non-amyloid form of Rnq1. Most importantly, however, the analysis of soluble Rnq1-GFP levels ([*pin*-]) revealed a downregulation in both *SSD1-v deg1* and *ssd1-d deg1* mutants relative to their respective wild type controls and this effect was clearly pronounced in the *ssd1-d* strain.

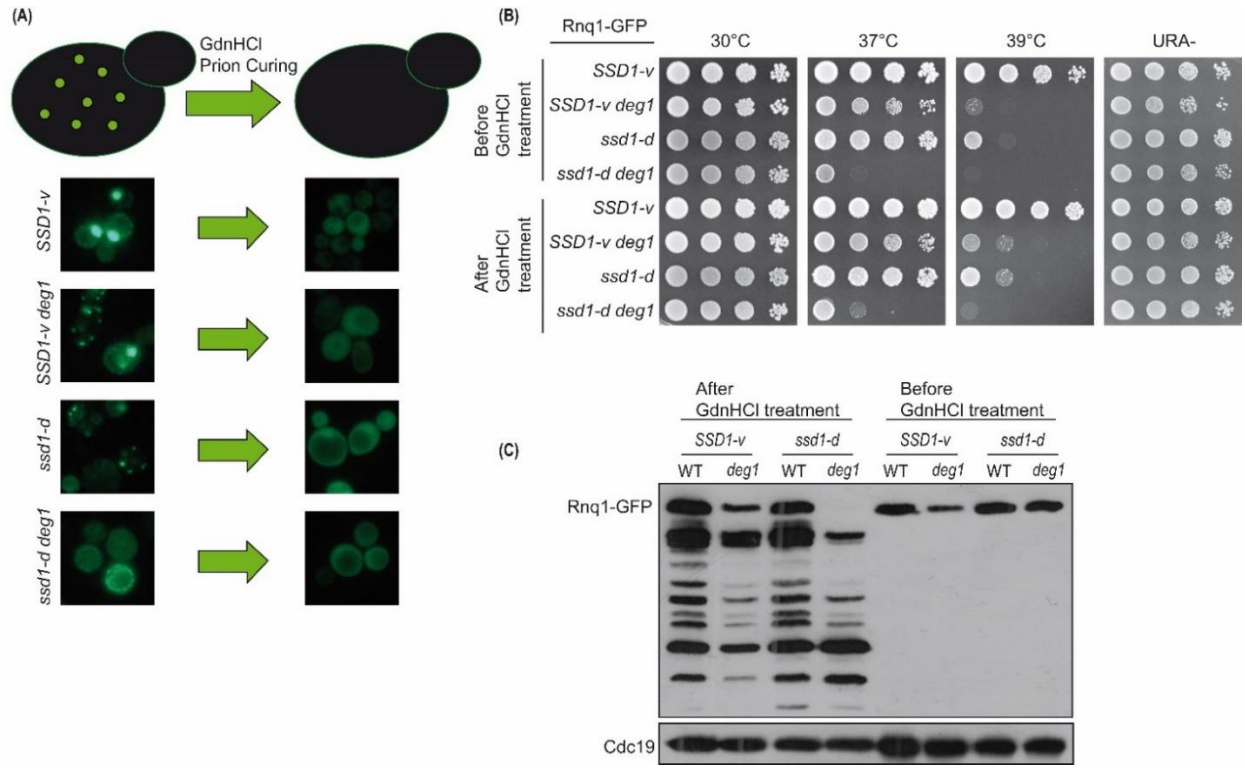

Figure 19. Analysis of Rnq1-GFP levels in [*PIN*+] (before GdnHCl treatment) and [*pin*-] (after GdnHCl treatment). (A) Rnq1-GFP tagged WT and *deg1* mutant in both *SSD1* backgrounds were treated with GdnHCl, and efficiency of prion curing was analyzed by fluorescence microscopy before and after the treatment. (B) [*pin*-] and [*PIN*+] WT and *deg1* mutants carrying the *RNQ1-GFP* expression construct were spotted on YPD and URA- plates. The YPD plates were incubated at indicated temperatures, and the URA- plate was incubated at 30°C for 48 h. (C) Total protein from indicated strains expressing Rnq1-GFP before and after GdnHCl treatment was used for Western analysis with anti GFP and anti Cdc19 antibodies.

5.4. Effect of *SSD1* on autophagy and protein aggregation in *deg1* mutants

In this chapter, the autophagy in WT and *deg1* mutant, in both *SSD1-v* and *ssd1-d* background, is compared. Following this experiment, the rapamycin is used to test the influence of the Deg1 dependent Ψ modification in *ssd1-d* background strain on autophagy, based on the inactivation of TORC1. This experiment is done to see how pseudouridine modification is related to the TOR-controlled starvation response. The rest of this chapter is invested in the differences in protein aggregation formation in both *SSD1* backgrounds and whether it is possible to rescue the higher protein accumulation with the expression of the dominant *SSD1* allele. Afterward, the formation of aggregates resulting from the entrance of a wrong amino acid in the ribosome is analyzed. At the end of this chapter, the influence of the Pus1 and Deg1 related pseudouridylation on the rapid tRNA decay (RTD) as a temperature-dependent mechanism, and the impact of RTD on the formation of protein aggregates in a Pus1 related Ψ modification mutant is tested. This result will provide the possibility of comparing the influence of different pseudouridine modifications on aggregation accumulation and whether there is a connection between RTD and protein aggregation.

5.4.1. Autophagy differences between *SSD1-v* and *ssd1-d* yeast strains

In a previous study, combined modification defects involving the *deg1* mutation in *SSD1-v* background strains were shown to result in the activation of different cellular starvation response, including the premature induction of autophagy (Bruch et al., 2020). As explained before, autophagy is negatively regulated by the TORC1 pathway, and TORC1 inactivation can activate autophagy. Since *deg1* phenotypes were found in this study to be enhanced by *ssd1* mutation, it appeared possible that changes in inadequate autophagy activation might correlate with the *SSD1* effect. To test the autophagy activation in the *deg1* tRNA modification mutant in both *SSD1-v* and *ssd1-d* backgrounds, a GFP-Atg8 processing assay, a common method used to test the autophagy activation in yeast studies, was performed in the presence and absence of rapamycin. As a result of autophagy-related vacuolar delivery of Atg8, a free GFP signal would appear on the Western blotting gel (Cheong & Klionsky, 2008). Rapamycin exposure is used in this assay as a well-established inhibitor of TORC1 to induce autophagy via TOR inhibition. In absence of rapamycin, WT cells of both

backgrounds display anti-GFP signal corresponding to full length GFP-Atg8 fusion protein and an additional signal corresponding to free GFP resulting from a basal processing of the fusion protein. As expected, rapamycin exposure drastically shifted the ratios of the two signals in both strain backgrounds towards the smaller free GFP, with only minor amounts of GFP-Atg8 detectable (Figure 20), consistent with robust TORC1 inactivation and activation of autophagy. In comparison to the untreated wild types, untreated *deg1* mutants of both backgrounds displayed increased levels of free GFP, consistent with a premature activation of autophagy to loss of tRNA pseudouridylation at positions 38 and 39. Hence, not only double tRNA modification mutants but also single *deg1* mutants in two distinct strain backgrounds apparently inadequately activate autophagy. Clearly, however, autophagy activation by loss of *DEG1* is weaker compared to the exposure to rapamycin, which might reflect a partial rather than complete inhibition of TORC1 upon loss of *DEG1*. Alternatively, other TORC1 independent ways of autophagy activation in *deg1* mutants cannot be excluded so far. However, it should be noted, that several other lines of evidence support a reduced TORC1 function in distinct tRNA modification mutants lacking Deg1 and/or Elongator function (Scheidt et al., 2014; Bruch et al., 2020). Based on intensity analysis, the degree of autophagy activation in the *ssd1-d deg1* mutant could be elevated as compared to *SSD1-v deg1* (Figure 20).

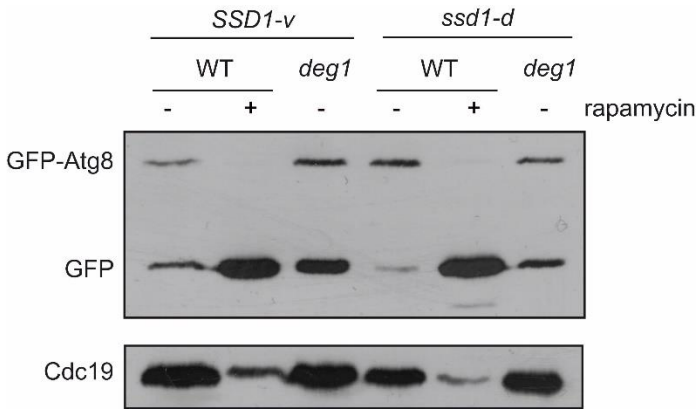

Figure 20. Autophagy induction assay using GFP-Atg8 fusion protein, in *deg1* mutants in *SSD1-v* and *ssd1-d* backgrounds. Total protein from indicated strains expressing GFP-Atg8; for WT in presence and absence of rapamycin; for Western analysis, anti GFP and anti Cdc19 antibodies were used. Due to ImageJ analysis, the free GFP induction level, compared to GFP-Atg8 signal in the *SSD1-v* background between the WT and *deg1* mutant is equal to 1.7, and this intensity level is equivalent to 7.1 in the *ssd1-d* background.

As observed, *deg1* mutation can lead to autophagy activation, which could be due to the inactivation of TORC1 in the absence of Ψ modification. To check whether this effect is related to the TORC1 function, the dependence of *deg1* mutation-induced autophagy on *ATG1* function was analyzed. *ATG1* encodes a kinase that plays an essential role in autophagy induction via TORC1 inhibition (Straub et al., 1997). To test whether *deg1*-mutation induced autophagy follows the canonical TORC1-controlled autophagy and therefore required *ATG1*, *atg1*, *deg1* single and *atg1 deg1* double mutants in the *ssd1-d* strain background were generated and analyzed. All strains were transformed with the GFP-Atg8 construct, and autophagy induction was investigated by Western blot analysis. The free GFP signal as observed previously in Figure 20 was entirely dependent on the presence of *ATG1*. Not only the rapamycin induced free GFP signal disappeared in atg1 but also the accumulation of free GFP in the untreated *deg1* mutant was lost in the *deg1 atg1* strain (Figure 21). The disappearance of the free GFP signal in *deg1 atg1* double mutant reveals that the autophagy induction as a result of the Ψ modification defect requires the Atg1 kinase activity, which is under the control of TORC1.

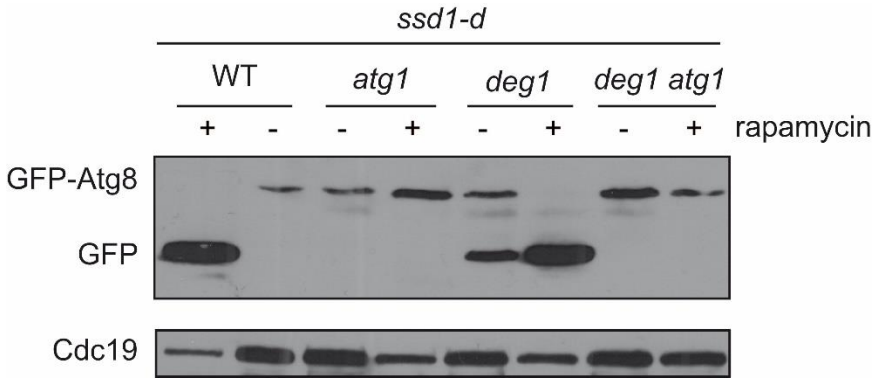

Figure 21. Effect of *atg1* mutation on autophagy induction in the *deg1* mutant. Total protein from WT, *atg1*, *deg1*, and *deg1 atg1* strains, expressing GFP-Atg8 were grown in the presence and absence of rapamycin. Western analysis was then performed using anti GFP and anti Cdc19 antibodies.

5.4.2. Role of different alleles of *SSD1* in protein aggregation

Some recent studies showed upset protein homeostasis as a significant outcome observable in yeast strains in the absence of $mcm^5s^2U_{34}$ and/or $\Psi_{38/39}$ modifications (Bruch et al., 2020; Klassen et al., 2016, 2017). The mentioned effect was most visible in double mutants missing parts of the $mcm^5s^2U_{34}$

modification combined with the *deg1* mutation or a complete lack of the $mcm^5s^2U_{34}$ modification (Nedialkova & Leidel, 2015; Klassen et al., 2016). The protein homeostasis defect of the tRNA modification mutants is assumed to cause the growth deficiency of the mutant strains at elevated temperatures since the temperature stress challenges the proteostasis mechanism. Due to the increased temperature sensitivity of *ssd1-d* and *ssd1-d deg1* strains compared to the wild type and *deg1* mutant in *SSD1-v* background, the possible changes in the amount of protein aggregates formed was scrutinized. It was considered that the possibility of the severe growth phenotypes in *ssd1-d deg1* might be related to increased amounts of protein aggregates since a similar effect for an *elp3 ncs2* mutant was observed in a recent study (Xu et al., 2019).

Total protein and protein aggregates from wild type and *deg1* mutants of *SSD1-v* and *ssd1-d* were extracted and analyzed on Nu-PAGE gradient gels. As indicated in Figure 22A, a *deg1* mutation aggravated the amount of formed protein aggregates in the presence of both *SSD1-v* and *ssd1-d* alleles. The wild type *ssd1-d* showed slightly more aggregates than *SSD1-v* and *ssd1-d deg1* displayed moderately more aggregates than *deg1* mutant in *SSD1-v* background (Figure 22A).

To check whether the absence of proper function in *SSD1* is causing the differences in protein aggregate formation, plasmids containing the *SSD1-v* allele were introduced into the *ssd1-d* wild type and *ssd1-d deg1* mutant strains, and the amount of formed protein aggregates was compared between the different strains. As presented in Figure 22B, the expression of the *SSD1-v* [*SSD1-v*] decreased the level of protein aggregates obtained from both *ssd1-d* and *ssd1-d deg1* strains. Hence, *ssd1-d* enhances the protein aggregation regardless of the defect in tRNA modification. However, the *ssd1-d* allele also aggravates the proclivity of the *deg1* mutant to form protein aggregates, and this effect might be applicable for the increased thermosensitivity of the *deg1* mutant in the *ssd1-d* background. Of note, the expression of the *SSD1-v* on a plasmid [*SSD1-v*] suppressed both temperature sensitivity and the induction of protein aggregates, which suggests a functional correlation between these two (Khonsari et al., 2021).

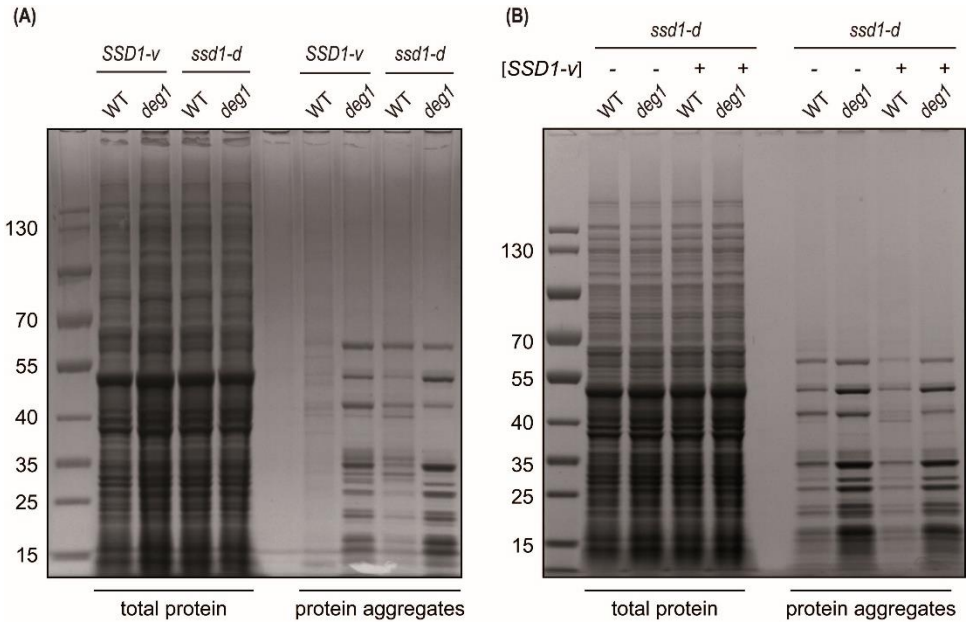

Figure 22. Impact of *deg1* mutation and *ssd1-d* allele on induction of protein aggregates. (A) Total protein and aggregates were extracted from WT and *deg1* mutants in *SSD1-v* and *ssd1-d* backgrounds. (B) Total protein and aggregates were extracted from *ssd1-d* wild type and *deg1* mutant in the presence (+) and absence (-) of plasmid-based *SSD1-v* [*SSD1-v*]. Samples were analyzed by Nu-PAGE and Coomassie staining.

5.4.3. Protein aggregation as a result of mistranslation

As mentioned, there are two models suggested for protein aggregate formation in tRNA modification mutants. One model refers to aggregate formation due to codon-specific ribosome pausing (Nedialkova & Leidel, 2015). The other model implies the formation of aggregates as a product of mistranslation, i.e. the acceptance of wrong tRNAs to the A site of the ribosome during translation. Such mistranslation events result in of the positioning of wrong amino acids in the final protein chain which increases misfolding of the protein and aggregation (Prokhorova et al., 2017). To test this effect, paromomycin as a mistranslation-inducing drug was used. Paromomycin binds to rRNA in the decoding center of the ribosome's A site, in a region in which two crucial rRNA residues are involved in detecting the correct pairing between codon and anticodon (Prokhorova et al., 2017) and Figure 23A. The region targeted by paromomycin is crucial for either accepting or rejecting incoming complexes of aminoacyl-tRNAs and eEF1A. Paromomycin was shown to increase the frequency of mistranslation in yeast cells (Prokhorova et al., 2017), but the effect on global protein aggregation as detected with the assay shown in Figure 23B remained to be studied. In theory, substantial mistranslation should increase protein aggregation. The WT and *elp3* mutant in the

SSD1-v background strains were used for this experiment. The *elp3* mutant was chosen for this experiment due to its paromomycin sensitivity, and its established effect on basal misreading and codon specific ribosome pausing (Patil et al., 2012; Nedialkova & Leidel, 2015; Joshi et al., 2018; Hawer et al., 2019). They were grown in the absence and presence of 200 µg/ ml of paromomycin, and total protein and protein aggregates were extracted from both strains grown in different conditions, and for comparison, analyzed by Nu-PAGE gel. The WT and *elp3* mutant were accumulating a low amount of aggregates, and when they were grown in the presence of paromomycin in the media, the aggregate fractions showed a smear-like pattern on the gel, and more bands were observed as compared to both strains grown in the absence of the drug in cell culture. This result shows that codon-specific ribosome pausing is not the only way that controls the aggregation formation, and mistranslation by the ribosome also plays a role in the appearance of protein aggregates. However, if the aggregate formation observed in the *elp3* mutant would mainly result from mistranslation, then application of the mistranslation inducing drug paromomycin on the *elp3* mutant could be expected to result in a synergistic increase of protein aggregation (enhanced basal mistranslation plus drug induced mistranslation), which was not observed.

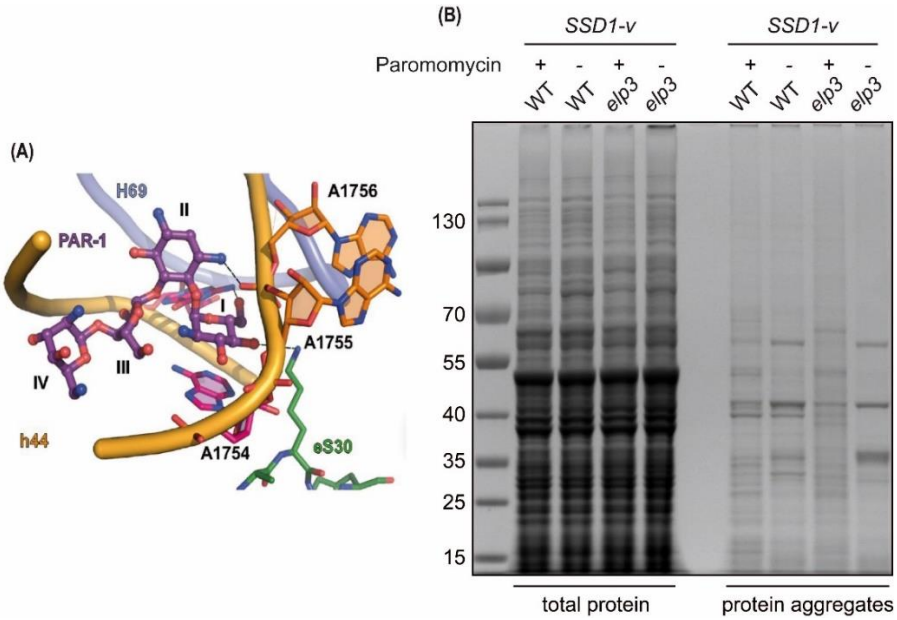

Figure 23. Paromomycin effect on the formation of the protein aggregates. (A) Binding of paromomycin (PAR-1) (Violet) to the rRNA (yellow) in the 80S ribosome from *Saccharomyces cerevisiae*. (the image is modified from (Prokhorova et al., 2017)). (B) Total protein and protein aggregates from *SSD1-v* WT and *elp3* mutant in the presence (+) and absence (-) of paromomycin. Samples were analyzed by Nu-PAGE and Coomassie staining.

5.4.4. Investigation of Rapid tRNA decay involvement in *deg1* and *pus1* phenotypes

Temperature sensitivity can be observed in various tRNA modification mutants. In some well-studied cases, this phenotype is caused by a temperature-dependent mechanism called rapid tRNA decay (RTD), which is responsible for destabilizing of specific tRNAs in higher temperatures, leading to the growth defect phenotype appearance (Alexandrov et al., 2006; Phizicky & Alfonzo, 2010). The degradation of tRNA by the RTD pathway involves Xrn1 and Rat1 exonucleases in the cytoplasm and nucleus, respectively. RTD can be inhibited in the absence of a sulfur assimilation gene called *MET22*. The accumulation of high concentrations of pAp (adenosine 3´,5´ bisphosphate) in *met22* as a sulfur assimilation byproduct inhibits both Xrn1 and Rat1, and thereby suppresses the growth defect of RTD related tRNA modification mutants (Phizicky & Hopper, 2010). Both *pus1* and *deg1* mutants are known to exhibit a clear temperature sensitive growth phenotype (Lecointe et al., 1998; ; Klassen & Schaffrath, 2018; Khonsari & Klassen, 2020), see Figure 24, and the latter mutant was found in this thesis to accumulate protein aggregates (Figure 22), a potential involvement of RTD in these phenotypes was then investigated. The *pus1* and *deg1* mutations were combined with a *met22* deletion to reveal the potential involvement of RTD in phenotypes associated with the tRNA modification genes. Growth phenotypes were compared between the WT and single mutants next to the generated double mutants. As shown in Figure 24, the thermosensitivity of the *pus1* mutant is moderately suppressed by *met22* mutation, which supports the assumption that some Pus1 modified tRNAs become destabilized by RTD in the absence of the modification. A similar phenotypic rescue was not observed for the *deg1* mutant in the same genetic background, which shows that pseudouridine modification at $U_{38/39}$ is linked to a *MET22*-independent temperature sensitivity phenotype (Figure 24).

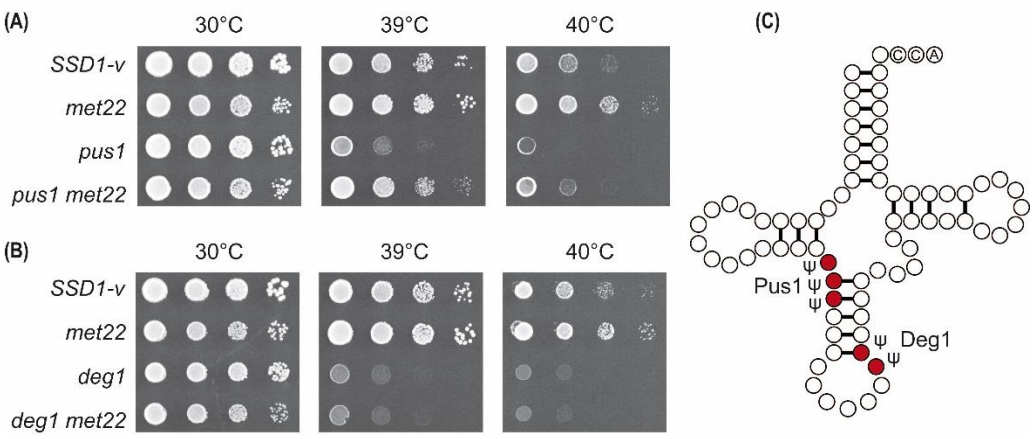

Figure 24. Thermosensitive growth phenotype of *pus1* and *deg1* in the absence of *MET22*. (A) Growth of wild-type (BY4741), *met22* and *pus1* single mutants, and *pus1 met22* double mutants at elevated temperatures. (B) Growth of wild-type (BY 4741), *met22* and *deg1* single mutants, and *deg1 met22* double mutants at elevated temperatures. All plates were incubated at the relevant temperature for 48 h. (C) Scheme indicating position and required genes for Ψ modifications in tRNA.

Since the *pus1* mutants exhibit a similar temperature sensitive growth phenotype as for the *deg1* strain, it was valuable to know, whether this too might be linked to protein aggregate induction. In addition, since the phenotype is suppressed by *met22* mutation, a potential rescue of protein aggregation was investigated. In general, a specific decay of individual tRNA by RTD seemed well suited to induce codon specific ribosome slowdown, which is considered to be one key trigger for protein aggregation of U_{34} tRNA modification mutants (Nedialkova & Leidel, 2015). Aggregates were extracted from WT, *pus1*, *met22*, and *pus1 met22* strains, and analyzed as before. As shown in Figure 25, there was a little difference between the WT and both single or the double mutant. Based on this result, the RTD-related temperature sensitivity of *pus1* mutants and the rescue by *met22* mutation does not imply a *MET22* dependent induction of protein aggregates in the *pus1* mutant. In future studies, well established cases of RTD could be analyzed for protein aggregate induction and suppression by *met22* mutation, to obtain more insight into the functional links between protein aggregation and codon specific ribosomal slowdown.

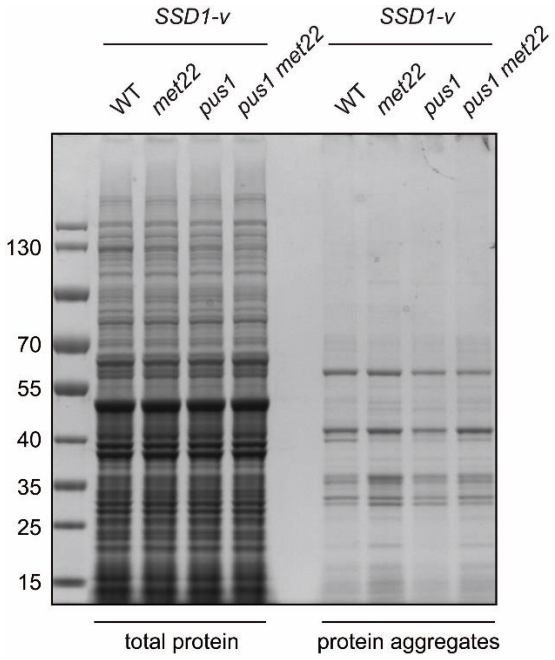

Figure 25. Protein aggregation in WT, *pus1*, *met22*, and *pus1 met22*. Total protein and aggregates were extracted from all mentioned strains in *SSD1-v* background, and samples were then analyzed by Nu-PAGE and Coomassie staining.

5.5. Role of tRNA modification and *SSD1* in the regulation of yeast life span

As mentioned, tRNA molecules play an important role in aging and may significantly influence the life span when modified. Here the influence of some of these modifications in different *SSD1* backgrounds is tested to see how each of these modifications influence chronological aging and if they are capable of rescuing it. In this chapter, the influence of negative interaction of ncm^5/mcm^5 modifications in the U_{34} position, with Ψ modification at positions 38 and 39 of the tRNA, is tested on the chronological aging of the cells. Furthermore, the influence of autophagy and ubiquitination on aging is as well studied compared to each other under the influence of the negative interaction of tRNA modifications. Finally, at the end of this chapter, the relation between protein aggregation and chronological aging is studied in a strain lacking chaperon 40's protein, Zuo1, which expectedly produces more protein aggregates. This experience gives better information about protein aggregation's influence on cells' aging and shows whether protein aggregation formation is harmful to the cells or not.

5.5.1. Establishing the chronological aging assay using a yeast *snf1* mutant

In the yeast *Saccharomyces cerevisiae*, Snf1 is the equivalent of AMPK in multicellular eukaryotes (Woods et al., 1996). Multiple cellular regulations are happening under the influence of downstream targets of Snf1 in yeast. Snf1 is also needed for entering the stationary phase of growth, and is known that the absence of Snf1 in yeast causes an accelerated aging (Wierman et al., 2017). To establish the aging assay for further experiments, WT and *snf1* mutant strains in the BY4741 background were used. The accelerated aging in *snf1* could already be observed between the first and second plating (Figure 26), see Materials and Methods 7.2, which can be related to the requirement of Snf1 activity throughout the conversion occurring in the diauxic shift for optimal chronological aging.

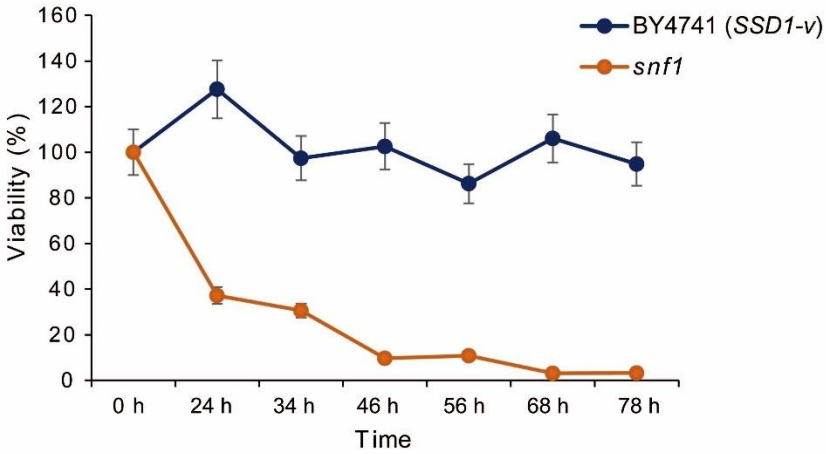

Figure 26. Comparison between chronological aging in WT and *snf1* mutant in BY4741 background. Both strains were grown in 2 ml of minimal media overnight and were inoculated the next day at 8:00 a.m. into 10 ml fresh minimal media with OD 600 of 0.1. Cultures were then incubated at 30°C on the shaker for 3 days; each culture was plated on two YPD media separately (once at 8:00 a.m. and once at 0:00 p.m.) and were incubated at 30°C for 48 h and the colony forming unit (CFU) was counted for each plate after the incubation time. For each sample 3, biological replicates were considered.

5.5.2. Genetic interaction between tRNA modification genes and its influences on aging

tRNAs can undergo modifications and might influence the life span by affecting the stability of tRNA, codon recognition, or aminoacylation. To see the influence of tRNA modification on chronological aging in the yeast *Saccharomyces cerevisiae*, the influence of the absence of Ψ modification at positions 38 and 39, and the lack of ncm^5/mcm^5 modifications in U_{34} position, was tested in the *SSD1-v* strain BY4741, in which the effect of simultaneous removal of both modifications can also be tested. As a result, both modifications were not influencing the chronological life span (CLS) in this background (Figure 27A). It should be noted, that the absence of Deg1 dependent pseudouridylation at positions 38 and 39 caused an enhanced accumulation of protein aggregates compared to WT (Figure 22).

As observed before, the combined absence of tRNA modification genes, such as *ELP3* and *DEG1*, or *URM1* and *DEG1*, influences the temperature sensitivity in a more severe way compared to the single mutant strains. To see the influence negative interaction of tRNA modification genes on aging, the WT strain's chronological life span was measured next to the chronological life span of *elp3* and *deg1* single mutants as well as an *elp3 deg1* generated double mutant in a BY4741 background. Interestingly, the *elp3 deg1* double mutant aged faster in comparison to the other three strains. The accelerated aging was already visible on day 5, with the drastic change of cell viability to less than 20%. Furthermore, this strain almost lost its viability at day 12, the time point in which the other strains showed viability between 40-50% (Figure 27A). This shows that the negative interaction between ncm^5/mcm^5 at U_{34} of the tRNA, and Ψ at $U_{38/39}$ (Figure 27B), does not only increase the temperature sensitivity but also accelerates chronological aging phenotype. It is also shown that in *SSD1-v* background, the *elp3 deg1* double mutant accumulates more aggregates compared to *elp3* and *deg1* single mutants (Klassen et al., 2016). However, *deg1* single mutant was also forming more aggregate proteins compared to the WT strain. Thus, the accelerated aging phenotype could be due to other genetical factors, and is not directly related to the formation of protein aggregates.

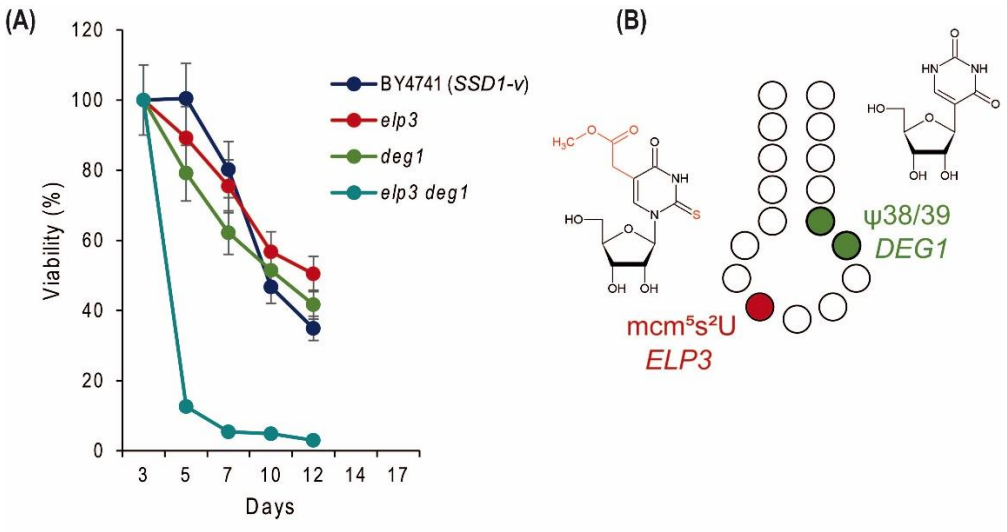

Figure 27. Chronological aging in a combined tRNA modification mutant lacking *ELP3* and *DEG1*. (A) The chronological aging assay was performed for WT, *elp3*, *deg1*, and *elp3 deg1* double mutant. All strains were inoculated at day 0 with OD600 of 0.1 and were incubated on a shaker at 30°C for 12 days. (B) the place of *ELP3* related ncm^5/mcm^5 modification, and *DEG1* related Ψ modification on a tRNA molecule.

5.5.3. Chronological aging in *SSD1-v* and *ssd1-d* backgrounds in comparison

Previously, a comparison between the chronological aging of *SSD1-v* and *ssd1-d* strains was performed, and *ssd1-d* was correlated with a shorter chronological lifespan compared to *SSD1-v* (Li et al., 2009). In this work, the CLS assay was as well performed in both BY4741 (*SSD1-v*) and W303 (*ssd1-d*) strains. This experiment confirmed that the *ssd1-d* strain ages faster than the *SSD1-v* strain (Figure 28A). The results presented in Figure 27 revealed that in the *SSD1-v* strain, absence of either *Deg1* or *Elp3* dependent modifications alone did not shorten CLS, in contrast to the combined absence of both, which drastically shortened CLS. Since this thesis revealed that other phenotypes of *deg1* mutants were aggravated in the *ssd1-d* strain background, the CLS of different tRNA modification mutants got determined in this background. For this approach, different single tRNA modification mutants, including two thiolation mutants (*uba4* and *ncs6*), lacking the thiol-group of the mcm^5s^2U modification, as well as *elp3* and *deg1* mutants, lacking ncm^5/mcm^5 and $\Psi_{38/39}$ modifications respectively, were generated in the *ssd1-d* background and the chronological aging assay was performed for this set of strains. Both thiolation mutants showed a similar aging period in comparison to the WT

ssd1-d, and the *elp3* single mutant as well showed no changes in chronological lifespan (Figure 28B), similar to the results obtained in the *SSD1-v* background strain (Figure 27). Interestingly, however, a *deg1* mutant in the *ssd1-d* background showed accelerated aging, which was not observed in the *SSD1-v* strain (Figure 27). Hence, in contrast to the *SSD1-v* strain background, the *DEG1* loss of function appears to accelerate aging in the background of an *ssd1-d* strain.

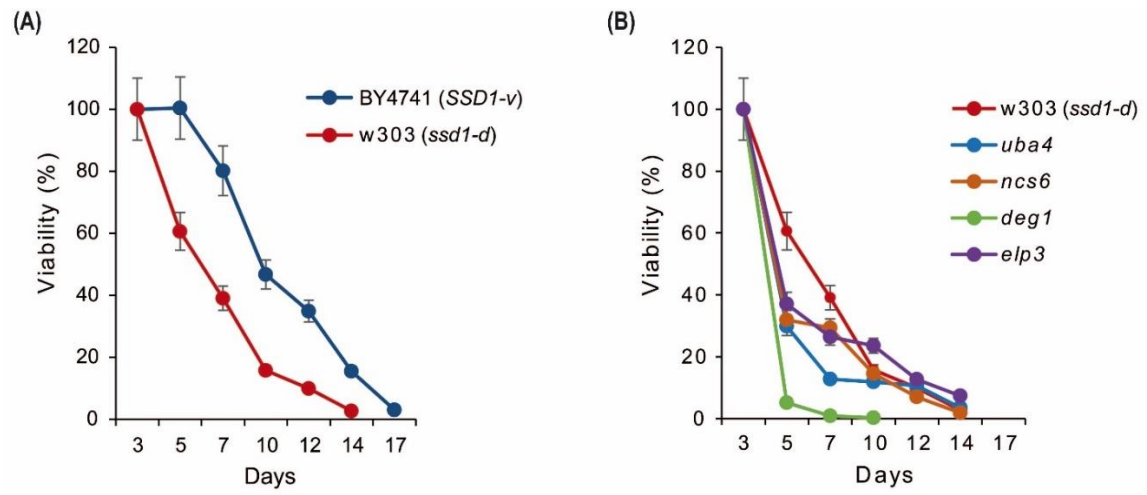

Figure 28. Chronological aging in both *SSD1* background strains. (A) a chronological aging assay was performed for both WT strains in a period of 17 days. *ssd1-d* strain's viability reaches 0% on day 14, while *SSD1-v* reaches this level on day 17. (B) Chronological aging assay was performed for WT and variable tRNA modification mutants of *ssd1-d* strain. *uba4* and *ncs6* represent thiolation mutants, *elp3* represents a strain lacking ncm^5/mcm^5 modification, and *deg1* mutant lacks the pseudouridine modification at positions 38 and 39 of the tRNA. All mentioned modification mutants are generated in the *ssd1-d* background, and they all showed an almost similar aging behavior compared to WT except for the *deg1* mutant.

To see how thiolation mutants influence chronological life span in different *SSD1* backgrounds, the chronological aging assay was performed for the *SSD1-v* wild type, *uba4* and *ncs2* mutants, both of which are lacking the thiol-group of the mcm^5s^2U modification. The mentioned mutants presented a similar aging pattern to *SSD1-v* wild type strain (Figure 29). Based on the chronological life span of thiolation mutants in both *SSD1* backgrounds it can be assumed that these specific thiolations do not significantly affect the chronological aging in *Saccharomyces cerevisiae*.

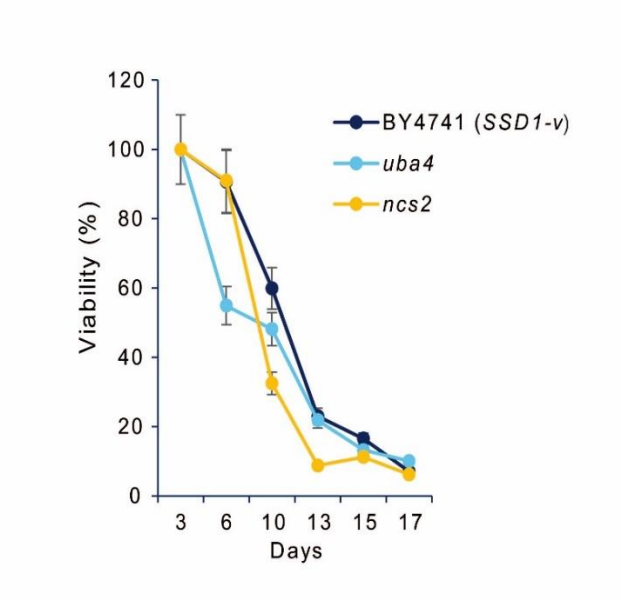

Figure 29. Chronological aging of thiolation mutants in *SSD1-v* background strain. The chronological aging assay was performed for WT and two thiolation mutants in *SSD1-v*. *uba4* and *ncs2* represent thiolation mutants, and the thiol-group of the mcm^5s^2U modification is absent in both strains.

5.5.4. Chronological aging in *deg1* mutants in different *SSD1* background strains

Since both *ssd1-d* and *deg1* mutations can enhance the protein aggregation, and because this effect might be linked to the long-term survival in the stationary phase, the effects of *ssd1-d* and *deg1* individually or combined were analyzed on chronological aging. A chronological aging assay was performed for both *SSD1* strain backgrounds in the presence and absence of the *deg1* mutation over a time range of 17 days in the stationary growth phase (Figure 30A). As anticipated, the *ssd1-d* strain presents a faster loss of viability compared to the *SSD1-v* strain. On day 7 of cell growth in the stationary phase, the viability rate was less than 50% in the *ssd1-d* cultures. Meanwhile, *SSD1-v* cultures needed 10-12 days to reach the same growth level. The *deg1* mutant in the *SSD1-v* background shows a similar viability pattern to the *SSD1-v* wild type, and the *deg1* mutant in the *ssd1-d* strain presents a severely accelerated aging phenotype compared to *ssd1-d* wild type strain (Figure 30A). Thus, as opposed to the *SSD1-v* strain, the absence of functional *DEG1* gene seems to affect the aging in the *ssd1-d* background strain.

To analyze if the differential effects of *deg1* mutation in *ssd1-d* and *SSD1-v* strains were specifically due to the difference in the allelic forms of *SSD1*, *SSD1-v* plasmids were introduced in *ssd1-d* wild type and *ssd1-d deg1* mutant strains, and their chronologic aging was measured. As indicated in Figure 30B, the introduction of the *SSD1-v* plasmid [*SSD1-v*] ameliorated the viability in the stationary phase over time, consistent with the established role of *SSD1* in the survival during the stationary phase. Anyhow, *ssd1-d deg1* [*SSD1-v*] still showed accelerated aging over time in comparison to *ssd1-d* [*SSD1-v*] wild type control. Thus, the result shows that *DEG1* represents a gene relevant to aging in the *ssd1-d* background, but not in the *SSD1-v* strain and this is likely because of other differences between these two strains, and not due to their none similar *SSD1* locus (Khonsari et al., 2021).

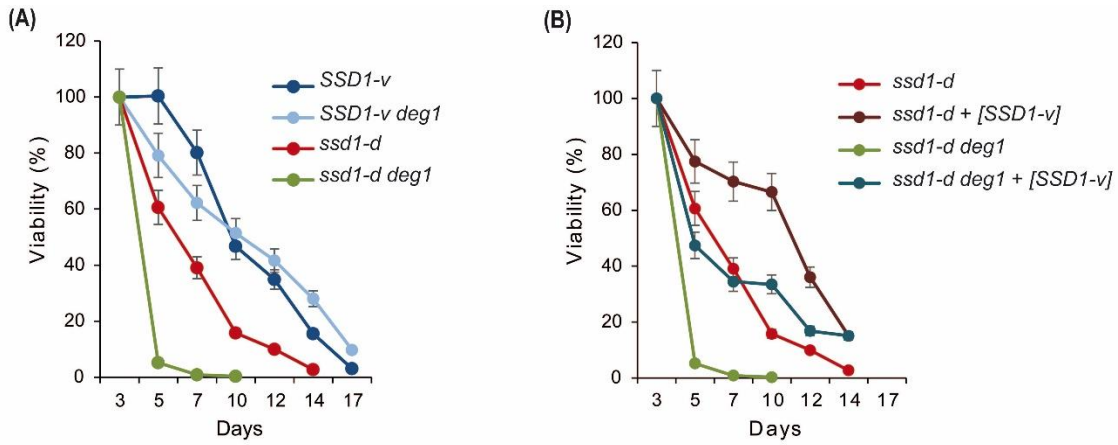

Figure 30. The role of *SSD1* and *DEG1* on chronological aging. (A) Chronological aging was tested for the *SSD1-v* wild type, and the *deg1* mutant compared to *ssd1-d* and *ssd1-d deg1* strains over a 17 days' time range. Viability (%) indicates the determined colony forming units (CFU) per ml normalized to the respective value at day 0. The average of three independent cultures together with the standard deflection is given. (B) As in (A) but with shown strains containing or lacking the plasmid-based *SSD1-v* [*SSD1-v*].

5.5.5. Role of autophagy and the proteasome in shortened CLS of a tRNA modification mutant

During the aging process, the proteome loses its quality, and it is not as functional as it is in the earlier phase of life (E. T. Powers et al., 2009). Damaged proteins and protein aggregates will be degraded either by autophagy or the ubiquitination proteasome system (UPS) (Finley, 2009; Wong & Cuervo, 2010). To see the influence of these two protein degradation mechanisms on chronological aging in

the yeast *Saccharomyces cerevisiae* lacking ncm^5/mcm^5 and $\Psi_{38/39}$ modifications, the ATG1, and *UBR2* genes were deleted in a fast dying double mutant *elp3 deg1*. Atg1 is a serine/threonine kinase required in autophagy, and Ubr2 is the cytoplasmic ubiquitin-protein ligase (E3) (De Groot et al., 2001; Díaz-Troya et al., 2008). The chronological aging of *elp3 deg1 atg1* and *elp3 deg1 ubr2* triple mutants was tested next to the *elp3 deg1* double mutant and the WT as a control. All mutant strains showed an accelerated aging as expected (Figure 31A). With a closer look at the viability of these strains at day 5, *elp3 deg1 atg1* showed the lowest viability in comparison to the other two mutants, and the *ubr2* deletion was slightly rescuing the accelerated aging in *elp3 deg1* double mutant (Figure 31B). This shows that the ubiquitination form of protein degradation is beneficial for the cells lacking both ncm^5/mcm^5 and $\Psi_{38/39}$ modifications. It is known that autophagy is important for the cells' survival in the stationary phase of growth and a faster death for the mutant strain is expected (Ruckenstuhl et al., 2014). The deletion of Ubr2 in the *elp3 deg1* double mutant causing a slight rescue could be due to the higher activity of the proteasomes in this strain as a result of absence of Rpn4, the regulatory protein for the proteasome gene expression.

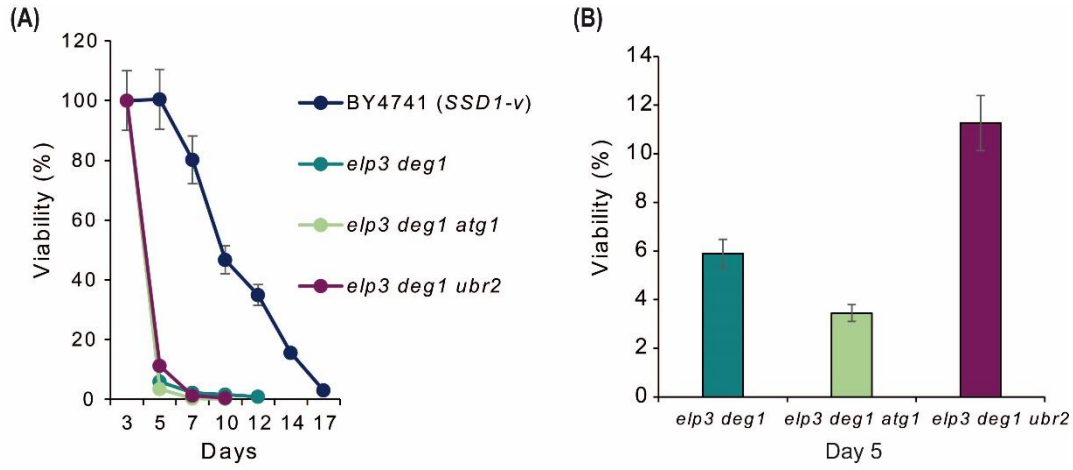

Figure 31. Comparison of different protein degradation mechanisms in the presence of ncm^5/mcm^5 and $\Psi_{38/39}$ modifications. (A) The chronological aging assay was performed for WT, *elp3 deg1*, *elp3deg1 atg1* and *elp3 deg1 ubr2* mutants. (B) Comparison of the viability of *elp3 deg1*, *elp3 deg1 atg1* and *elp3 deg1 ubr2* at day 5.

5.5.6. Lack of *ZUO1* induces protein aggregation without affecting chronological lifespan

Protein homeostasis needs to be ideally and constantly controlled not to result in protein aggregates formation. Cellular protein degradation mechanisms and molecular chaperons play a central role in this controlling system (Hartl et al., 2011; Preissler & Deuerling, 2012). In eukaryotic cells, a group of molecular chaperones responsible for cytosolic folding of the proteins includes two chaperone systems, binding close to the ribosome's exit tunnel, the exit site of the nascent peptide chain during translation. Hsp70 and Hsp40 are members of this system, and Zuo1 (Zuotin) belongs to the Hsp40 protein family (Weyer et al., 2017). The absence of functional Zuo1 or other components of the ribosome associated complex (RAC) involved in folding of the nascent polypeptide induces protein aggregation in yeast (Koplin et al., 2010; Bruch et al., 2020) and composition of protein aggregates in mutants lacking U_{34} modification or a functional RAC were found to be highly similar (Nedialkova & Leidel, 2015). However, it remained unknown, whether protein aggregates observed in tRNA modification mutants are functionally involved in shortened life spans. To test this, it was analyzed whether protein aggregates induced by dysfunctional RAC also qualify as a life span reducing condition. Thus, the chronological aging assay was performed for a *zuo1* mutant next to the WT. It was observed that the *zuo1* mutation did not cause any changes in the process of chronological aging (Figure 32A). To see the influence of the absence of Zuo1 compared to the absence of a tRNA modification mutant, the total protein and protein aggregates of WT, *zuo1* and *elp3* mutants were then extracted. As expected, the *zuo1* single mutant accumulated significantly more protein aggregates than the wild type. The *elp3* single mutant showed only modest induction of protein aggregates, which is consistent with a previous study (Nedialkova & Leidel, 2015) and this work (Figure 23 and 32B). Due to the wild type aging phenotype, despite the presence of protein aggregation in *zuo1* mutants, it can be assumed that increasing protein aggregation does not always result in shortened chronological life span. Thus, some other genetic factors must influence the normal aging of *zuo1* mutant regardless of the higher amount of protein aggregation formation in this strain compared to the WT.

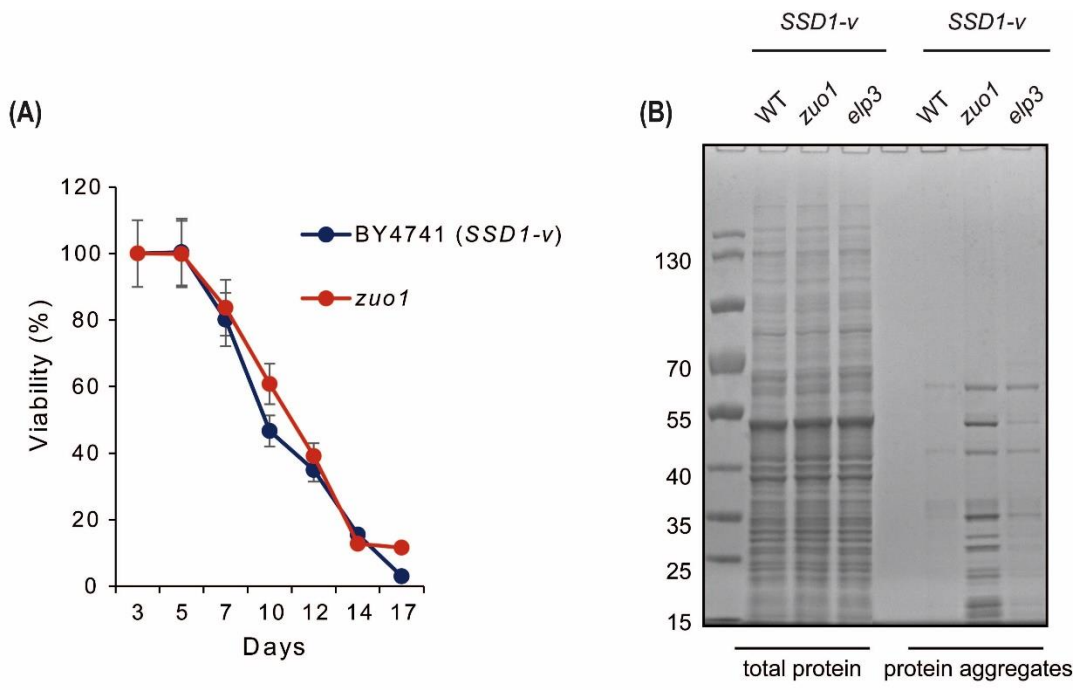

Figure 32. The relation between aging and protein aggregation formation. (A) A chronological aging assay was performed for a BY4741 WT and the *zuo1* mutant, generated in the same background. (B) Total protein and protein aggregates were extracted from the WT, *zuo1* and *elp3* mutants. All strains belong to the BY4741 (*SSD1-v*) background. Samples were then analyzed by Nu-PAGE and Coomassie staining.

6. Discussion

6.1. Role of Ssd1 in growth phenotypes of tRNA modification mutants

The RNA binding Ssd1 protein plays an essential role in cell wall remodeling in the yeast *Saccharomyces cerevisiae*. Ssd1 is an RNA binding protein, and it binds to the cell wall protein encoding mRNAs, and delivers them to the cells' growth sites (Hogan et al., 2008; Jansen et al., 2009; Kurischko et al., 2011). A recent study tested the impact of different alleles of *SSD1* on an *elp3* modification mutant, lacking the ncm^5/mcm^5 modifications in the U_{34} position. As a result, it was observed that the *elp3* temperature sensitivity (*ts*) phenotype was enhanced in the W303-1B strain containing the *ssd1-d* allele (Xu et al., 2020). Earlier in this work, it was explained that the maintenance of the translational capacity of $tRNA^{Lys}UUU$ and $tRNA^{Gln}UUG$ requires a functional Elongator complex, and a functional $tRNA^{Gln}UUG$ additionally requires the Deg1 protein; consistently, the strong negative genetic interaction between *ELP3* and *DEG1* genes was shown to be due to the additive functional impairment of $tRNA^{Gln}UUG$ (Han et al., 2015; Klassen et al., 2016; Klassen & Schaffrath, 2017; Bruch et al., 2020). This negative genetic interaction was mainly studied in the *SSD1-v* yeast background. In this thesis, the interaction between genes responsible for modifications in the anticodon stem-loop of the tRNA is studied in both *SSD1* backgrounds to better understand the correlation between tRNA modification and *SSD1* locus.

In this research, both *elp3* and *deg1* mutants showed a more pronounced phenotype at aggravated temperatures in the *ssd1-d* background. These mutants are both more temperature-sensitive in the W303-1B background than in BY4741 (Figure 10A), and in addition, they also show a more severe growth reduction in the presence of the TORC1 inhibitor drug, rapamycin (Figure 11A). Furthermore, the genetic interaction between *ELP3* and *DEG1*, and *ELP3* and *URM1*, demonstrated a synthetically sick phenotype in the *SSD1-v* background (Klassen et al., 2016). These negative genetic interactions are shown in this study to create synthetically lethal strains in the *ssd1-d* background (Figure 12). Multiple experiments were performed to test whether this observation is due to the *SSD1* locus variation and or involves other genetic differences. First, the *SSD1-v* allele [*SSD1-v*] was ectopically expressed in the *ssd1-d* strains (Figure 10B). If the phenotypic difference between the two strains used is exclusively due to loss of

Ssd1 function in the *ssd1-d* strain background, phenotypes are expected to be suppressed to the levels observed in the *SSD1-v* strain. The results showed a *ts* rescue for the *ssd1-d* strains upon introduction of *SSD1-v*, which is evidence of the effect of *SSD1* locus on this phenotype. Then, the *SSD1* gene was deleted, and the influence on the *ts* phenotype was tested. If the aggravated phenotypes in the *ssd1-d* strain are due to loss of function of mutation in *SSD1*, a complete deletion of this gene in the *SSD1-v* background should mimic the phenotype observed in *ssd1-d*. As a result, the complete loss of *SSD1* led to a growth phenotype comparable to the strain carrying *ssd1-d* (Figure 34). However, the observed phenotype for *ssd1* strain lacking additional tRNA modification defects is in part similar to the phenotype of *elp3* and *deg1* mutants, revealing that *ssd1* mutation alone induces phenotypes overlapping with the tRNA modification defects (Figure 10A, Figure 34). This suggests that related cellular effects of these mutations cause the *ts* phenotype of *elp3* and *deg1* and *ssd1* mutants, and synthetic phenotypes of *ssd1-d elp3* and *deg1* double mutants are not necessarily due to a tRNA-modification-specific effect of *ssd1*. In previous work, the phenotypic variation of *elp3* mutants by the different *SSD1* alleles was interpreted as a specific modulation of the tRNA modification defect induced phenotype. It should be noted, however, that the *ts* phenotype of *ssd1* is clearly apparent only at 39°C and the previous study tested *ts* phenotypes only up to 37°C (Xu et al., 2019). Hence, the *ts* phenotype of *ssd1* single mutants was not explicitly noticed.

To test if the synthetic lethality in *elp3 deg1* and *urm1 deg1* double mutants can be rescued by the overexpression of $tRNA^{Gln}UUG$ or expression of *SSD1-v*, the plasmid shuffling method was repeated once with the overexpressed $tRNA^{Gln}UUG$ and an empty vector, and once with the expressed *SSD1-v* and its suitable empty vector. The overexpression of $tRNA^{Gln}UUG$ could rescue the lethality in *urm1 deg1* and *elp3 deg1* double mutants following prolonged incubation of 5-FOA plates. The latter is a *URA3* counter selective medium used for removal of the plasmid transiently providing *ELP3* and *URM1* genes during the plasmid shuffling process. This delayed rescue of lethality in these strains compared to the WT or the respective single mutant strains shows that $tRNA^{Gln}UUG$ overexpression provides a rescue of the synthetic lethal genetic interaction between these genes in the *ssd1-d* background but growth of the resulting double mutant strain, is severely delayed. The expression of *SSD1-v* could also rescue the *urm1 deg1* lethality following prolonged incubation (Figure 35A) but could not

rescue the *elp3 deg1* double mutant's lethality even after a long-time incubation (Figure 35B). Hence, *SSD1-v* expression can rescue the lethality of *urm1 deg1*, while it cannot rescue the *elp3 deg1* (Figure 35). The reason behind the suppression of *urm1 deg1* double mutant lethality after overexpression of the $tRNA^{Gln}UUG$, and the inability to rescue the *elp3 deg1* mutant in the same conditions, respectively, might be related to the fact that also *urm1 deg1 SSD1-v* shows a weaker negative phenotype in comparison to the *elp3 deg1* double mutant. This could be attributed to a more severe translational defect of $tRNA^{Gln}UUG$ lacking $\Psi_{38/39}$ in combination with the mcm^5 part of mcm^5s^2U compared to a situation where $\Psi_{38/39}$ and the s^2U part of mcm^5s^2U are missing (Klassen et al., 2016). Elevated levels of the hypomodified tRNA are thought to partially compensate the translational inefficiency in the double mutants. However, it is possible that this compensation requires a certain degree of functionality of the hypomodified tRNA. If the functional defect is too severe, as is likely the case for *elp3 deg1* double mutant, the compensation by elevated cellular tRNA abundance might not be adequate to enable sufficient translational activity. In support, overexpression of $tRNA^{Gln}UUG$ provided a much smaller phenotypic suppression in the viable *SSD1-v elp3 deg1* strain compared to the *SSD1-v urm1 deg1* mutant (Klassen et al., 2016). Expression of *SSD1-v* provides a small rescue of *ssd1-d urm1 deg1* double mutant but no rescue of the *ssd1-d elp3 deg1* strain. This indicates that the lethality of the *elp3 deg1* double mutant in the *ssd1-d* strain is caused by other genetic differences between the utilized strains.

Phenotypic similarity of *elp3*, *deg1* and *ssd1* mutants can also be explained by the comparable effects of these mutations on the cell wall integrity. Both *ssd1* and *elp3* strains present aggravated sensitivity to Calcofluor-white (CFW), a fluorescent blue dye and a cell wall stressor (Frohloff et al., 2001). It was also shown that *elp3* mutant phenotypes can be suppressed moderately by upregulating the cell wall integrity (CWI) pathway genetically (Xu et al., 2019) or by stabilizing cells osmotically, consistent with parts of the growth defects being caused by cell wall damage (Frohloff et al., 2001). It was observed that the *deg1* mutant, similar to an *elp3* strain in the *ssd1-d* background presents a suppressed growth phenotype following osmotic stabilization, pointing to possible similar effects of *elp3* and *deg1* mutations on cell wall integrity (Figure 36). If *ssd1*, *elp3* and *deg1* mutation each individually induced cell wall defects with the help of different mechanisms, these could be increased in the respective double mutants with a single tRNA modification defect and the absence of the *SSD1* gene or the

presence of *ssd1-d* allele. To emphasize this idea, a spot assay on CFW plates was performed, and similar to *elp3* strain, the *deg1* mutant also showed an increased in CFW sensitivity. An *ssd1 deg1* double mutant showed additivity, not only rapamycin and temperature sensitive phenotypes but also CFW sensitivity (Figure 34). While the molecular background for rapamycin sensitivity of different tRNA modification mutants is not fully investigated, it is notable that modification mutants involving *elp3* and *deg1* mutations present signs of lower TORC1 activity (Bruch et al., 2020; Scheidt et al., 2014). Since TORC1 also plays a role in the maintenance of cell wall integrity (Reinke et al., 2004; Torres et al., 2002), it is possible that chronic cell wall integrity defects occurs in the tRNA modification mutants due to lowered TORC1 activity. These defects could then be further aggravated due to the loss of Ssd1 and could cause the detected set of pleiotropic phenotypes in presence of *ssd1-d* allele.

To explore whether *ssd1* mutation generally enhances temperature sensitivity caused by mutation of distinct tRNA modification genes, additional examples in which the loss of a tRNA modification is linked to a known *ts* phenotype were tested. The *pus1, trm1* and *ncl1* mutants in both *ssd1-d* and *SSD1-v* strain backgrounds were selected. Unexpectedly, these mutants do not generally exhibit enhanced phenotypes in the *ssd1-d* W303-1B strain as compared to *SSD1-v* BY4741. Contrariwise, even less temperature sensitivity effects were observed for *pus1, trm1* and *ncl1* in W303-1B. To check whether other phenotypes associated with these genes are similarly weakened, the responses of *trm1* and *pus1* strains to the anticancer drug 5-FU were analyzed. As expected based on previously published results (Gustavsson & Ronne, 2008), both *pus1* and *trm1* exhibit strongly increased sensitivity against a combination of 5-FU and mild heat stress and this phenotype was observed in *ssd1-d* and *SSD1-v* strain backgrounds. However, the relative strength of this effect is weakened rather than enhanced in W303-1B *pus1* and *trm1* mutants compared to the similar strains in BY4741 (Figure 16). Hence, phenotypic enhancement by the truncated version of *SSD1* seems to be refined to two specific modification defects (*elp3* and *deg1*), inducing a pleiotropic set of phenotypes overlapping with *ssd1*. An important fact is the CFW sensitivity that is shared between *ssd1*, *elp3* and *deg1* but not for instance with other pseudouridine synthase defects (Figure 34B). CFW sensitivity is common to mutants with defects in cell wall maintenance (Wojda et al., 2007; Miles et al., 2019). Since this trait is shared among the tRNA modification mutants

deg1, elp3 and the RNA binding protein defective *ssd1* mutant, the negative genetic interaction between these genes might result from additive cell wall damage. The *ts* phenotype in some other pseudouridylation modification mutants like *pus1* mutant can be related to some other factors like RTD, as was observed in an experiment in *SSD1-v* background (Figure 24). Thus, the phenotypic variation of tRNA modification defects by *ssd1* could be limited to the tRNA modifications that play a role in cell wall integrity and might therefore show additional defects of the cell wall. While direct cell wall damage has not been proven for tRNA modification mutants, reduced secretion of a cell wall protein was described for Elongator mutants (Abdullah & Cullen, 2009). It is possible that certain, but not all tRNA modification defects result in a disproportionately high expression defect of protein that need to be co-translationally inserted into the ER-secretory pathway. This would include many cell wall proteins and could in turn explain cell wall integrity loss and associated phenotypes.

6.2. Influence of Ssd1 and Deg1 on the expression of the Gln-rich prion protein Rnq1

The $tRNA^{Gln}UUG$ decoding efficiency is highly dependent on the $\Psi_{38/39}$ (Borchardt et al., 2020; Han et al., 2015; Sokołowski et al., 2018). Due to this finding, the differences between *ssd1-d deg1* mutant phenotypes and *SSD1-v deg1* strain could be related to a further elevated decoding defect of $tRNA^{Gln}UUG$ in the *ssd1-d* background. To analyze this option, expression efficiencies of Rnq1 in the different strains used in this study was tested. Rnq1 is a Gln-rich protein, and its mRNA translation efficiency was shown to be reduced when $\Psi_{38/39}$ was not present (Klassen et al., 2016). While the temperature sensitivity phenotype of *deg1* mutants was observed to be enhanced in the presence of the *ssd1-d* allele (Figure 10), the expression level of an Rnq1-GFP protein was not more severe in the *ssd1-d* strain compared to *SSD1-v* (Figure 17). On the opposite, in *SSD1-v*, *deg1* mutation reduced Rnq1-GFP levels, but this effect was not observed in the *ssd1-d* strain background. Therefore, *ssd1-d* does not explicitly enhance the effect of modification loss at the level of translation and rather potentially suppresses it. At this stage, it remains unknown why the relative reduction of Rnq1-GFP signal strength is actually decreased in the *ssd1-d deg1* strain. Of note, all yeast strains used thus far are carriers of the [PIN^+] prion, which implies that Rnq1 adopts an amyloid

fold and is in the aggregated prion conformation. An impact of the strain background on the amyloid aggregate formation potential of the protein might contribute to the observed differences between *deg1* mutants. It appeared possible that total protein extracts which are usually prepared involving a centrifugation step to remove cell debris might underrepresent the large prion aggregates known to form in [*PIN*$^+$] strains (see Materials and Methods 7.14 and 7.18). If there are strain specific changes in the aggregate sizes of Rnq1 expressed in *deg1* mutants, determination of Rnq1 levels using a standard protocol mainly detecting soluble Rnq1 could lead to biased results.

To explore this possibility, total protein preparations were subjected to a protocol used to enrich protein aggregates. Aggregated and soluble protein fractions were obtained from WT and *deg1* mutants of both background strains, and western analysis was performed to detect the abundances of Rnq1-GFP in each of these different fractions. The soluble total protein fraction showed the same result as before. However, in the protein aggregate fractions, both *ssd1-d deg1* and *SSD1-v deg1* showed drastically lower levels of Rnq1-GFP as compared to the same fraction from the respective wild type control (Figure 18A). Thus, if solely the aggregate fraction is taken into consideration, *deg1* mutation has comparable effects on the levels of Rnq1-GFP in both strain backgrounds. Since prion proteins can from aggregates of widely differing sizes, the analysis of soluble fractions might therefore indeed provide biased results when strains differ in the sizes of protein aggregates. It is as well possible that smaller aggregates remain in the soluble fraction, depending on the centrifugal forces applied to separate cell debris and aggregates from the total protein preparations. A difference between the aggregate sizes was in fact observed using fluorescence microscopy of the WT and the *deg1* mutant in both background strains (Figure 18B).

To further investigate the effect of the *deg1* mutation on the expression of Rnq1 without such bias, the prion was entirely removed and differences of Rnq1-GFP level in the fully soluble state were compared. The [*PIN*$^+$] prion can be eliminated resulting in [*pin*$^-$] strains by repeated exposure of cells to low amounts of GdnHCl (protein denaturant) or by deleting the gene encoding the Hsp104 chaperone. In this study, strains were passaged twice on medium containing 3mM of GdnHCl. The efficiency of prion curing was assayed using fluorescence microscopy, which revealed abundant foci formation of Rnq1-GFP in all parental [*PIN*$^+$] strains but

changed to homogeneous signal distribution after the curing procedure. When Rnq1-GFP levels were detected in the [*PIN*$^+$] strains, the total protein extract (soluble fraction) showed the same result before GdnHCl treatment as was expected and observed. However, after the treatment, a considerable difference was observed in the *ssd1-d* background (Figure 19C). In *ssd1-d* [*pin*$^-$], *deg1* mutation resulted in a drastic loss of Rnq1-GFP fusion protein, which is in strong contrast to the results obtained for [*PIN*$^+$]. Since Rnq1-GFP does not form aggregates in [*pin*$^-$], different aggregate sizes might indeed have biased the results obtained in the previous [*PIN*$^+$] strain and a translational defect of Rnq1-GFP mRNA is possibly present in both, *ssd1-d* and *SSD1-v* strains lacking *DEG1*. Moreover, now the relative signal loss upon *deg1* deletion appears heavily increased in the *ssd1-d* strain as compared to the *SSD1-v* strain. Thus, *ssd1-d* might aggravate the translational defect caused by *deg1* mutation. It should be noted, however, that changes in mRNA levels in the different strains cannot be ruled out at present and other genetic differences than the *SSD1* locus might have also affected the Rnq1-GFP levels. In addition, it was observed that all [*pin*$^-$], but none of the [*PIN*$^+$] strains accumulated significant amounts of Rnq1-GFP degradation products. It is likely, that the amyloid aggregate state of Rnq1-GFP prevented degradation of the fusion protein, which was in turn enabled upon curing of the prion. Therefore, not only differences in translational efficiency but also changes in Rnq1-GFP stability and turnover might have contributed to the observed differences in Rnq1-GFP levels in the different strains. However, based on the considerable difference of the level of Rnq1-GFP in WT and *deg1* mutant in the *ssd1-d* background, it is likely that *ssd1-d* can specifically aggravate the influence of the lack of modification at the translation level.

6.3. Influence of *ssd1* mutation on autophagy induction in the absence of *DEG1*

Autophagy is a recycling and degradative process in the cell, and it can be induced due to starvation, organelle damage, DNA damage, hypoxia, and pathogen infection (Paglin et al., 2001; Rich et al., 2003; Papandreou et al., 2008). Autophagy induction is usually beneficial for cells and can reduce the speed of aging (Ruckenstuhl et al., 2014). However, on the other hand, too much autophagy is also harmful to the cells, as shown in a study on Parkinson’s disease (Spillantini et al., 1998). In the yeast *Saccharomyces cerevisiae,* cells undergo autophagy along with an activation of starvation transcriptional programs during the stationary phase and due to the low

amount of nutrients (Thevelein et al., 2000; Smets et al., 2010). During this process, the translation rate is also reduced to preserve energy and limiting cellular resources. Based on a recent study, the moderate difference between mRNA levels in the double mutant's stages of growth shows the importance of the combined presence of tRNA modifications, in the case of autophagy, as a result of starvation in early stages of life (Bruch et al., 2020).

Here the effect of Deg1 dependent pseudouridine modification, on the autophagy induction, under the influence of the *SSD1* locus is studied. The GFP-Atg8 fusion protein as a widely used autophagy reporter (Cheong & Klionsky, 2008) was employed for this purpose. When autophagy is activated, this reporter is processed and free GFP released, which is detectable via western blot analysis. As a control, rapamycin is used to inhibit TOR and activate autophagy. The GFP-Atg8 fusion reporter was expressed in the two wild type strain used in this study and the corresponding *deg1* single mutants. Protein extracts were obtained from exponentially growing cells and analyzed by Western blot. Both *deg1* mutants displayed increased GFP-Atg8 processing and the *deg1* mutant in the *ssd1-d* background accumulated more free GFP than the *deg1* mutant in the *SSD1-v* background (Figure 20). This indicates that autophagy is already activated in exponential growth phase when *DEG1* is missing and this effect is elevated in the *ssd1-d* background as compared to the *SSD1- v* strain. It is known that TORC1 negatively regulates autophagy (Yorimitsu et al., 2007), and it seems possible that the absence of pseudouridine modification at positions 38 and 39 of the tRNA results in an inactivation of TORC1 and, as a result activates the autophagy. This autophagy induction occurs in the log phase of growth of *deg1* mutants, when nutrients are still abundantly present. Therefore, the activation of autophagy, possibly connected to inactivation of TORC1 occurs prematurely and might represent a mis-sensing of starvation. Of note, combined tRNA modification mutants involving the *deg1* mutation were recently shown to activate distinct transcriptional starvation response in addition to inappropriate autophagy (Bruch et al., 2020). The results obtained in this thesis demonstrate that lack of *DEG1* is already sufficient to induce autophagy.

To further explore whether inappropriate autophagy activation in the *deg1* mutant occurs in the conventional TORC1-controlled manner, the requirement of Atg1, a subunit of a kinase complex that is directly targeted by TORC1 for autophagy activation in the tRNA modification mutant was tested.

In addition, autophagy induction was tested in the presence and absence of the rapamycin which inhibits TORC1. As expected, the deletion of *ATG1* disabled autophagy, and even in the presence of the autophagy inducing drug there was no sign of GFP-Atg8 processing and therefore autophagy induction was prevented (Figure 21). The *deg1* mutant, as was observed before, showed autophagy even without the presence of rapamycin. In the double mutant *deg1 atg1*, the autophagy induction was entirely lost, as was observed in *deg1* single mutant. This means that the improper autophagy in the *deg1* mutant indeed depends on the Atg1 kinase as does autophagy triggered by TORC1 inhibition. The Atg1 kinase is under the control of TORC1. Hence, the loss of pseudouridine modification at positions 38 and 39 of the tRNA activates autophagy likely as a TOR controlled starvation response. The mechanism, how tRNA modification defects could trigger autophagy and TORC1 inhibition remains elusive at present. One option for such process could involve an accumulation of non-aminoacylated tRNA in response to the tRNA modification defect. It was demonstrated that reduced tRNA charging can indeed directly reduce TORC1 activity (Kamada, 2017), and an accumulation of non-charged tRNA could represent a suitable starvation measure that is monitored by TORC1. It should be noted that so far, no accumulation of non-aminoacylated tRNA in *deg1* mutants was described. In general, however, tRNA modification loss can affect the efficiency of recognition between amino-acyl-tRNA synthetase and its substrate.

6.4. Effect of Ssd1 on protein aggregation in tRNA modification mutants

Protein aggregation appears due to misfolding or improper folding of the protein. Different models are suggested for the formation of protein aggregates in the absence of specific tRNA modifications. Due to (Nedialkova & Leidel, 2015), in the absence of wobble uridine modifications, codon translational rates are non-optimal, and a codon-specific ribosome slow down during the process of translation was detected. Such reduction in the speed of translation is assumed to be responsible for misfolding the nascent protein and subsequent aggregate formation. However, during this study, the possibility of another tRNA related reason for the formation of the protein aggregates is as well tested. It seems that the acceptance of wrong tRNA to the ribosomal A-site is as well leading to the formation of the protein chain containing wrong amino acids, and is followed up by formation of the protein aggregates (Figure 23). In some recent studies, disturbed protein homeostasis has been observed due to the absence of

$mcm^5s^2U_{34}$ and/or $\Psi_{38/39}$ modifications in *SSD1-v* background (Klassen et al., 2016, 2017; Bruch et al., 2020). Based on the observed temperature sensitivity of the WT and *deg1* mutant in both *SSD1* backgrounds (Figure 1A), the possible relation between this phenotype and protein aggregation was tested. Since Ssd1 also has a documented role in protein disaggregation by influencing the ability of heat shock protein Hsp104 to bind protein aggregates (Mir et al., 2009), the phenotypic variation of *deg1* and *elp3* could be at least partly mediated by effects on protein aggregation. Indeed, protein homeostasis defects of an *elp3 ncs2* double mutant (Xu et al., 2019, 2020) and a *deg1* single mutant (Figure 22) were enhanced in the presence of the *ssd1-d* allele. It is not relevant to conclude that the presence of the truncated version of *SSD1* only by itself is increasing the amount of the observed cellular protein aggregates (Figure 22A). Hence, Ssd1 could prevent protein aggregation in a mechanistically different way compared to the tRNA modifications, which can be explained by the described effect on Hsp104 function (Mir et al., 2009; Parsell et al., 1994). This independent effect on protein homeostasis could explain the additive phenotypes of *ssd1-d* overlapping with *elp3* or *deg1* mutations. On the other hand, *ssd1-d* can increase the tendency of *deg1* mutant for protein aggregates induction, and this effect might be related to the higher temperature sensitivity of the *deg1* mutant. The suppression of both temperature sensitivity and protein aggregation with the expression of the *SSD1-v* plasmid is also shows a robust functional correlation between the two (Figure 22B).

In some cases, tRNA modifications are known to prevent rapid tRNA decay (RTD) (Alexandrov et al., 2006). This surveillance mechanism monitors correct tRNA folding and modification. Especially at elevated temperatures RTD initiates degradation of hypomodified tRNA via exonucleases. A mutation in the *MET22* gene blocks RTD and suppresses the temperature sensitivity of tRNA modification mutants in which RTD is activated (Alexandrov et al., 2006). Of note, neither Pus1 nor Deg1 dependent modifications were tested for a potential function in the prevention of RTD, despite their previously established temperature sensitive phenotypes. Hence, it was of interest to test whether the *ts* phenotype of either *pus1* or *deg1* are RTD related. Since *met22* mutation suppresses RTD and rescues RTD related *ts* phenotypes, *deg1* and *pus1* mutation were combined with a deletion in *MET22* and tested for phenotypic suppression. The temperature sensitivity of *pus1* could be partially rescued by *MET22*

deletion, but this was not the case for the *deg1* mutant. Hence, the Pus1 dependent pseudouridylation at positions 1, 26, 27, 28, 34, 36, 65, and 67 (Simos et al., 1996; Motorin et al., 1998; Behm-Ansmant et al., 2003, 2006) could be involved in the protection from RTD, but pseudouridine modification at positions 38 and 39 seems not to fulfill such role . Further work will be required to elucidate which tRNA may become destabilized in *pus1* mutants. In all cases studied so far, RTD was found to selectively target only a few specific tRNAs (mostly $tRNA^{Val}IAC$ and $tRNA^{Ser}UGA/CGA$) (Alexandrov et al., 2006). According to the model of protein aggregate induction by ribosomal slow-down at specific codons, RTD of selected tRNAs could also create a situation of ribosomal slown-down and potentially could induce protein aggregation. To see the relation between the RTD-related Pus1 dependent modifications and protein aggregation, total protein and protein aggregates of a single and double mutant of *pus1* and *met22* were extracted. However, no difference between the amount of aggregates accumulation of the single and double mutants was observed (Figure 25), although the temperature sensitivity rescue of the *pus1* single mutant in the absence of *MET22* raises the expectation of seeing less protein aggregation formation in the *pus1 met22* double mutant. This result suggests that under standard conditions, the Pus1 dependent tRNA pseudouridylation is not a modification that plays an important role in the maintenance of protein homeostasis and prevention of protein aggregation in the yeast cell, despite the genetic evidence that it may be involved in protection from RTD. It may be possible, that elevated temperature is required to trigger RTD in *pus1* mutants and therefore protein aggregation may be different when cells are grown at mild heat stress. However, at regular temperature, Pus1 dependent modification is clearly distinct from the other pseudouridine synthase focused on in this study (Deg1) in that it is not required to be protected from protein aggregation.

6.5. The influence of tRNA modification in different *SSD1* background strains on chronological aging in *Saccharomyces cerevisiae*

tRNA modifications have an important role in translation and protein homeostasis. Hence, they might also be important for aging. While baker's yeast is an attractive model system to study the influence of individual genes on aging, published data from different study provide inconsistent results about modification genes involved in formation of identical tRNA modifications (Simos et al., 1996; Huang et al., 2008; Jackman & Alfonzo, 2013). The absence of some of these modifications increase the life span, some decrease it, and many were not found to impact life span (Eisenberg et

al., 2009; Marek & Korona, 2013; Garay et al., 2014; McCormick et al., 2015; Campos et al., 2018; Yu et al., 2021). It is already known that the lack of individual modifications has a minor effect on cells growth compared to the absence of multiple modifications. It has also been observed that in some cases, the phenotypes are only visible in the case of the lack of two modifications at the same time (Sokołowski et al., 2018). The $\Psi_{38/39}$ modification and ncm^5/mcm^5 modifications at wobble uridine position of $tRNA^{Gln}UUG$ in the *SSD1-v* strain are good examples for causing no changes in the chronological lifespan individually (Figure 29). However, the absence of both of these modifications in combination changes the chronological aging in yeast and causes accelerated aging. In a previous research, it has been observed that cells lacking both Ψ and ncm^5/mcm^5 modifications show changes in cell morphology, and a budding site position defect can be observed. It was also seen that these cells are multinucleate (Klassen et al., 2016). These changes in cell growth due to the absence of these two modifications might be a reason for this accelerated aging phenotype.

The chronological aging occurs faster in the *ssd1-d* background than the *SSD1-v* strain (L. Li et al., 2009) and Figure 28A. The influence of both $\Psi_{38/39}$ modification and ncm^5/mcm^5 modifications were as well tested in *ssd1-d* background strain. The Elp3 dependent modification was not changing the chronological aging in this background since *elp3* mutants showed an aging phenotype similar to the WT strain. On the other hand, the lack of pseudouridine modification in the *deg1* mutant caused a faster aging in *ssd1-d* background strain (Figure 28 and 30). It was previously shown that *SSD1* mutations alone, could influence the transcript levels of genes responsible for longevity and reduce chronological lifespan (Li et al., 2009). In addition to that, proper CWI signaling -for which Ssd1 is important- helps the long-term survival in the stationary phase (Cao et al., 2016). Thus, the additive effects of *deg1* and *ssd1* mutations on the CWI and mRNA levels in longevity genes could explain the *ssd1-d*-specific aging effect of the *deg1* mutation. Moreover, the clearance of protein aggregates during chronological aging (Peters et al., 2012) could affect the long-term stationary phase survival. Therefore, the *ssd1-d deg1* mutant's shortened lifespan might partly be related to the enhanced amount of protein aggregates (Figure 23). However, due to the reduced chronological aging of the W303-1B *deg1 ssd1-d* [*SSD1-v*] mutant compared to the *ssd1-d* [*SSD1-v*] control (Figure 30B), other genetic differences between the *SSD1-v* and *ssd1-d*

strains used in this study are obviously involved in the short-lived phenotype observed in W303-1B *deg1* but not in BY4741 *deg1* strain.

The influence of s^2U modification was also tested on chronological aging next to Ψ and ncm^5/mcm^5 modifications in the *ssd1-d* background strain. The *uba4* and *ncs6* mutants lacking s^2U modification (Han et al., 2015; Klassen et al., 2015), showed the same aging phenotype as the WT strain. This is evidence of the lack of influence of this type of modification in the *ssd1-d* background, and it means that thiolation modification is not necessary for *ssd1-d* strain's chronological aging. However, the slow-growing *urm1 deg1* double mutant in *SSD1-v*, a lethal strain in the *ssd1-d* background, shows that a presence of thiolation modification and pseudouridine modification is significant for cell's growth in higher temperatures.

As explained, damaged proteins and protein aggregates can be removed from the cell by autophagy or by proteasomal degradation (Finley, 2012; Wong & Cuervo, 2010). The *elp3 deg1 atg1*, *elp3 deg1 ubr2* and the *elp3 deg1* mutant strains showed an accelerated aging as expected. When comparing these strains, the *UBR2* deletion was rescuing the accelerated aging slightly and the lack of *ATG1* was leading to the faster death of the cells. Autophagy is a known aging influencing factor (Ruckenstuhl et al., 2014) and by removing the Atg1 as the kinase of the autophagy mechanism, the cell is no longer capable of getting rid of the excess aggregates by autophagy mechanism. Thus, cells lacking autophagy in their stationary phase will die faster, especially in the absence of both ncm^5/mcm^5 and $\Psi_{38/39}$ modifications. The removal of Ubr2 in a strain lacking both mentioned modifications is influencing the UPS mechanism. The removal of the Ubr2 enhances the proteasome activity. Thus, cells lacking Ubr2 are containing a more active site for degrading the defect proteins and are having a higher chance of survival during the stationary phase.

6.6. Protein homeostasis impact on cells aging in tRNA modification mutants

As explained, the *elp3 deg1* double mutant is lethal in *ssd1-d* and is a fast dying strain in the *SSD1-v* background. The influence of two mechanisms by which cells get rid of protein aggregates was tested by removing a member of each complex. The *elp3 deg1 atg1* triple mutant was generated to see the influence of autophagy in a strain containing tRNA modification cross-talks, and *elp3 deg1*

ubr2 for seeing the influence of ubiquitination proteasome system. In a previous study, it is shown that protein aggregates can be separated into two separated compartments regarding their aggregation state and their ubiquitination status. The soluble misfolded proteins, which are also ubiquitinated, will accumulate in a JUNQ in which there is a high concentration of proteasomes. In contrast, other non-ubiquitinated aggregates are sequestered in a perivacuolar inclusion called IPOD, which will be removed from the cell later on by autophagy (Kaganovich et al., 2008). It was observed that the *elp3 deg1* double mutant is accumulating a higher amount of aggregates than the single mutants (Klassen et al., 2016). It was as well observed that this strain shows an accelerated aging phenotype (Figure 27). By considering that the accumulation of protein aggregates in the cell is not beneficial for cells survival, an accelerated aging is expected in case of the removal of the mechanisms which are helpful for getting rid of protein. This was tested by generation of two triple mutants. In an *elp3 deg1* double mutant with the deletion of *ATG1* an accelerated aging was observed due to the deactivation of autophagy in the cell as expected. In the other generated strain, the removal of Ubr2 in *elp3 deg1* mutant was enhancing the proteasome activity since it prevents the ubiquitination and degradation of Rpn4, the regulatory protein for the proteasome gene expression, which leads to the rescue of chronological aging in this strain. Hence, both autophagy and UPS are playing important roles for cells survival during the stationary phase in a yeast strain lacking both ncm^5/mcm^5 and $\Psi_{38/39}$ modifications.

To further investigate the influence of protein homeostasis, the chaperons´ importance was also tested in the presence and absence of ncm^5/mcm^5 tRNA modification. As explained Zuo1 is the protein of the Hsp40 chaperon, a member of a chaperon complex close to the exit site of the translated peptide chain on the ribosome (Weyer et al., 2017). The chronological aging phenotype of a *zuo1* mutant was surprisingly similar to the WT even though the same mutant could accumulate more aggregates compared to WT (Figure 32). Hence, the Zuo1 as a member of the chaperon complex plays an essential role in aggregates formation, and the removal of it will lead to the formation of more protein aggregates (Bruch et al., 2020; Klassen et al., 2020). On the other hand, the *elp3* mutant strain also showed a similar chronological aging phenotype compared to WT in the same background. The same strain, however, is not forming so much aggregates. These results can lead us to the conclusion that the cell

might protect itself from toxic proteins. This could be tightened up to the fact that protein aggregates can get stored in the mentioned inclusion bodies. So, it might be that in *zuo1* mutant, there is more protein aggregate formation observable but the fact that these aggregates are toxic for the cells makes them get transferred either in JUNQ or IPOD, which may protect the cell from the harmful effect of these aggregates in some levels. In the long term, like during the stationary phase which is measured by the time a cell can stay viable, there will be other explanations for the survival of cells that accumulate aggregates more than WT but can last as long as *zuo1* mutant. Due to a study, oxidatively damaged proteins are controlled so that the newly produced cell is kept free of damaged proteins during the cytokinesis (Aguilaniu et al., 2003). However, this study was mainly on replicative aging in yeast. It can also help explain survival during chronological aging. The followed-up experiments suggest that oxidatively damaged proteins turn into aggregates recognizable by the heat shock protein Hsp104p (Erjavec et al., 2007). In another study, it was suggested that the Hsp104p containing aggregates would attach to actin cables rather than bind to the microtubule, and due to the flow of actin cables away from the bud tip of the daughter cell, these aggregates are not capable of entering the newly formed cells (Liu et al., 2010). The fact that the aggregates cannot enter the daughter cells might be why every time the mother cell containing the toxic aggregate divides, the daughter stays preserved. Hence the division can usually go until the natural death of cells in replicative aging, and this could indirectly influence the stationary phase of growth and can be observed in chronological aging, as was observed in a comparison between the *zuo1* and the WT strains.

The formation of protein aggregates was also observed in tRNA modification mutants, but as explained, they also might be stored in the JUNQs and IPODs. The observed aging phenotype can be due to some other genetical factors which partially can be related to the cell wall integrity defects which was observed in *ssd1*, *elp3*, and *deg1* mutants. In the end, for the future investigations, the *SSD1-v* in the BY4741 strain was replaced with *ssd1-d*, and the *ssd1-d* in the W303-1B strain was replaced with *SSD1-v* allele by CRISPR-CAS9 plasmid (Figure 37).

7. Materials and Methods

Table 2. Yeast strains used or generated in this study

Strain	Genotype	Reference
***S. cerevisiae* BY4741**	*MATa, his3Δ, leu2Δ, met15Δ, ura3Δ*	Euroscarf, Frankfurt
S. cerevisiae elp3	BY4741 *elp3Δ::KanMX4*	Euroscarf, Frankfurt
S. cerevisiae urm1	BY4741 *urm1Δ::KanMX4*	Euroscarf, Frankfurt
S. cerevisiae deg1	BY4741 *deg1Δ::KanMX4*	Euroscarf, Frankfurt
S. cerevisiae pus1	BY4741 *pus1Δ::KanMX4*	Euroscarf, Frankfurt
S. cerevisiae pus4	BY4741 *pus4Δ::KanMX4*	Euroscarf, Frankfurt
S. cerevisiae pus6	BY4741 *pus6Δ::KanMX4*	Euroscarf, Frankfurt
S. cerevisiae pus7	BY4741 *pus7Δ::KanMX4*	Euroscarf, Frankfurt
S. cerevisiae trm1	BY4741 *trm1Δ::KanMX4*	Euroscarf, Frankfurt
S. cerevisiae trm8	BY4741 *trm8Δ::KanMX4*	Euroscarf, Frankfurt
S. cerevisiae ncl1	BY4741 *ncl1Δ::KanMX4*	Euroscarf, Frankfurt
S. cerevisiae ssd1	BY4741 *ssd1Δ::KanMX4*	Euroscarf, Frankfurt
S. cerevisiae met22	BY4741 *met22Δ::KanMX4*	Euroscarf, Frankfurt
S. cerevisiae snf1	BY4741 *snf1Δ::KanMX4*	Euroscarf, Frankfurt
S. cerevisiae ubr2	BY4741 *ubr2Δ::KanMX4*	Euroscarf, Frankfurt
S. cerevisiae zuo1	BY4741 *zuo1Δ::KanMX4*	Euroscarf, Frankfurt
***S. cerevisiae* W303-1B**	*MATα {leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15}*	(Fiorentini et al., 1997)
S. cerevisiae elp3	W303-1B *elp3Δ::SPHIS*	this study
S. cerevisiae urm1	W303-1B *urm1Δ::SPHIS*	this study
S. cerevisiae deg1	W303-1B *deg1Δ::SPHIS*	this study
S. cerevisiae pus1	W303-1B *pus1Δ::SPHIS*	this study
S. cerevisiae trm1	W303-1B *trm1Δ::SPHIS*	this study
S. cerevisiae trm8	W303-1B *trm8Δ::SPHIS*	this study
S. cerevisiae ncl1	W303-1B *ncl1Δ::SPHIS*	this study
S. cerevisiae atg1	W303-1B *atg1Δ::SPHIS*	this study
S. cerevisiae uba4	W303-1B *uba4Δ::SPHIS*	this study
S. cerevisiae ncs6	W303-1B *ncs6Δ::SPHIS*	this study

Table 3. Oligonucleotides used in this study

Oligonucleotide	Sequence	Target
koELP3fw	AGTCCTAAAAGCACCTAAGGAAAATCGAAGAACA CCCTGACAAAGCAGCTGAAGCTTCGTACGC	pUG27/ *ELP3*
koELP3rv	AAAACCGGCCATGTCGGCGGCACATAAAAGTTC TATTTACCTTTAGCATAGGCCACTAGTGGATCTG	pUG27/ *ELP3*
Elp3fw	CGATAAGACAGTGAGAGAAGG	*ELP3*
Elp3rv	AACACATGCAGCAGTTACTCC	*ELP3*
koURM1fw	CAATACTGATTTCTGATACTAAAACGAGATAGGT TAATAGCAAAATCGGGCAGCTGAAGCTTCGTAC GC	pUG27/ *URM1*
koURM1rv	CTTTATATATATATATGTAGCTGCTTCTTAAAAAT TATTTGCTGCTATTTGCATAGGCCACTAGTGGAT CTG	pUG27/ *URM1*
urm1fw	CACATACCGGATTATGTTCCTTCCC	*URM1*
urm1rv	ACTGGAATGGTAGAGGTCTTTTGGG	*URM1*
koDeg1fw	GGTGCCCACATGCAATCTTTACTGCCCTACTATA ACCTCCCTTGACAGCTGAAGCTTCGTACGC	pUG27/ *DEG1*
koDeg1rv	GAAATATAGTCTTCAAGGTTATATTATACAGGTTT ATATATTATTGCATAGGCCACTAGTGGATCTG	pUG27/ *DEG1*
Deg1fw	TGGGCTCAGCTCATCTTG	*DEG1*
Deg1rv	GTGTCGGTCGTCCAATATC	*DEG1*
koPUS1fw	ATAAAGGACAATAAAGTGCTAGTAAATAACAATTAT AAGTGATATCAAGGCAGCTGAAGCTTCGTACGC	pUG27/ *PUS1*
koPUS1rv	ATGTCAATGCCTTAGAAATTAAAGTTGGTAAGAAAG AAGGAAAGGGCAACGCATAGGCCACTAGTGGATCTG	pUG27/ *PUS1*
pus1fw	GATGCGGGTAACTATTAGCC	*PUS1*
pus1rv	GCGCAATGAGCTTTCCAAGG	*PUS1*
koTRM 1fw	ACAGATCCTGAGCAGTCATAAGTTGATACCTTTCCTC TTACAATGTAGATCAGCTGAAGCTTCGTACGC	pUG27/ *TRM1*
koTRM1rw	GGGCGGATCCTTAGTTTCTTACGTTTTAGCTCTAACA CTAATCAAAATTCGCATAGGCCACTAGTGGATCTG	pUG27/ *TRM1*
trm1fw	TCTGCTATTGTGCCGCTATG	*TRM1*

trm1rv	CATACATACTGCCCTCCTG	*TRM1*
koNCL1fw	TCTAACACTTCCTTTTATCTACACTGTAATCCGAAGA ATACACTATAAGGCAGCTGAAGCTTCGTACGC	pUG27/ *NCL1*
koNCL1rv	AATAATATACATTTACTTTACAGTGGAGGGGATAAGA AACATGATAACTAGCATAGGCCACTAGTGGATCTG	pUG27/ *NCL1*
ncl1fw	CGCTGAGTTCTTCCAAAGAC	*NCL1*
ncl1rv	CCAACTCCGCAGGTCTTTCG	*NCL1*
koTRM8fw	TAGATTCAGCAGTTCCCATAGGATAAAATTTTCAAGC GTTTATTGTTAAGCAGCTGAAGCTTCGTACGC	pUG27/ *TRM8*
koTRM8rv	TAAGAAATAGTTATGTATATGTGGTAAATTGTTCTAG TTATACATCTATGgCATAGGCCACTAGTGGATCTG	pUG27/ *TRM8*
trm8fw	GGATGTGGAGTCAACTACAG	*TRM8*
trm8rv	TGCGGCTCAAATAACCTGGC	*TRM8*
koATG1fw	ACCCCATATTTTCAAATCTCTTTTACAACACCAGACG AGAAATTAAGAAACAGCTGAAGCTTCGTACGC	pUG27/ *ATG1*
koATG1rv	AAATATAGCAGGTCATTTGTACTTAATAAGAAAACCA TATTATGCATCACGCATAGGCCACTAGTGGATCTG	pUG27/ *ATG1*
atg1fw	GGCAAAGGAGATAGGAGAATA	*ATG1*
atg1rv	CGTAAAGCATTTCGAGAGTA	*ATG1*
koUBA4fw	TTAAGCTGATGCCGTTGACTGCAAAAGGAAGTAAAT AGAAGTCAATAACACAGCTGAAGCTTCGTACGC	pUG27/ *UBA4*
koUBA4rv	GGGATAAAAAAAAATAAAGTTACATATACACGTTAT ACATGTATAGGTCAAGCATAGGCCACTAGTGGATCTG	pUG27/ *UBA4*
uba4fw	TCCAGCTACGCTATCTCAAG	*UBA4*
uba4rv	TACCGTAGCGGCAAAGAATC	*UBA4*
koNCS6fw	AAAATTTTGGCGATGAGACGATATGGTAAGAGTAAA GCAAAGGAACCGTCCAGCTGAAGCTTCGTACGC	pUG27/ *NCS6*
koNCS6rv	TATATTATATTATGTTACGCTGCATTCTTCTACTGCGA GCTATATATATGGCATAGGCCACTAGTGGATCTG	pUG27/ *NCS6*
ncs6fw	GGCGATGAGACGATATGGTAAG	*NCS6*
ncs6rv	TGCGCGTTCTAATGGAGGGC	*NCS6*

Table 4. Plasmids used in this study

Plasmid	Selectable genes	Reference
pUG6	Amp^R, *kanMX (Tn 903)*	(Gueldener et al., 2002)
pUG27	Amp^R, *SpHIS5 (S. pombe)*	(Gueldener et al., 2002)
pUG72	Amp^R, *KlURA3 (K. lactis)*	(Gueldener et al., 2002)
pUG73	Amp^R, *KlLEU2 (K. lactis)*	(Gueldener et al., 2002)
$ptRNA^{Gln}UUG$	Amp^R, *KlLEU2 (K. lactis)*	(Klassen et al., 2016)
pHA-URM1	*(HA)3-URM1* cloned in *pRS426SmaI*	(Furukawa et al., 2000)
pFF8	(Klassen et al., 2015)	(Klassen et al. 2015)
YCplac111	Amp^R, pMB1 ori, *ScLEU2, ARS1-CEN4*	(Gietz & Sugino, 1988)
pSH47	Amp^R, *CEN6/ $ARSH_4$; $GAL1^p$-Cre-$CYC1^T$; URA3*	(Prein et al. 2000)
pPL091	SSD1 (JK9-3da allele) in pRS315	(Reinke et al. 2004)
pPL092	SSD1 (JK9-3da allele) in pRS316	(Reinke et al. 2004)
pPL093	SSD1 (W303a allele) in pRS316	(Reinke et al. 2004)
pAH145	Cas9, SSD1 guide RNA	Alexander Hammermeister

7.1. Growth Culture for *Saccharomyces cerevisiae*

To grow yeast cells, yeast extract peptone dextrose (YPD) and yeast nitrogen base (YNB) mediums were used as complete and minimal media respectively. To prepare liquid YPD media, 2% (w/v) glucose and 3% (w/v) yeast extract peptone were used, and to prepare agar plates 2% (w/v) agar was added to mentioned ingredients. For preparation of liquid minimal media, 0.67% yeast nitrogen base (YNB), 2% (w/v) glucose and regarding the auxotrophic marker 2 mg/ml Adeninsulfate, 2 mg/ml L-Histidin, 12 mg/ml L-Leucin, 2 mg/ml L-Methionin, 2 mg/ml L-Tryptophan oder 2 mg/ml Uracil were used, and to prepare agar media 2% (w/v) agar was added. Stress tolerances were tested by spotted dilutions on YPD plates supplemented with (1.25 nM, 2.5 nM and 5 nM) of rapamycin, (1 µg/ml, 5 µg/ml and 10 µg/ml) of 5-fluorouracil, (7.5 mg/ml and 60 µg/ml) calcofluor white and 0.9 M sorbitol. Thermal stresses were performed by incubating YPD plates between 30°C to 40°C and results were documented after

48-72 h (Digital camera DMC-FZ, Panasonic Lumix, Hamburg, Germany). Cell cultures used for aging experiments were prepared based on (Hu, J. et al., 2013).

7.2. Chronological Aging Assay

Yeast chronological lifespan (CLS) was determined according to (V. D. Longo et al., 2012). To establish the aging assay in this study, WT and *snf1* mutant strains in the BY4741 background were used. Both strains were inoculated at 10 ml of minimal media with the OD600 of 0.1 at 8:00 a.m. on day zero. Then each sample was plated on two separate yeast extract peptone dextrose (YPD) plates for three days, once at 8:00 a.m. and once at 6:00 p.m. due to the known accelerated aging in *snf1* mutant. For further experiments, freshly streaked colonies were inoculated into a preculture consisting 2 ml synthetic complete dextrose (SDC) medium and incubated at 30°C overnight. Main cultures were prepared as described in Erlenmeyer flasks covered with aluminum foil. The optical density of the main culture was measured until the stationary phase was reached, considered as time point day zero with an initial survival of 100% (Longo et al., 2012). To determine viability, cells from each flask were diluted and plated on two YPD plates, which were incubated at 30°C until colonies appeared. Colony counts were used for calculating colony forming units per ml (CFU/ml) for each culture and time point (0-17 days). Relative viability represents the CFU/ml value normalized to the value obtained for day zero. Each strain was analyzed using three independent cultures that were cultivated in parallel.

7.3. Spot Assay and Phenotypic Analyzes

Spot assay was performed in order to phenotypically examine yeast strains for either temperature or drug sensitivity. For this purpose, cells were diluted in 50 ml of sterile water and an OD600 of 2 was set as the start suspension for serial dilution preparation. This was then diluted 1:10, 1:100 and 1:1000 in a micro test plate 96 well, R (SARSTEDT) using a multichannel pipette, and drops were then transferred to Agar plates using a spotting tool (replica plater for 96 well plates).

7.4. Agarose Gel Electrophoresis

The separation of DNA took place in 1% agarose (w/v) gels. To prepare the gels, the appropriate amount of agarose was boiled in TAE buffer (40 mM Tris-HCl;

20 mM HAc; 1 mM EDTA, pH 8.0) for the complete dissolve of the agarose, the solution was then stored at 65°C and was poured into a gel chamber (Gel electrophoresis chamber, Biozym Scientific GmbH, hessisch Oldendorf, Germany) when needed. The electrophoretic separation of the DNA was performed at 130 V in the time needed regarding the size of the DNA fragment. TAE was used as the running buffer and after electrophoresis, the agarose gel was transferred to an ethidium bromide bath (0.0002% (w/v) EtBr) for 20 minutes and DNA fragments were detected using a UV-light transilluminator (Light table, Intas, Göttingen, Germany). The fragment size was determined by comparison with the Gene Ruler 1 kb Plus DNA Ladder (Thermo Fisher Scientific).

7.5. Gel Purification and Sequencing

After running the agarose gel and observation of the expected DNA fragment the bands were cut out with a scalpel on the UV table. The DNA was then eluted from the gel with the help of NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel). Purification was handled according to the manufacturer's instructions. Samples were then prepared based on instructions on (Eurofins genomics, LightRun Tube) and were sent for sequencing.

7.6. Polymerase Chain Reaction (PCR)

The polymerase chain reaction is originally based on the method by (Mullis et al., 1986) which allows the in vitro amplification of the DNA. The PCR was used in various areas and a PCR program adapted according to the manufacturer's instructions was used for each DNA polymerase. The PCR conditions were adapted to the size of the expected product as well as the size of the used primers. All reactions were taken place in a LabCycler from SensoQuest. After running the program, the PCR product was checked using agarose gel electrophoresis. For an overview, a PCR program is shown as an example, as it was used for the DreamTaq DNA polymerase (Thermo Fisher Scientific) to create deletion cassettes based on (Gueldenet et al., 2002):

3-10 minutes at 96°C (Preheating)	
30 seconds at 96°C (Denaturation)	35 Cycles
30 seconds at 56°C (Annealing)	
1 minute per kb at 72°C (Elongation)	
10 minutes at 72°C (Final Elongation)	
Unlimited time at 15°C to keep DNA products stable	

Ingredients used for the preparation of a 200 µl PCR reaction mixture:

4 µl of the 10 µm forward and reverse primers each; 20 µl of a 2mM dNTPs; 20 µl of DreamTaq Green DNA buffer; 1 µl of Plasmid from (e.g. pUG plasmid system); 1 µl of DreamTaq DNA polymerase and 150 µl sterile H_2O.

After the cycler was done DNA was precipitated using DNA precipitation protocol as explained below and it is verified on a 1% (w/v) agarose gel using 3 µl of the product mixed with 7 µl of water and 2 µl of DNA loading buffer.

7.7. DNA Precipitation

In resulted solution after the polymerase-chain-reaction nucleic acids are surrounded by a hydration shell which can be withdrawn by heavily polar substances to reach precipitation of nucleic acids. The solution is treated with two volumes of 100% isopropanol and mixed thoroughly, then the mixture is incubated at -20°C for 30 minutes or overnight, afterwards it gets centrifuged for 15 minutes at 15.000 rpm. The supernatant is then discarded and 500 µl of cold 70% Ethanol is added to wash remaining salts from the nucleic acid pellet. After another 15 minutes of centrifugation at 15.000 rpm the supernatant is discarded and the pellet is dried for 10 minutes at 50-60°C using a heat block. The dried pellet is then suspended in 40 µl water (for a 200 µl PCR product) and the concentration of the suspension is then measured using Epoch.

7.8. Bacteria Culture

Escherichia coli cells were grown on Lysogeny broth (LB) medium by incubation at 37°C (Incubator E24R, New Brunswick Scientific, Enfield, USA). 100 µg/ml of ampicillin was added to the medium for cells carrying the plasmid.

7.9. Plasmid Isolation from *E. coli*

The FastGene® Plasmid Mini Kit (NIPPON Genetics, Dueren, Germany) was used to isolate plasmids from *E. coli*. The plasmids were isolated according to the manufacturer's instructions.

7.10. Transformation

Lithium acetate was used for weakening the cell wall and allowing the DNA uptake by the cell for starting the transformation. To increase the efficiency of transformation a carrier DNA was used to competitively prevent the DNA fragment from degradation. Cells were streaked on YPD plates and were incubated at 30°C for 48 h. They were then inoculated in 1 ml sterile water and centrifugated (Centrifuge 5424, Eppendorf, Hamburg, Germany) at 4000 rpm for 2 minutes. The supernatant was then discarded and the pellet was inoculated in 2 ml of 0.1 M lithium acetate and was incubated at 30°C for 30 minutes. Cells were centrifugated at 4000 rpm for 2 minutes and the supernatant was then discarded and the pellet was then carefully resuspended in 240 µl 50% PEG4000, 36 µl 1M lithium acetate and 50 µl of carrier DNA, the previously prepared deletion cassette (35 µl) or plasmid (2 µl) were added at this stage and were incubated at 42°C (Thermomixer comfort, Eppendorf, Hamburg, Germany) for 45 minutes. Cells were then centrifugated at 5000 rpm for 2 minutes and the gained pellet was then resuspended in 80 µl sterile water and was pipetted (Pipettes HTL, ABIMED, Langenfeld, Germany) on selective media and spread out using Drigalski spatula, and plates were incubated at 30°C for 48 h.

7.11. Gene Deletion in *Saccharomyces cerevisiae*

Yeast cells carrying deletion mutations (Table 2), were generated using plasmid-based knockout cassettes (Table 4), with (*SpHIS5*, *KlURA3*, or *KlLEU2*) auxotrophic marker genes or antibiotic resistance (*KanMX*) and were amplified

using the PCR method (Gueldener et al., 2002). To secure targeted gene selection, up-and downstream of the objective gene's flanking regions with approximately 50 nucleotides were used (oligo table 3) (Gueldener et al., 2002) and transformed as explained (Gietz & Schiestl, 2007). Replacements were then verified by PCR using forward and reverse primers located outside of the target genes (Table 3).

7.12. Glycerol Stock

To have the opportunity to use the generated yeast strains at any time point, freshly streaked colonies were inoculated in 70 ml sterile water mixed with 30 ml of 85% glycerol and were stored at -80°C (Freezer MDF-454V, Sanyo, Muriguchi, Japan).

7.13. Plasmid Construction and Shuffling

To perform deletion of *DEG1* in *urm1Δ::SpHIS3* or *elp3Δ::SpHIS3* strains in the *ssd1-d* background, these were first transformed with pFF8 (*ELP3;* (*Klassen et al., 2015*) or pHA-URM1 (*URM1;* (*Furukawa et al., 2000*). Following this, *DEG1* was deleted by using a PCR-generated deletion cassette (*deg1Δ::SpHIS5*) and *URA3* plasmids pFF8 or pHA-URM1 eliminated by growth on minimal media containing uracil and 5-fluoro-orotate (1 µl/ml). Expression of *SSD1-v* involved pPL091 (*LEU2*)- and pPL092 (*URA3*)- (Reinke et al., 2004), and overexpression of *ssd1-d* involved pPL093 (*URA3*)-. Overexpression of $tRNA^{Gln}UUG$ was performed using pRK55 plasmid (Klassen et al., 2016). Rnq1 was expressed as a GFP fusion from plasmid p1332 (Nakavashiki et al., 2005).

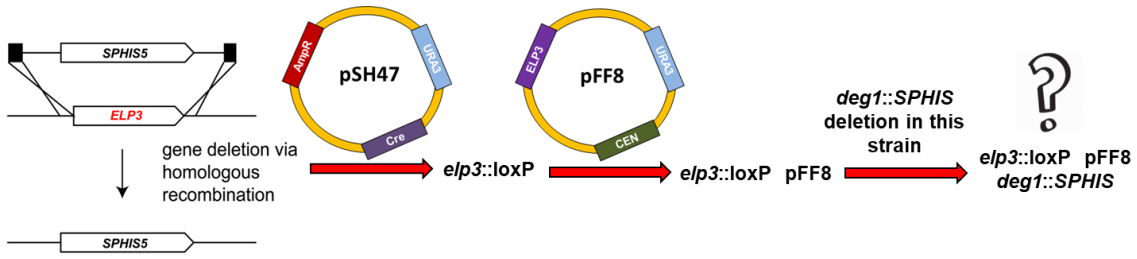

Figure 33. Generating *elp3 deg1* double mutant via Plasmid shuffling. The *elp3* mutant is generated via homologous recombination. Afterward the pSH47 is getting expressed in *elp3*::*SPHIS* mutant. Then the *elp3::*loxP colonies which are not capable of growing on HIS^- plates will be plated two times on 5-FOA plates, and afterward pFF8 plasmid will be expressed in the appeared colonies. At this stage, the deletion of *deg1*::*SPHIS* in *elp3*::loxP pFF8 is being performed and the viability of the generated strain will get tested.

7.14. Protein Isolation and Purification

Freshly streaked cells were grown in 50 ml YPD and if needed in selective liquid media overnight, they were inoculated in 50 ml of the same media with OD600 of 0.1 in the morning and were harvested at OD600 of 1 in 50 ml falcon tubes at 4000 rpm for 2 minutes at 4°C (Centrifuge 5810 R, Eppendorf, Hamburg, Germany). The supernatant was then discarded and cells were suspended in 15 ml sterile water and were centrifugated again at 4000 rpm for 2 minutes at 4°C. the supernatant was then discarded and the pellet was inoculated in 1 ml cold sterile H_2O and was transferred to 1.5 ml screw cap microtube and was vortexed (Vortex Genius S3, IKA Labortechnik, Staufen, Germany) and then centrifuged again at 4000 rpm for 2 minutes at 4°C and after removing the supernatant 300 µl of buffer A (10 mM K-HEPES, pH 7,0; 10 mM KCl; 1,5 mM $MgCl_2$; 0,5 mM PMSF; 2 mM Benzamidin) and 200 µl glass beads were added. The yeast cells were mechanically disrupted by vigorous vortexing for 1 minute by bead beater (Mini-Beadbeater-16, Biospec Products). Vortexing with bead beater was repeated for additional four more times. The samples were incubated on ice for 5 minutes between the individual digestion steps, after the last bead beating step cells were centrifuged at 13000 rpm for 5 minutes at 4°C for getting rid of bigger cell particles. The supernatant was then transferred to a 1.5 ml microtube and was centrifuged again at 13000 rpm for additional 20 minutes at 4°C for getting rid of cell debris. The supernatant containing the protein extract was then transferred to a new 1.5 ml microtube and the protein concentration was determined according to Bradford assay as explained below. For further processing of the extracts using SDS-PAGE as explained below, protein extracts were mixed with 5-fold Laemmli buffer (50% (v/v) glycerol; 25% (v/v) βME; 0.25 M Tris-HCl, pH 6.8; 10% (w/v) SDS; 0.2% (w/v) bromophenol blue) boiled at 99°C for 10 minutes and were boiled for additional 2 minutes at 99°C.

7.15. Bradford Assay

The protein concentration of a total protein extract was determined by the method introduced by Bradford (1976) using Bradford reagent which was calibrated before the use (Bio-Rad Laboratories, München, Germany). 990 µl of the 1:5 diluted dye concentrate was mixed with 10 µl of the 1:25 diluted

protein extract. The OD595 was measured after 15 minutes of incubation in the dark. The protein concentration could be determined by comparing the measured OD595 with a calibration line that was previously created.

7.16. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

To separate proteins according to their molecular weight, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used based on Laemmli (1970). For this purpose, a gel with two different phases was used, stacking gel (5% (w/v) acrylamide; 0.125 M Tris-HCl, pH 6.8; 0.2% (w/v) SDS) and a separating gel (9% - 15% (w/v) acrylamide; 0.375 M Tris-HCl, pH 8.8; 0.2% (w/v) SDS). In addition to the samples, the PageRuler ™ Plus Prestained Protein Ladder (ThermoFisher Scientific) was as well loaded in the gels as a size standard. The separation took place using a Running buffer (192 mM glycine; 25 mM Tris; 0.1% (w/v) SDS) filled the SDS electrophoresis tank (Mini-PROTEAN® Tetra Vertical Electrophoresis Cell, Bio-Rad), by applying a constant voltage of 200 V. The electrophoresis was performed until the desired separation of the samples was achieved. The detection of the proteins was then possible by the western blotting method.

7.17. Western Blot

After separation of the proteins using SDS-PAGE, they were transferred to a PVDF membrane (Immobilon®-P Transfer Membrane, pore size 0.45 µm, Merck Millipore) using the transferring system by (Trans-Blot® Turbo™, Bio-Rad) for 20 minutes. The PVDF membrane was then blocked for 1 h at RT with TBSTM (20 mM Tris; 137 mM NaCl; 0.2% (v/v) Tween 20; 5% (w/v) milk powder). The membrane was incubated overnight at 4 ° C. with the primary antibody which was dissolved in TBSTM. After the antibody incubation, it was washed three times for 5 minutes with TBST (20 mM Tris; 137 mM NaCl; 0.2% (v / v) Tween). The membrane was then treated with a secondary antibody, likewise dissolved in TBSTM, for 2 hours at room temperature. It was then washed three times with TBST again and protein detection with the aid of a chemiluminescence reaction took place via the horseradish peroxidase coupled to the secondary antibody. For this purpose, the membrane was treated with the WesternBright Chemilumineszenz Substrate (Biozym) according to the manufacturer's

instructions. Finally, the signals were made visible by applying and developing an X-ray film (LucentBlue X-ray film, Advansta) in the darkroom.

7.18. Protein Aggregate Isolation

Cells were grown as explained in the protein isolation and purification chapter and harvested at OD600 of 1 and were stored at -80°C if not used immediately. Protein aggregates were then extracted based on (Koplin et al., 2010). Pellets were suspended in 5 ml of lysis buffer (20 mM sodium phosphate, PH 6.8; 10 mM DTT; 1mM EDTA, 1mM PMSF; 0.1% tween; 3 mg/ml Zymolyase; 2 protease inhibitor complete mini-tablets (Roche)) and chilled at RT for 30 minutes. Cells were then opened using sonication (Branson Sonifier W-250) on ice and at 4°C room for 9 times at level 4 with a duty cycle of 50% for 40 seconds. Samples were kept on ice for 4 minutes between sonication cycles. Cells were then centrifuged at 200 rcf for 20 minutes at 4°C (SiGMA 3K30 laboratory centrifuge). 4 ml of the supernatant was then transferred to Nalgene® centrifuge tubes and 0.5 ml of it was transferred to microtubes for protein adjustment using Bradford assay; all samples were kept on ice during the whole time. 80 µl of the adjusted proteins were transferred to 1.5 ml microtubes and were set aside, rest of the adjusted samples were then centrifugated at 16000 rcf for 20 minutes at 4°C and the supernatant was discarded carefully afterward. The pellet was then washed using 5 ml 2% N-P40-buffer (20 mM sodium phosphate, PH 6.8; 1 mM PMSF; 2% Nonident P40; 1 protease inhibitor complete tablet (Roche)). Sonication was repeated as explained before for 6 times and cells were again centrifuged. The washing step was repeated without sonication and pellets were washed for one last time using washing buffer (20 mM sodium phosphate, 6.8 PH, 1mM PMSF; 1Protease inhibitor complete mini-tablet (Roche)). Samples were then sonicated on the ice at 4°C room for 4 times at level 2 with a duty cycle of 65% for 35 seconds and were centrifugated at 16000 rcf for 20 minutes at 4°C for the last time. The supernatant was then removed carefully and slowly due to the instability of aggregate pellets which were then dissolved in 20 µl sterile H_2O and transferred to 1.5 ml microtubes and 5 ml laemmli buffer was added to this mixture. For the 80 µl total protein 20 ml of the laemmli buffer was added. The buffer was boiled for 10 minutes at 99°C and after adding it to samples they were boiled for additional 2 minutes at this temperature. The same amount of protein aggregates and total protein was then loaded in a Nu-PAGE gradient gel and the gel was running at 100V for 2 h using NuPAGE™ MOPS SDS Running Buffer. The gel was then analyzed using a ready Coomassie protein stain (InstantBlue™ Protein Stain).

8. References

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10. Appendix

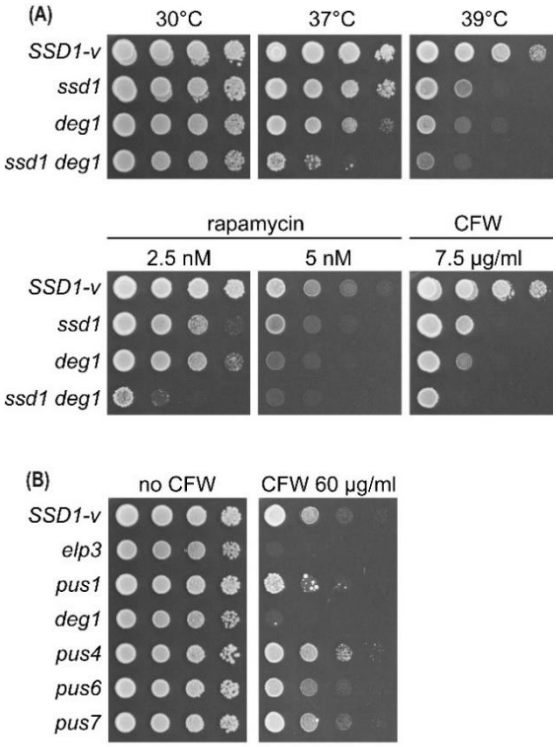

Figure 34. Drug sensitivities of gene deletion mutants in the *SSD1-v* background. (A) *SSD1-v* wild type, *ssd1*, and *deg1* deletion mutants were serially diluted and spotted on YPD plates containing no drug or the indicated amounts of rapamycin or calcofluor white (CFW). Drug-free plates were incubated at the indicated temperatures and rapamycin or CFW plates were incubated at 30°C for 48 h (B) *SSD1-v* wild type and indicated deletion mutants were serially diluted and spotted on YPD plates without or with 60 µg/mL CFW and incubated at 30°C for 48 h. All strains are in the BY4741 background.

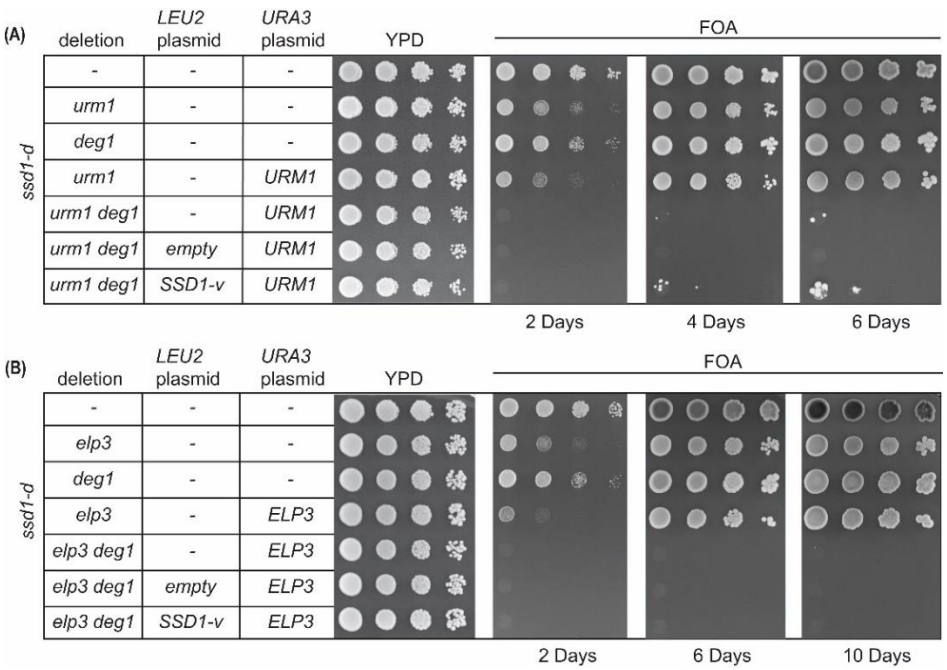

Figure 35. *SSD1-v* expression and the possibility of rescuing the synthetic lethality of combined tRNA modification mutants. All strains are in the W303-1B background and contain indicated gene deletions and in addition either no plasmid (-), empty *LEU2* vector YCplaC111 (empty), *LEU2-SSD1-v* single copy (s.c.) vector pPL091, or single copy *URA3* vectors providing *ELP3* and *URM1*, respectively. (A) *SSD1-v* expression rescue of *urm1 deg1*. (B) The *SSD1-v* expression shows no rescue of *elp3 deg1*. Strains were serially diluted and spotted on YPD and FOA plates. YPD plates were incubated for 48 h and the FOA plates were incubated as indicated at 30°C.

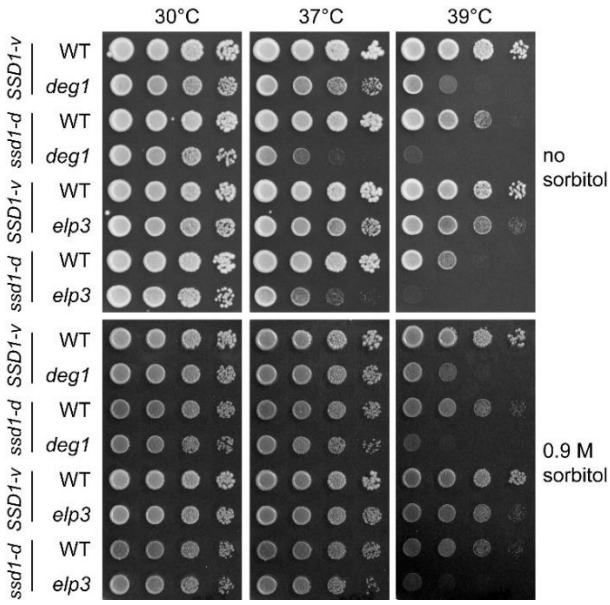

Figure 36. Suppression of temperature sensitivity of *elp3* and *deg1* mutants by osmotic stabilization. Indicated strains in *SSD1-v* and *ssd1-d* backgrounds were serially diluted and spotted on YPD plates without and with 0.9 M sorbitol. Plates were incubated at the indicated temperatures for 48 h.

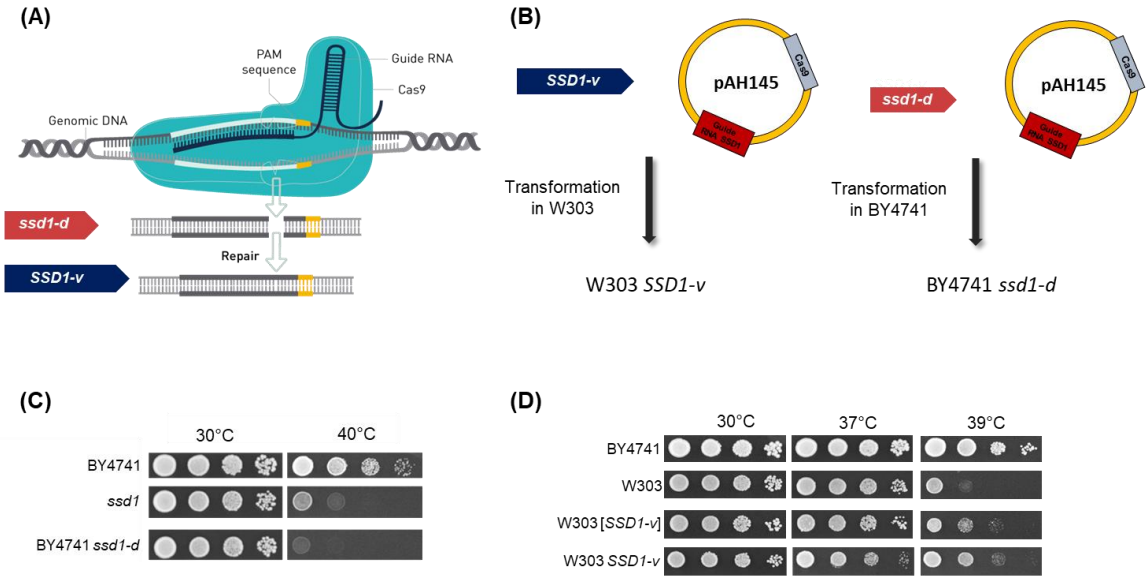

Figure 37. Generation of BY4741 *ssd1-d* and W303-1B *SSD1-v* using CRISPR-CAS9 plasmid. (A) Genome engineering scheme of CRISPR-CAS9. The Cas9, guide RNA, and PAM sequence are marked and it's shown how the *ssd1-d* is repaired and replaced with *SSD1-v* in W303-1B strain. This figure is modified from Genome editing (CRISPR/Cas9) | Diagenode. (B) the generation of W303-1B *SSD1-v* and BY4741 *ssd1-d* using CRISPR- CAS9 plasmid [pAH145]. (C) Temperature sensitivity phenotype of BY4741 in comparison to *ssd1* knock out strain in the same background and the newly generated BY4741 *ssd1-d*. all strains were spotted on YPD media and were incubated for 48 h in 30°C and 40°C. (D) Temperature sensitivity phenotype of BY4741, W303-1B, W303- 1B containing [*SSD1-v*] plasmid and a newly generated W303-1B *SSD1-v*. all strains were spotted on YPD plates and were incubated in 30°C, 37°C, and 39°C for 48 h.