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**Exploring the dynamics of the tRNA pool through  
systematic deletion of tRNA genes**

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# 1. Abstract

A powerful experimental tool to decipher the structure and function of biological systems is introducing a set of minimal genetic perturbations and exploring the functional and molecular consequences. To further the understanding of the tRNA pool composition and the way it effects the process of translation, I created a systematic deletion library of most of the 274 tRNA genes in the yeast *S. cerevisiae*. Analyzing the growth and the molecular phenotype of each tRNA deletion strain allowed me to elucidate the contribution of each tRNA gene to the tRNA pool, the translation process, and the cell's fitness. Creation of the tRNA deletion library revealed that most single-copy tRNA genes are essential, while two single-copy genes are partially dispensable. I further studied the wobble in these single copy tRNA genes and revealed that this mechanism is dependent on a methylation reaction which enhances the decoding between non-perfectly matching codon/anti-codon pairs. tRNA genes which are part of multiple copy families, are not essential, and show only mild phenotypes when grown in rich-medium. The backup for these tRNA deletions is probably provided by the remaining gene copies in the family. In order to expose additional phenotypes of the library's strains we screened across multiple stressful growth conditions. These screens exposed condition-dependent functions for additional tRNA genes, revealed by defects in either growth rate or growth yield. Interestingly I observed that in many families, deletion of different gene copies resulted in different growth defects, and I termed this phenomenon "differential contribution". Further analysis of differential contribution using double deletion strains revealed substantial growth aggravation for highly contributing tRNA genes, while tRNAs which contribute less did not. Molecular characterization of a selection of the tRNA deletion strains using expression microarrays revealed two distinct molecular signatures, dividing the tRNA deletions into two groups by the type of their cellular response. The cellular response to tRNA deletions which are part of multi-copy families included up-regulation of translation related processes. While deletions of tRNA genes that are part of low-copy families resulted in a cellular state of stress and strong indications for folding stress. To examine the possibility that tRNA deletion results in translation attenuation leading to protein misfolding, growth analysis was performed in the presence of several unfolding agents, combined with monitoring of misfolded protein formation in the cytosol. This analysis revealed an intriguing connection between proper translation and protein folding, which suggests that when tRNA supply is depleted, misfolding occurs. This deletion library represents the first systematic set of minimalist genetic perturbations in the translation machinery, and opens the way towards further characterization of this highly complex process.

## 2. Introduction

One of the strongest research paradigms in systems biology is the "tinkerer's approach" in which biological systems are characterized by systematic perturbations of most of their parts. The approach has been particularly powerful in a selection of model organisms, notably yeast<sup>1</sup>, worm<sup>2</sup> and mouse<sup>3</sup>, in which most of the genes in the genome were either deleted or inactivated. Such perturbation libraries revealed the functional contribution of most genes, sometimes under a diversity of growth conditions. Deletion mutant screens often expose the function of particular genes in specific conditions thus serving an important tool in genes functional annotation and evolutionary significance<sup>4</sup>. One of the prime conclusions from such systematic efforts was that the majority of the genes in any given genome appear "dispensable" at least when perturbed one at-a time, and in laboratory conditions. Most deletion and knockdown strains in most species hardly show a phenotypic effect. Such lack of essentiality is often interpreted to reveal a role for partially redundant genes and pathways that provide compensation and backup for the deleted gene<sup>5-9</sup>, either due to gene duplications<sup>10-12</sup> or due to functional convergence<sup>13</sup>. The genuine functional role of genes in such cases can then be exposed by coordinated deletion of pairs or larger sets of genes<sup>14-16</sup>. An additional reason for apparent lack of essentiality of genes under such screens is the limited exploration of the growth conditions space. In such a manner the essentiality of additional genes in the yeast genome was exposed<sup>17,18</sup>.

System-based approaches were applied thus far to several cellular processes<sup>19-21</sup>, including to the study of the translation process. Such studies included examination of translation profiles by ribosome density<sup>22-24</sup> performed across different conditions<sup>21,25</sup> and organisms<sup>26</sup> or by trough analysis of component in this process, such as ribosomal genes, and analysis of their attributes<sup>27,28</sup>. Nevertheless the contribution of the tRNA pool to the different aspects of translation was never examined using a systems approach. The translation of mRNA into protein is mechanistically divided into three major steps initiation, elongation and termination. Each of these steps is further broken into minor steps that use distinct sets of factors<sup>29</sup>. All steps are known to be regulated to adjust protein translation rates according to the requirements of the cell. The best understood mechanisms for regulating translation are at the initiation step<sup>30,31</sup>. The efficiency of translation elongation has been investigated thoroughly in individual genes<sup>32</sup>, and much less at the genome-wide level. From individual gene studies it became clear that the efficiency of the process depends on

the secondary structure of the mRNA<sup>33</sup>, on the codon usage<sup>34</sup>, and on the tRNA pool<sup>35–37</sup>. In particular it was shown that the availability of tRNAs needed for the translation of each gene affects protein production levels<sup>38</sup>. Thus the efficiency of translation elongation is mainly dictated by a dynamic interplay between the codon demand of the translated transcript and the cellular pool of tRNA molecules.

The idea that intracellular concentrations of tRNAs play an important role in the dynamics and regulation of protein synthesis was suggested by Ames and Hartman as early as 1963<sup>38</sup>. Since then there have been numerous works that emphasized the importance of tRNA concentrations and the role of codon anti-codon interaction in the dynamics of translation<sup>39–41</sup>. The idea that the rate of translation might vary along a gene was also implicit in these studies but until the work of Varenne *et al.* (1984) there was no experimental study of the phenomenon<sup>35</sup>. The results obtained in this study demonstrated two important points, first the rate-limiting step in the elongation cycle of polypeptides is tRNA selection, and the stochastic search of the ternary complex aminoacyl-tRNA-EF-Tu-GTP, the preceding steps transpeptidation and translocation are much faster. A second point states that as different tRNAs exist in *E. coli* in different intracellular concentrations, the rate of translation varies along the mRNA. tRNA genes may be present in multiple copies in a genome, e.g. *S. cerevisiae* has 274 tRNA genes encoding 42 different isoacceptors. The presence of multiple copies of a particular gene, especially those that are transcribed by RNA polymerase III, seems to be common in eukaryotes<sup>42</sup>. The end product of expression in these cases is RNA, therefore the requirement for large quantities of encoded products can be met by multiple rounds of transcription and/or by multiple copies of the gene<sup>43</sup>. All tRNAs are transcribed initially as precursors containing 5' leader and 3' trailer sequences that must be removed by processing. tRNAs are transcribed by RNA Pol III which, along with RNA Pol I, dominates cellular transcription, combining to exceed 80% of total RNA synthesis in growing cells. Due to the energy devoted to tRNA transcription, and the need for coordination of tRNAs and ribosomes function, their transcription must be regulated in response to nutrient availability and environmental conditions<sup>44</sup>. Inappropriate regulation of tRNA expression such as tRNA<sub>i</sub><sup>Met</sup> promotes cell and cancer in mice<sup>45,46</sup>. The Regulation of Pol III transcription has been the focus of multiple studies<sup>47–49</sup>, where it was found to be negatively regulated by the protein Maf1, which is conserved throughout eukaryotes<sup>50,51</sup>, which is under the regulation of the PKA and TOR pathways<sup>52,53</sup>. tRNA promoters are composed of two highly conserved sequence elements that reside within the transcribed

region. The proximal A box resides within 10-20 bp of the transcription start site. The location of the more distal B box is far more variable, partly because some tRNAs include introns of various length and variable stem regions<sup>42,54,55</sup>. All tRNAs are transcribed initially as precursors containing a 5' leader and 3' trailer sequences that must be removed by post-transcriptional processing<sup>44,56</sup>. Pre-tRNA also undergo a complex set of base modifications that are carried out by a set of enzymes that recognize specific features of tRNA structure<sup>44,57</sup>. In addition, eukaryotic tRNAs have oligomer CCA added to their 3' ends by specialized nucleotidyl-transferase<sup>58</sup>. A subset of the tRNAs also contain intervening sequences, which are removed by a dedicated set of tRNA splicing enzymes<sup>59</sup>. During their processing tRNA molecules are exported, and reimported into the nucleus, and eventually end up in the cytoplasm, where they must undergo aminoacylation to participate in protein synthesis<sup>60-62</sup>. The long maturation process of tRNA molecules generates a situation in which there are numerous forms of tRNA molecules in the cell, corresponding to various stages of maturation. To date not much is known about the regulation of tRNA gene transcription, i.e., whether it is constitutive and constant, or differentially regulated under certain conditions. An experimentally verified case of differential regulation exists in the mulberry silk worm, *Bombyx mori*. During the fifth instar larval stage, which is characterized by increased synthesis of silk fiber protein there is an increase in the tRNA population which decodes the most frequently represented codons of this protein<sup>36</sup>. This tRNA (tRNA<sub>1</sub><sup>Gly</sup>) is encoded by a multigene family comprising 20 gene copies, all of which have identical sequences and therefore the same internal control elements (A and B boxes); yet they are differentially transcribed. Certain *cis*-sequence elements are present in the 5' upstream regions of the tRNA genes<sup>63-65</sup> sequence that could differentially regulate their transcription and result in differential expression of seemingly identical copies. The difference in expression lies at least in part at the level of nucleosome packing at the tRNA genes hindering or enabling transcription differentially on the various tRNA gene copies<sup>66</sup>.

To date only a few studies made an attempt to quantify the level of the various types of tRNA molecules. The studies by Ikemura T. 1981, quantified a large number of *E. coli* tRNA molecules after these were separated using two-dimensional polyacrylamide gel electrophoresis and purified<sup>39</sup>. In a similar manner half of the tRNA molecules from *S. cerevisiae* were quantified<sup>40</sup>. The most extensive experimental work in this area has been conducted in *E. coli*. Absolute tRNA abundance was measured at conditions that allow various growth rates in which it was observed that tRNA isoacceptors corresponding to

abundant codons increase in concentration as the growth rate increases but not as dramatically as might be anticipated <sup>67-69</sup>. Current measures of translation efficiency of genes often assume that the tRNA pool is constant throughout the life of a cell and organism or across tissues and cell types <sup>70</sup>, though recent measurements reveal a more complex picture <sup>71</sup>. Using tRNA-dedicated arrays it was shown that the availability of tRNAs may change dynamically throughout the life of an organism <sup>72</sup>. Although existing technologies measure individually some of the key steps in processing and amino-acid loading of the tRNAs <sup>62,73,74</sup>, a complementary approach would be to completely delete tRNA genes and ultimately expose the effect of each tRNA gene on the phenotype. In addition, while most expression measurement methods, such as microarrays do not allow differentiating between the role of identical tRNAs genes from the same anti-codon family, deletion analyses of each such tRNA can expose their individual contribution to fitness. Thus existing studies clearly exposed the need to assay essentiality of each tRNA under various conditions. Yet despite the above, and the central role of the tRNAs in translation so far no systems-genetic study was carried out to methodologically decipher the role of each tRNA gene in translation.

A dynamic tRNA pool would grant the cell the ability to adapt the translation efficiencies to better fit the immediate needs by changing the supply of certain tRNAs. Yet very little is known about potential dynamic shaping of the tRNA pool in response to environmental changes. Gaining the ability to measure the levels of each tRNA isoacceptor across different conditions in the cell will allow exploration of possible dynamics within the tRNA pool. In addition this ability will provide comprehensive information about the tRNA supply to the translation machinery in changing environmental conditions.

In my study I focused on the stage of translation elongation and the tRNA pool using a global genomic approach. Gaining such comprehensive information about the contribution of each tRNA gene to the cellular tRNA pool across different conditions can provide novel information which will allow improved predictions of translation efficiencies. Gaining a better understanding of the dynamic interplay between the codon demand of a transcript and the cellular pool of tRNA molecules, responsible for supplying these demands, will ultimately allow the development of a dynamic model of the cells' proteome across various environmental conditions.

### 3. Summary of main findings

To study the tRNA pool I created a systematic deletion library in which 203 cytosolic tRNA genes were deleted from the genome of the yeast *S. cerevisiae*. I used two robotics-based screening methods to measure and score various growth parameters for all tRNA deletion strains. I evaluated the contribution of each tRNA gene to fitness, using the colony size method in rich medium in addition to 13 stressful conditions that included conventional biotic stresses and translation inhibitors. I followed this analysis by screening the deletion library using an OD based method in rich medium as well as 4 stressful conditions. These screens revealed that single gene-copy tRNAs which are non-essential often exhibit severe phenotypes, while tRNA genes which are members of multi-copy families are non-essential and exhibit mild growth defects under most conditions. Screens of the library using both methods revealed differential contribution between identical gene copies of same isoacceptor. Using genetic interactions I further studied this phenomenon for the *tR(UCU)* family. This analysis revealed growth aggravation for double deletions which contained "major" contributing copies versus "minor" copies which did not exhibit such aggravation. A similar analysis was used to decipher the backup through wobble observed for the two single copy tRNA deletions. Double deletion between the single copy *tR(CCU)I* and the tRNA-modifying enzyme *TRM9* revealed aggravating epistatic interaction which suggested a biochemical basis for backup through wobble. In contrast to the phenotypic level characterization, which showed relatively minor effects over-all, a molecular level investigation revealed a much more extensive response in which entire gene modules changed their expression levels in response to many of the deletions. Two distinct molecular signatures were observed, dividing the tRNA genes according to the number of copies in the isoacceptor family. While tRNA deletions which belong to multi-copy families displayed up-regulation of translation related processes including ribosome biogenesis, rRNA processing and tRNA processing. tRNA deletions of low-copy families resulted in an opposite response in which translation related processes were somewhat down regulated. For these tRNA deletions the expression profile demonstrated stress to various extent, indicated by up-regulating genes involved in response to stimulus, protein quality control and protein folding. To further investigate the possibility of unfolding stress, upon tRNA deletion, I chose two different routes, the first used the OD based method to screen the strains in the presence of various folding agents, while the second was more direct and monitored the

state of the quality control machinery in the cell. Both these methods revealed that deletion of certain tRNA genes leads to accumulation of misfolded proteins in the cell and to saturation of the cellular quality control machinery. A short lab-evolution period was sufficient to evolve a bypass to the deletion of the single-copy *tr(CCU)J*, which had the most drastic effect on growth phenotype. Sequencing of the evolved strains from four independent repetitions of the evolution experiment revealed a strikingly simple genetic change. I found that in each repetition of the experiment, a single tRNA gene which regularly provide backup through wobble to the deleted tRNA gene, was mutated by a single base substitution in the anti-codon loop to become identical to the deleted gene. The experiment constitutes an intriguing case in which the simplest evolutionary change, that can be easily predicted, recurs as the optimal evolutionary solution.

## 4. Materials and methods

### 4.1. Yeast strains

- *S. cerevisiae* strain Y5565(*MAT $\alpha$  can1 $\Delta$ ::MFA1pr-HIS3 mfa1 $\Delta$ ::MFA1pr-LEU2 lyp1 $\Delta$  ura3 $\Delta$  leu2 $\Delta$* ) was used as a source strain on the basis of which library construction in addition to all double deletion constructs was made.
- *S. cerevisiae* strain BY4743(*MAT $\alpha$ / $\alpha$  his3 $\Delta$ 1/his3 $\Delta$ 1 leu2 $\Delta$ 0/leu2 $\Delta$ 0 LYS2/lys2 $\Delta$ 0 met15 $\Delta$ 0/MET15 ura3 $\Delta$ 0/ura3 $\Delta$ 0*) was used for analysis of tRNA essentiality in tetrad analysis.
- The *S. cerevisiae* ORF deletion library <sup>75</sup> was used for growth experiments of tRNA flanking gene deletions.

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### 4.2. Yeast plates and liquid media

Unless indicated all yeast growth experiments were performed on a rich medium (YPD) (1% yeast extract, 2% peptone, 2% D-glucose) preformed at 30°C. All plates contained in addition to the specified growth medium 18 g/L Agar (Difco).

- Growth experiments performed in a minimal medium (SCD) contained Yeast Nitrogen Base (w/o amino acids) 0.67%, 0.15% Synthetic complete amino-acid mix, and 2% D-glucose. These experiments were also performed at 30°C.
- Growth experiments in rich medium with low glucose (0.5%), were performed on rich medium supplemented with 0.5% glucose instead of 2%.
- Growth experiments performed in rich medium supplemented with 1% galactose included 1% galactose instead of glucose.
- Growth experiments performed in 0.5NaCl contained 0.5M NaCl which was supplemented to rich medium.
- Growth experiments to examine unfolding were performed as follows:
  - Rich medium supplemented with 5mM azetidine-2-carboxylic acid (sigma)
  - Rich medium supplemented 1.5 $\mu$ g/ml Tunicamycin (sigma) dissolved in DMSO. Control was performed by supplementing rich medium with the appropriate amounts of DMSO.
- Minimal medium supplemented with 1.5mM DTT (Roche).

To test the effect of various stress conditions in the colony size method various stressful agents and carbon sources, were used as indicated below.

Rich medium was supplemented with the following:

10mM Copper (Sigma)

10mM H<sub>2</sub>O<sub>2</sub>

0.06% MMS (Sigma)

300ng/μl Cyclohexamid (Sigma)

1.2M and 0.5M NaCl

In addition rich medium without carbon source was supplemented with the following:

3% glycerol

0.5% glucose(Difco)

1% galactose (Sigma)

In addition growth on rich medium was examined in 37°C and 40°C.

### **4.3. Bioinformatics analysis of *S. cerevisiae* tRNA genes**

As a preparatory stage to the deletion library construction some bioinformatics analysis regarding the number and location of *S. cerevisiae* tRNA genes was needed. For that we integrated data between three databases, the genomic tRNA database, the Saccharomyces genome database (SGD) and the National Center for Biotechnology Information (NCBI). A total of 274 tRNA genes were found in the genome, each was scanned for presence of additional genomic features (such as LTR, ORF etc. ) in the flanking 600 base pairs. This was crucial as we needed to examine which additional features would be co-deleted with the tRNA gene. In addition 500 base pairs flanking the tRNA were scanned using a sliding window of 45 base pairs. Each window was scored for similarity to the yeast genome using BLAST. As a default a cut-off of BLAST E-value of 0.01 was used as a maximum, which corresponds to ~35% identity in 45 base pair segment. This step was essential in order to ensure that the homology sequences used for deletion would be unique to one tRNA gene at a time.

### **4.4. Creation of a tRNA deletion library**

The tRNA deletion library was constructed in a similar way to the *S. cerevisiae* gene-deletion library<sup>76</sup>. The DNA template for the recombination event was created in a two steps sequential PCR. The first PCR created the basic template for the recombination while the second PCR served to lengthen the produced template from the first PCR so it contains 45 base pairs homologues to the desired tRNA deletion. The first PCR reaction used an 83 base pairs upstream primer and an 82 base pairs downstream primer to amplify the HPH antibiotics 'cassette'. These primers contained a genomic sequence that flank either the 5' or 3' end of the tRNA (directly proximal and distal to the start and end of the gene

respectively), 18 and 17bp of sequence common to all gene disruptions, a 20 base pairs unique sequence (the 'molecular bar-code' TAG) and 22 base pairs of sequence, homologous to the HPH gene. The template for this PCR reaction was the pAG32 plasmid, encoding the hygromycin B phosphotransferase conferring resistance to the antibiotic hygromycin B, from the DEL-MARKER-SET (EQUOSCARE)<sup>77</sup>. The second PCR reaction used two tRNA gene specific 45 base pair primers, to extend the tRNA specific homology of the first PCR product to contain 45 base pairs. The product of the second PCR served for yeast transformation.

#### **4.5. Construction of a wild type strain with HPH resistance**

To ensure that the resistance marker does not influence the growth of the deletion strains and enable proper comparison between the tRNA deletion strains and the wild type strain, I created an additional wild-type strain. In this strain the HPH gene, conferring resistance to the antibiotic hygromycin B, was introduced into the genome of the Y5565 strain, to match the resistance of the library strains. The gene was added 1000 bp up-stream of the tL(CAA)G3 gene. For all further analyses this strain was considered the “wild-type”.

#### **4.6. Creation of tRNA double deletions**

Double deletion of tRNA genes was constructed in a similar way to the creation of the tRNA deletion library strains. A strain from the tRNA deletion library was used for the deletion of an additional tRNA gene by means of homologues recombination. The sequential PCR was repeated for the appropriate tRNA gene using the same primers used for the creation of the tRNA deletion library. The templates for the first PCR round were either the pAG25 plasmid encoding the nourseothricin N-acetyl-transferase and conferring resistance to the antibiotics nourseothricin, or the pFA6a-kanMX6 encoding the kanamycin gene and conferring resistance to the antibiotics G418<sup>78</sup>.

#### **4.7. Deletion of the *TRM9* gene**

Deletion of the *TRM9* gene was conducted using homologues recombination. Using gene specific primers, homologous to the start and stop of the *TRM9* gene and flanked by sequence homologues to the pFA6a-kanMX6 served to create the template for the deletion. Colonies were verified as for the tRNA deletion library.

#### **4.8. Verification of tRNA deletion strains**

For each tRNA deletion strain, five different colonies were selected, and verified by two PCR reactions. The junctions of the antibiotics 'cassette' were amplified using two primer combinations. Each primer combination contained a primer homologous to sequence within antibiotics 'cassette' of either the promoter or terminator of the marker gene, and a tRNA specific primer, homologous to sequence upstream or downstream of the tRNA deletion. Only colonies from which the correct products were amplified served for further analysis. For multiple tRNA deletions a third PCR reaction was added for verification. This reaction used the two tRNA gene-specific primers to validate the absence of an amplified product from colonies selected as positive. Three positive colonies were selected for each deletion strain and were used for further analysis. The colonies were usually technical replicates of a single transformation.

#### **4.9. Verification of lethality of tRNA deletions**

To examine essentiality of suspected tRNA genes, the tRNA gene was deleted from a diploid strain BY4347 in a similar manner to the construction of the tRNA deletion library. Resulting colonies were verified, grown on rich medium and allowed to undergo sporulation in a minimal medium containing 1% potassium acetate, for three days at 25°C. The resulting tetrads were then dissected using a Micromanipulator (Singer). Spores were then allowed to germinate at 30°C and scored on plates containing the appropriate antibiotics.

#### **4.10. Construction of complementation plasmids**

In order to reintroduce into the cell a tRNA deleted gene I created a set of "complementation plasmids". Complementation plasmids were constructed for multiple tRNA genes using gene specific primers homologous to 250 base pairs up and down stream of the tRNA gene. Genomic DNA of Y5565 was used to amplify the desired tRNA gene, flowed by cloning to the pRS316 centromeric plasmid<sup>79</sup>. The construction of the multi-copy plasmids of *tR(UCU)E* and *tR(UCU)K* were performed as indicated above, and cloned to the pRS426 plasmid containing a 2 micron Origin of replication, providing about 20 copies in haploid strains<sup>80</sup>. This plasmids were constructed by Idan Frumkin a fellow student in the lab.

#### **4.11. Growth measurements using optical density**

Cultures of both wild type and tRNA deleted strains were grown for two days at 30°C into

the deep stationary growth phase. Cultures were then diluted (1:50) into a divided robotic flask (8 rows) containing various types of fresh media(as detailed in section 1.2). The wild type strain (Y5565+HPH) was placed in the last row of the flask as a control. The growth experiments were performed using a robotic system (TECAN). The robotic system includes a liquid handling system, appropriate for 96 well plates. The flasks were used by the robotic system to build 96 well plates. Each row in the flask corresponded to a row of 12 wells in the 96 well plates. Each 96 well plate contained a wild type strain in addition to seven deletion strains. Mineral oil was added to the 96 plates prior to incubation at 30°C. Plates were grown for 20 hours or longer, and the population growth was monitored every 30 minutes using a multi-well spectrophotometer at 600 nm (INFINITE200). The OD reads served for growth analysis and extraction of growth parameters. In each experiment 12 technical repetitions were performed for each strain. In addition all growth experiments were repeated a least twice to corroborate growth values.

#### **4.12. Reproducibility of OD based growth measurements**

To examine the reproducibility of OD based growth measurements, I analyzed the results using the Spearman rank correlation coefficient. The results I used were from two sets of measurements of the entire tRNA deletion library in rich medium. I analyzed two sets of growth parameters, distinguishing between growth rate and growth yield. The results obtained in this analysis indicated a very strong and significant correlation between the two sets of measurements with  $\rho=0.935$  for growth rate and  $\rho=0.936$  for growth yield.

#### **4.13. Growth measurements using colony size**

The entire library was spotted on three YPD plates, each plate contained 96 colonies<sup>81</sup>. In each plate the first row comprised of strains from the ORF deletion library which exhibit a growth defect in one of the examined stress conditions (representing positive control). The third and eighth columns were of the wild type strain, and were used for normalization. Each colony from these plates was replicated four times to construct plates of 384 colonies. Using the Singer Rotor DHA bench top robot and disposable 384 replicators, the plates were replicated to plates of various media and stress conditions as detailed in section 1.2. Plates were allowed to grow at 30°C until colonies reached their full size. Each plate was scanned twice using a commodity desktop reflective scanner. The colony size was evaluated using Balony yeast colony image analysis software. The data retrieved by the program served for scoring each colony, and eventually each strain, for colony size average and deviation from

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the standard.

#### 4.14. Double deletion epistasis calculations

The epistasis between any two deletion strains was calculated according to a conventional nonscaled measure of epistatic interactions according to the following equation<sup>82</sup>:

$\varepsilon = W_{XY} - W_X W_Y$  In which  $W_X$  and  $W_Y$  represent the growth values (separating growth rate and growth yield) of the single deletions and  $W_{XY}$  represents the growth values of the corresponding double deletion. The results of the calculation indicate the nature of the epistasis,  $\varepsilon = 0$  indicates no epistasis,  $\varepsilon < 0$  aggravation and  $\varepsilon > 0$  buffering.

#### 4.15. Genome-wide expression experiments

Overnight cultures were diluted into a fresh rich medium and grown to a cell concentration of  $1.5 \times 10^7$  cells/ml. Cells were then harvested frozen in liquid nitrogen, and RNA was extracted using MasterPure™ (EPICENTER Biotechnologies). The quality of the RNA was assessed using the BIOANALYZER 2100 platform (AGILENT); the samples were then processed and hybridized to Affymetrix yeast 2.0 microarrays using the Affymetrix GeneChip system according to manufacturer's instructions. The Array measurements were performed on three batches each containing a wild type strain and deletion strains. Background adjustment was done using the Robust Multi-array Average (RMA) procedure with Quintile normalization. Each strain was compared to the wild-type in its batch to minimize day and batch effects. Each tRNA deletion was assayed by a single array, tR(CCU)J was assayed in two biological repetitions.

#### 4.16. Expression of tRNA flanking genes

To examine the change in expression of tRNA nearby ORFs, and to verify that there is no apparent effect of the deletion of the tRNA on the expression of these ORFs I examined the expression change for a set of 13 tRNA gene deletions using expression microarray. A significant change in the expression level of a flanking ORF was observed for only one of 13 tRNA deletion strains examined, in the *tY(GUA)J2* gene (**Table 1**). In this tRNA deletion strain one of the flanking genes located 1kB downstream from the tRNA gene, is an essential gene, *CDC8*, which is significantly down regulated at the tRNA deletion strain compared to the wild-type. An additional strain *tW(CCA)M* shows a significant up-regulation of a nearby gene *FAR3* compared to the wild type strain. Consequently the effect of these nearby genes should be considered once analyzing the expression profile of the relevant strains. Yet it

should be mentioned that GO analysis performed in these strains did not expose enrichment in processes relevant to these genes.

Strain	Upstream gene	Fold change	Downstream gene	Fold change2
$\Delta tL(GAG)G$	YGR106C	-0.089	YGR108W	-0.364
$\Delta tM(CAU)C$	YCR018C	0.026	YCR019W	-0.022
$\Delta tR(UCU)E$	YEL011W	0.067	YEL009C	0.085
$\Delta tC(GCA)B$	YBR208C	0.601	YBR210W	0.036
$\Delta tR(CCU)J-1$	YJR054W	0.298	YJR055W	-0.639
$\Delta tR(CCU)J-2$	YJR054W	0.286	YJR055W	-0.804
$\Delta tW(CCA)M$	YMR050C	-	YMR052W	1.490
$\Delta tW(CCA)M$ yield	YMR050C	-	YMR052W	0.946
$\Delta tY(GUA)J2$	YJR056C	0.155	YJR057W	-1.422
$\Delta tL(CAA)G1$	YGL159W	0.235	YGL158W	-0.070
$\Delta tL(CAA)G2$	YGL040C	0.024	YGL039W	0.440
$\Delta tP(AGG)C$	YCR005C	-0.767	YCR007C	-0.125
$\Delta tR(UCU)M2$	YML071C	-0.413	YML070W	-0.350
$\Delta tH(GUG)G1$	YGL205W	0.767	YGL203C	0.084
$\Delta tV(AAC)J$	YJL037W	-0.127	YJL036W	-0.220
$\Delta tR(CCU)J/trm9$	YJR054W	0.739	YJR055W	-0.708
$\Delta trm9$	YJR054W	0.564	YJR055W	0.318

**Table 1 - Expression changes of ORFs flanking tRNA genes**

The above table indicates for each tRNA deletion the flanking genes, and the fold change (minus sign indicates down regulation and no sign indicates up regulation) for each gene obtained in the microarrays. For each tRNA deletion strain examined by microarrays, the Y names of the surrounding genes along with their expression values in the micro-arrays

#### 4.17. Bioinformatics analysis of expression data

The expression data retrieved for the 13 tRNA deletion strains was subjected to several analysis. To examine GO enrichment we used the Gorilla tool<sup>83</sup> on a ranked gene list. Correlations to the "compendium" database<sup>84,85</sup> compared change in expression of the tRNA deletions to those of the "compendium" deletions and growth conditions calculating both Spearman and Pearson correlation coefficients. The codon usage was calculated for the expression profile of all 13 tRNA gene deletions and correlations were calculated using both Pearson and Spearman correlation coefficients for each of 65 codons. The initiator methionine and three stop codons were excluded from the analysis.

#### 4.18. Monitoring folding stress in the cell

Monitoring the folding stress in the cell was performed using the CHFP-VHL plasmid (mCherry), kindly provided by Dr. Daniel Kaganovich<sup>86</sup>. Selected tRNA deletion strains, and the wild-type were transformed with the plasmid and selected on SD-URA plates. Overnight cutlers were grown on SC+2% raffinose, were diluted (1:10) into SC+2% galactose to induce the transcription of the CHFP-VHL. Cells were visualized using an Olympus IX71 microscope controlled by Delta Vision SoftWoRx 3.5.1 software, with X60 oil lens. Images were captured by a Photometrics Coolsnap HQ camera with excitation at 555/28 nm and emission at

617/73 nm (mCherry). Images were scored using the ImageJan Image Processing and Analysis software. For examination of folding under AZC stress, following induction with galactose cells were supplemented with 2.5mM AZC (Sigma) incubated for 30 minutes at 30°C, and visualized as before.

#### **4.19. Lab evolution experiments**

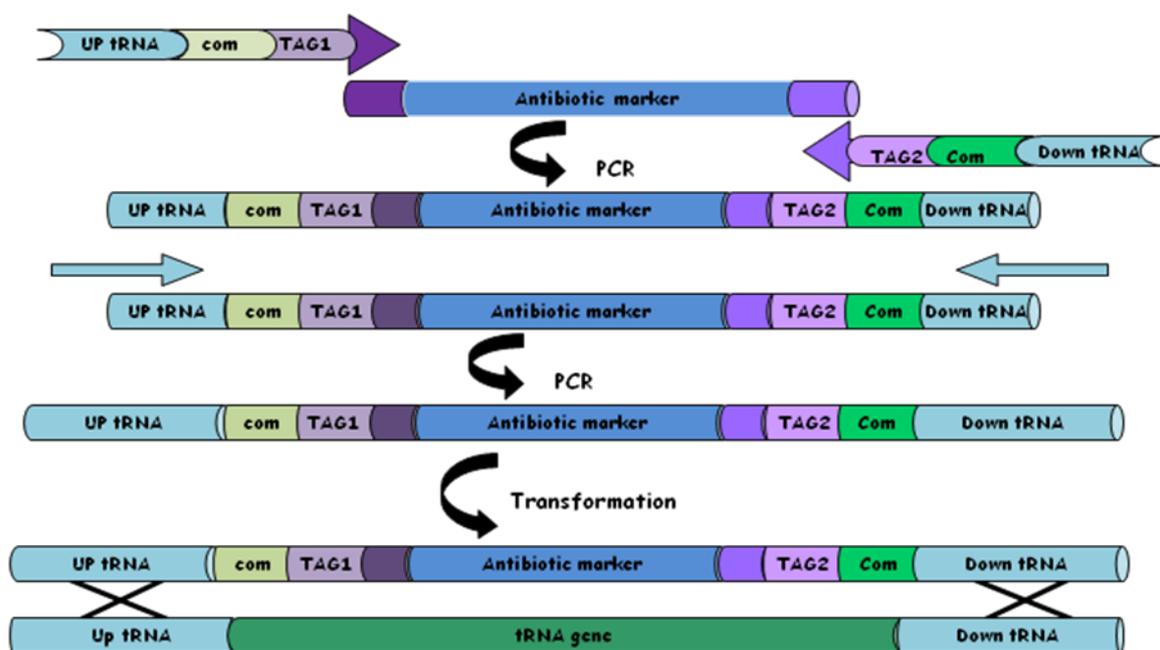
The lab evolution experiment was carried out by serial dilution of the  $\Delta tR(CCUG)$  strain grown on rich medium at 30°C. Cells were grown until reaching stationary phase under the relevant condition and then diluted by a factor of 1:120 into fresh media (about 7 generations per dilution). The evolving population was monitored by growth analysis using an OD based method, for changes in growth parameters. The lab evolution was repeated daily until no growth differences were detected between the wild type and evolved populations. This state was reached in all evolved population, after approximately 40 days. To sequence the tR(UCU) genes in the evolved population, genomic DNA was extracted using the MasterPure™ Complete DNA purification Kit (epicenter), the genomic DNA then served for PCR amplification, using tRNA gene specific primers, for all 11 gene copies. The resulting PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN), and sequenced using Automated DNA sequencing is done using a 3730 DNA Analyzer from ABI.

## 5. Results

### 5.1. Creation of a tRNA deletion library

#### 5.1.1. Construction of an *S. cerevisiae* tRNA deletion library

In *S. cerevisiae* there are 274 tRNA genes corresponding to 41 different tRNA isoacceptor molecules, in addition to an initiator methionine tRNA. In my study I aimed to delete each and every one of the 274 cytosolic tRNA genes in the *S. cerevisiae* genome. The tRNA deletion library was constructed in a similar way to the *S. cerevisiae* gene-deletion library<sup>76</sup>, by means of homologous recombination using an antibiotics 'cassette' as a marker. The antibiotics resistance gene in each resulting mutant was flanked by two distinct 20-nucleotide sequences that can serve as molecular 'bar-codes' to uniquely identify each deletion mutant. Each tRNA deletion "cassette" was constructed using two sequential PCR reactions (**Figure 1** and Materials and Methods)<sup>1</sup>.



**Figure 1 - Schematic representation of a tRNA deletion process.**

In this schema the first two PCR rounds needed to create the template for deletion are shown, in addition to the transformation and deletion by homologous recombination. The first PCR round was performed using a plasmid with antibiotic marker, as the DNA template, and primers containing only 23 base pairs out of the 45 base pairs chosen upstream and downstream of each tRNA gene. In the second PCR the product of the first PCR round was lengthened to contain 45 base pairs homologous to sequences flanking the tRNA gene. The product of this PCR round served for transformation. The deletion event is the result of homologous recombination.

In the first PCR reaction the gene conferring resistance to hygromycin B, was amplified. In a second PCR reaction the product of the first reaction was further amplified and prolonged to contain 45 base pairs homologous to the sequences flanking the tRNA gene (**Figure 1**). The

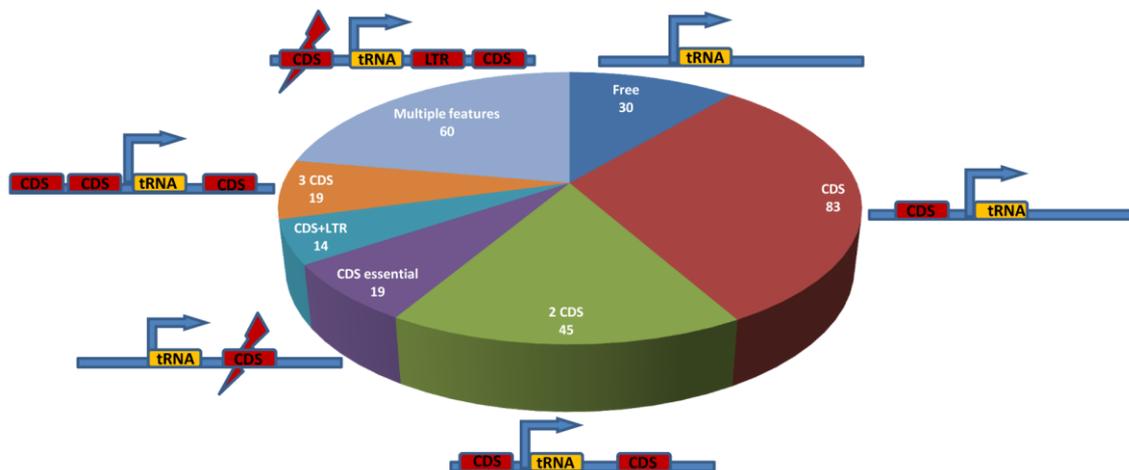
length and sequence homology of the PCR products were chosen in a way which would maximize the targeting specificity and recombination efficiency during an event of mitotic recombination of the gene disruption cassette<sup>78,87</sup>.

### **5.1.2. Bioinformatics analysis of *S. cerevisiae* tRNA genes**

The creation of the tRNA deletion library is based on the ability to specifically delete each tRNA gene by homologous recombination. This deletion strategy required a relatively long (45 base pairs) homologous sequence from each side for the recombination event to occur with high efficiency. The length of the homologous sequence was chosen based on relevant experiments in which it was shown that the efficiency of the process relies on the extent of the flanking homologous sequence<sup>87-89</sup>.

The sequences of the tRNA genes are highly similar as a whole, and even more so when looking at different isoacceptors of the same amino acid, were the differences might be less than a few base pairs dispersed along the gene. Different copies of the same isoacceptor are usually completely identical in their sequence. Given that the tRNA genes are short (about 73 base pairs) and relatively similar to each other, it was clear that the deletion by homologous recombination had to be based on the flanking sequences upstream and downstream to the tRNA genes. This is in contrast to the ORF-deletion library<sup>75</sup> in which sufficiently unique sequence within the deleted genes could be obtained that alleviated the need to target additional flanking sequences. The inherent compromise in our approach was thus the need to affect also the flanks of each deleted tRNA gene, which often contained potentially functional features. Thus in the design of the tRNA deletion library two main concerns had to be met. The first concern was that the deletion of each tRNA gene will not damage the integrity of other proximal genomic features. The second concern was to keep the gene specificity, preventing cases in which the chosen sequence similarity would lead to low specificity, resulting in nonspecific targeting of other tRNA genes. To tackle both concerns a number of bioinformatics analyses were performed, the first of which mapped the genomic sequences in a region of 600 base pairs upstream and downstream of all tRNA genes, identifying all the genomic features in these areas. Potential near-by genomic features include protein coding genes (CDS), long terminal repeats (LTR), autonomously replicating sequences (ARS), other tRNA genes, retrotransposons and transposable elements. To assess the extent of potential effect on near-by CDSs I used published data to estimate the length of upstream and downstream un-translated and regulatory regions of

CDSs<sup>90,91</sup>. This bioinformatics analysis made it clear that the tRNA genes are mostly flanked by CDSs or LTR sequences. Yet these genomic features flanking the tRNA genes are randomly distributed and there is no tendency for a specific amino acid or isoacceptor to contain such features in the near surrounding. In addition almost every tRNA family has at least a single copy which is depleted of any genomic features in the flanking 500 base pairs ("free") (**Figure 2**). In the analysis I divided the CDS into essential and non-essential, according to the effect of their deletion on the fitness (information from the *S. cerevisiae* gene-deletion library found in SGD<sup>76</sup>). As a whole there are 330 CDSs flanking the tRNA genes, most of them (61%) are categorized as non-essential.



**Figure 2 - Genomic features surrounding the tRNA genes in *S. cerevisiae*.**

The pie chart shows the genomic features surrounding all 274 tRNA genes in *S. cerevisiae*. The genomic feature or combinations on genomic features are indicated on each slice alongside the number of tRNA genes which are surrounded by them. The illustrations around the pie chart manifest the genomic architecture for some features including the tRNA gene. The arrow indicates the transcription start site of the tRNA gene. CDS with a thunder indicate essential CDSs.

There are tRNA genes which are flanked by two CDSs on both sides (**Figure 2**). Ten tRNA genes are overlapped with a CDS sequence. Most of these overlapping CDSs are considered "dubious" by the Saccharomyces Genome Database (SGD), thus less likely to encode for protein, and thus less likely to impose a problem upon deletion of the overlapping tRNA gene. Out of these 10 there were 5 cases of tRNA genes overlapped by CDSs that were deleted in the *S. cerevisiae* gene-deletion library. All of these cases resulted in a viable phenotype. Relying on this analysis I created the tRNA deletion library in batches according to the nature of flanking genomic features. I started from the section of tRNA genes which were "free" of genomic features, and proceeded to more challenging cases of tRNA genes that reside near a CDS or LTR. Finally I tackled the creation of the most challenging cases in which multiple genomic features surrounded the tRNA gene. A total of 69 tRNA genes were

not deleted as they comprise of genes with highly complicated genomic architecture and their deletion would most likely effect additional genes in the genome (see section 5.2.4).

## **5.2. Primer design for *S. cerevisiae* tRNA deletion library**

A second bioinformatics analysis was aimed at primer design. In this analysis a region of 500 base pairs upstream and downstream of the tRNA genes was scanned in an attempt to identify segments of 45 base pairs that can serve as primers for specific tRNA gene deletion. The analysis was performed using a sliding window and scanned the flanking sequences, in windows of 45 base pairs. The sequence of each window was then scored for similarity to the genome using BLAST against the yeast genome to exclude primers with potential sequence matches to off-target sequences. As a default I used a cut-off of BLAST E-value of 0.01, which correspond to ~35% identity at 45 bps, above which a match is not expected to give rise to effective DNA hybridization and homologous recombination event<sup>1,76,88</sup>. There were a number of special cases for which the primer design was not trivial. Such special cases were when genomic features, mainly LTRs and CDSs, are in very close proximity to the tRNA gene and tRNA gene duplications, an entire region including a tRNA gene which was duplicated multiple times in the genome. When designing primers for such tRNA gene deletions I had to relay in many cases on sequences from within the tRNA gene. In extreme cases when no primer was found, a less stringent search cut-off was used.

### **5.2.1. Verification of tRNA gene deletions**

Extensive verification of each deletion strain was highly important to the construction of the library as a quality assurance stage. Cases of failed deletion may arise from two sources: the first is the potential insertion of the resistance gene in a different location than the planned one thus resulting in antibiotics resistant colonies which are not deleted for the tRNA. The second may be due to an event of a pre-insertion duplication either of a fragment containing the targeted tRNA genes and flanking sequences, or of an entire chromosome, as was shown before in deletion of protein coding genes<sup>4</sup>.

The correct replacement of each tRNA gene was verified by multiple PCR amplifications (Materials and Methods). The recombination junctions of each deletion were verified by two primer combinations. For each tRNA deletion five different colonies were selected for verification using PCR prior to growth analysis characterizations. An effort was made to find at least 3 positive colonies for each tRNA gene deletion. For the purposes of growth analysis characterization at least two different colonies were assayed for each tRNA deletion.

### 5.2.2. Verification of lethal tRNA gene deletions

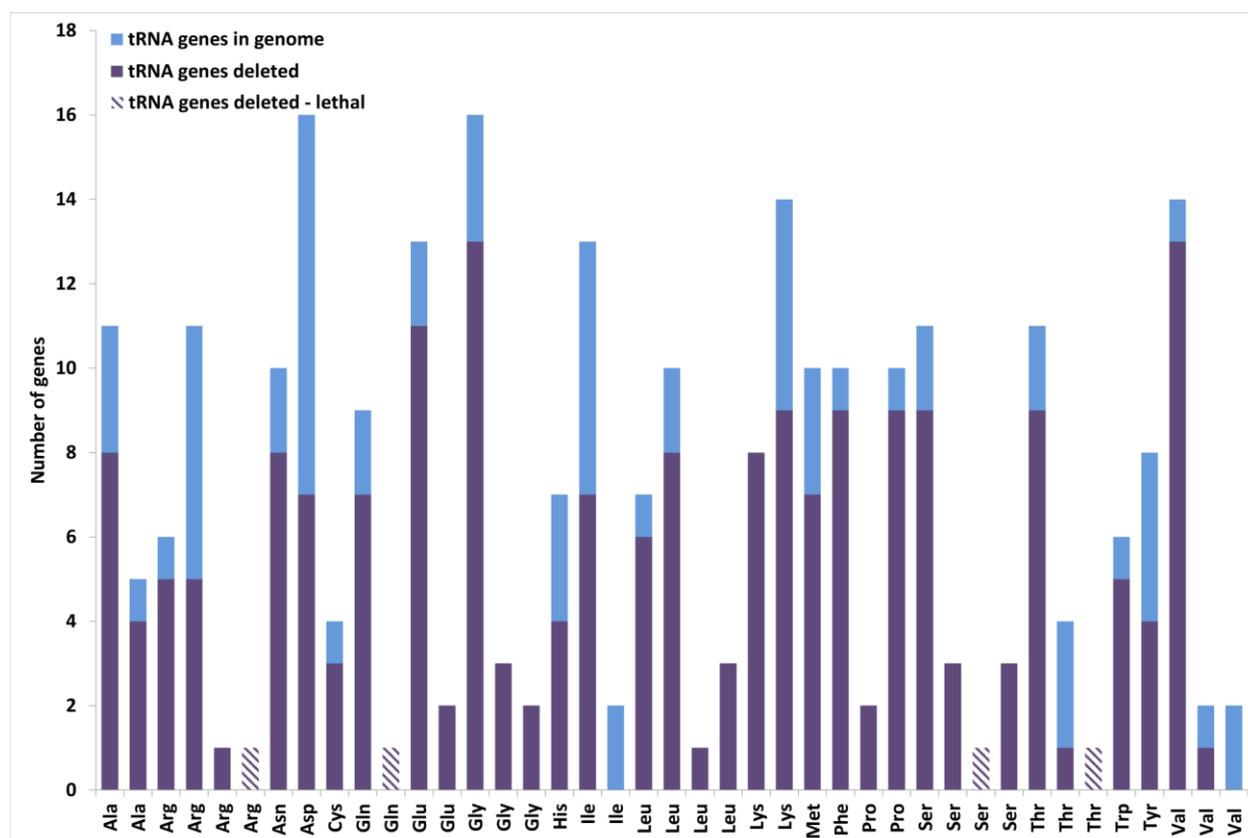
To assess cases of deletions that result in lethality and to avoid any incidences of false positives, each tRNA deletion strain which showed difficulties in production (very low transformation efficiency, difficulty in finding positive colonies by PCR) was created in a diploid strain and further analyzed by tetrad analysis to verify lethality (Materials and Methods). Four tRNA deletions were found to be lethal, *tT(CGT)K*, *tS(CGA)C*, *tR(CCG)L* and *tQ(CUG)M*, all representing cases of singleton tRNA genes. All these cases were described previously in the literature<sup>56,92</sup>.

### 5.2.3. Evaluation of the phenotypic effect caused by flanking genes

As most of the genomic features flanking the tRNA genes are CDSs, special attention had to be given to the possibility of mistakenly effecting these features or the sequences relevant for their proper expression control. To evaluate the extent to which deletions of tRNA genes affected expression of near-by genes I examined the genome-wide expression profile of several tRNA deletion strains using microarrays. In tRNA deletions which exhibit any growth defect in rich medium the expression of the flanking genes was examined. I found that apart from one tRNA deletion strain in gene *tY(GCA)J2*, in which indeed the near-by gene's expression was significantly reduced (table 1), the rest of the tRNA deletions appear not to elicit such an effect. To further tackle this concern from a different angle I used the ORF deletion library in cases where the near-by gene is a non-essential ORF. I chose a number of tRNA deletion strains, which exhibit a growth defect in either rich or minimal media, and compared the growth of the relevant ORF deletion strain to the growth of the relevant tRNA deletion strain. A total of 20 deletion strains from the ORF deletion library were examined, each flanking a different tRNA gene. The growth parameters of the ORF deletions were compared to those of the relevant tRNA deletion strains. I made sure to compare each deletion to the relevant wild-type with the same genetic background. One must bear in mind that this is a very strict control to compare the deletion of the entire ORF to that of a tRNA gene, thus if the ORF deletion shows no phenotype and the tRNA does it is safe to say that the deletion of the tRNA gene has no apparent influence on the mRNA or translation levels of the relevant ORF and it represents a genuine effect of the tRNA deletion itself. Reassuringly most (18/20) ORF deletions which were examined showed no growth phenotype compared to the relevant tRNA deletion strains. The conclusions from these controls indicates that most phenotypic defects associated to tRNA deletions represent genuine cases in which the tRNA itself, rather than other features in its surrounding was responsible for the phenotypic effect.

### 5.2.4. The *S. cerevisiae* tRNA deletion library - Final status

The tRNA deletion library contains 203 cytosolic tRNA deletions, including the four lethal tRNA strains. I constructed and verified the deletion of about 75% of the tRNA genes in the *S. cerevisiae* genome, and touched upon the 40 out of the 42 isoacceptor families (**Figure 3**). Reaching this point I decided to stop the construction of the library, given that the remaining tRNA gene deletions imposed some serious problems of primer design and possible effects on surrounding genomic features. Creating these deletions might not be beneficial for our purpose of establishing the effect of these tRNA gene deletions on the cell, as it would be hard to differentiate it from the effect caused by other genomic features.



**Figure 3 - Schematic representation of tRNA genes in the genome vs. deleted tRNA genes in the library.**

In this bar diagram each bar represents a tRNA iso-acceptor. The X axis indicates for each bar the amino acid identity, while the Y axis indicates the number of gene copies for each isoacceptor. The blue bars represent the copy number for each tRNA isoacceptor in the genome. The purple bars represent the number of tRNA genes deleted for each tRNA isoacceptor in the deletion library. Bars with dashed purple lines indicate essential tRNA genes.

### 5.3. Scoring growth phenotypes of tRNA deletion strains

We implemented two robotics-based means to screen and score mutant phenotypes under a diversity of growth conditions. The first method, which is based on measuring colony size<sup>93</sup> as a measure of growth ability, is very efficient and allows fast exploration of the entire library on a diversity of growth conditions. Fitness of strains in this common method is assumed to be proportional to the colony size<sup>14</sup>. We scanned by this means the entire library on 14 growth conditions (**Table 2**), these conditions represent rich and minimal media, a diversity of biotic stresses, drugs and translation inhibitor. In parallel we used a more sensitive screening method which is based on optical density (OD) measurements to obtain growth curves for the entire library during a 24 hours growth period. The obtained growth curves are a source of two fitness measures – the growth rate and the growth yield, i.e. OD at the entry into stationary phase. These two measures roughly correspond to the slope of the OD vs. time curve during the exponential phase and the OD level after diauxic shift respectively. Comparison of the results obtained in the two methods shows good similarity, although it is clear that the colony size method is less sensitive and thus depicts fewer phenotypes. While the OD-based measurement is more time-consuming this method exposed more tRNA deletion strains exhibiting a growth defect at any given condition. This method is capable of detecting subtle phenotypic defects in various phases of the growth, (lag phase, exponential and post-diauxic shift growth) which could not be easily depicted using the colony size method. A great deal of effort has been spent in calibrating the OD-based method and defining the noise cut-off and the level of reproducibility so that it can be used with high degree of confidence. Such calibrations were not performed for the colony size method. Nevertheless the colony base method was intended to provide low resolution screens, to aid in the selection of conditions for the high resolution OD screens, and provide a method for screening across conditions which are technically difficult using the OD based method. I thus describe first a screen of 14 conditions with the colony size method and then present results with higher resolution obtained with the OD-based method on a selection of the growth conditions.

In all measurements a wild-type strain was included for proper comparison. To facilitate proper comparison between the wild-type and the tRNA deletion strains, which carry a selectable marker, I introduced the same selectable marker to the wild-type too, yet without deleting any tRNA gene, and referred to the obtained strain as "wild-type" throughout.

Though the majority of the tRNAs are present in multiple gene copies in the genome, there are 6 tRNAs for which the genome contains only a single copy (in addition to 20 of the tRNA isoacceptors that are not present at all in the genome<sup>94</sup>). In agreement with previous studies<sup>56,92</sup>, I found that four out of the six single-copy tRNAs deletions are lethal, and two tRNA genes, *tR(CCU)J* and *tL(GAG)G*, are viable. None of the deletion strains that correspond to multi gene isoacceptor family (i.e. sets of two or more tRNA genes that share the same anti-codon identity) were lethal. We thus focus on the none-lethal deletion strains in all subsequent analyses.

#### **5.4. Screen of tRNA deletion phenotypes using colony size method**

In the colony size assay the entire library was represented on three different 96 plates. In each such plate two separate columns contained the wild type strain and served for normalization. As a technical control, the first row in each plate was composed of ORF deletion strains that are known to be defective on one or more of the examined conditions. Each such plate served as a template to build a corresponding 384 plate, in which each strain was represented by four separate colonies (Materials and Methods). The mean colony size and standard deviation of each quadruplet, representing a tRNA deletion strain, was calculated and served for scoring. Given the high degree of geographical effects and noise at the periphery of the plate<sup>81</sup> I excluded the two outermost rows and columns from this analysis. I began by characterizing the deletion library in rich medium (YPD).

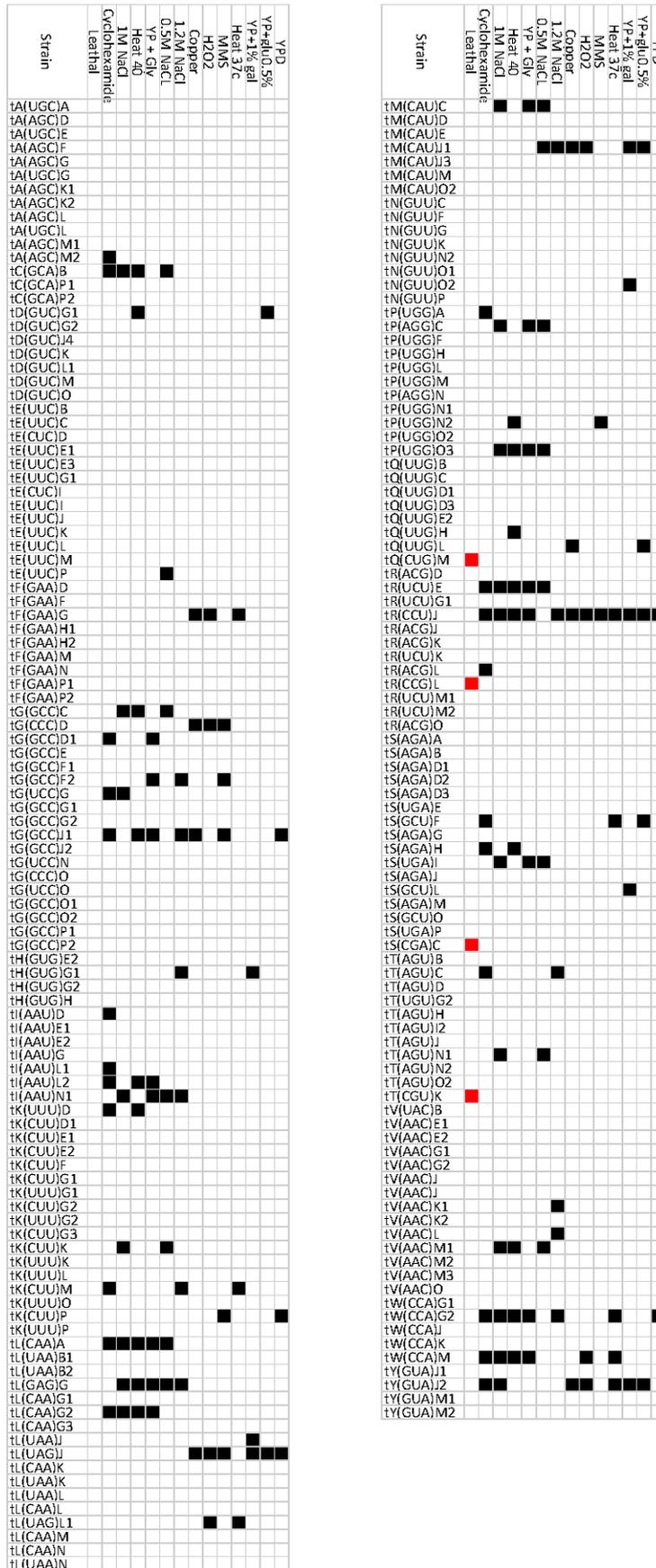
Examination of the tRNA deletion library in this condition exposed only a handful of the tRNA deletion strains (5 tRNA deletions) which showed a significant reduction in colony size compared to the wild-type; likewise, very few of the representative ORF deletion strains (1-2 ORF deletions) showed a significant phenotype in this condition. The five tRNA deletions which showed growth aggravation were *tR(CCU)J*, *tG(GCC)J1*, *tK(CUU)P*, *tW(CCA)G2*, and *tL(UAG)J*. These deletions are all of different amino acids, and apart from the deletion of *tR(CCU)J* which is a single copy deletion, are all part of multi-copy families. Three of these tRNA deletions (*tR(CCU)J*, *tG(GCC)J1* and *tW(CCA)G2*) were found to exhibit a growth rate defect, in rich growth medium using the OD based measurement of between 3-8% degrees, compared to the wild type. An additional strain *tL(UAG)J* exhibits a severe growth yield reduction compared to the wild type.

Stress	Concentration
Copper	10mM
H <sub>2</sub> O <sub>2</sub>	10mM
MMS	0.06%
Cyclohexamide	300µg/µl
YP+galactose	1%
YP+glycerol	3%
YP+glucose	2%
YP+glucose	0.50%
NaCl	0.5M
NaCl	1M
NaCl	1.2M
SC+glucose	0.02
Heat Shock	40°C
Heat Shock	37°C

**Table 2 – Growth conditions used for screening the tRNA library using the colony size method**

The growth conditions indicated above were used to screen the tRNA deletion library in the colony based method. For each stress the concentration is mentioned on the right, if multiple concentrations of the same stress were used they are all indicated. Growth experiments were performed at 30°C except for the heat shock for which temperatures are indicated in the table.

In order to expose additional potential tRNA genes with a functional role, and potentially find condition-specific essentiality for some of the tRNA genes, I screened the deletion library under a diverse set of stressful growth conditions. The set of challenges was designed to consist of biotic stresses that resemble those that yeast experience in the wild (heat-shock at 37°C or 40°C, oxidative stress mediated with H<sub>2</sub>O<sub>2</sub>, different concentrations of NaCl and DNA damage) in addition to growing on different carbon sources and translation inhibitor, see **Table 2**. As expected the growth experiments on stressful conditions exposed additional condition-specific functional contribution for an increasing number of the tRNA genes. For example, we identified a total of 52 tRNA genes (which constitute ~26% of the tRNAs represented in the library) that show an appreciable reduction in colony size in at least one of the 14 tested conditions. Examining each condition alone revealed that the highest number of tRNA deletion strains with decreased colony size was observed in Cycloheximide (translation inhibitor), heat shock and NaCl (**Figure 4**). In contrast, rich medium, showed the smallest number of strains with reduced colony size (five tRNA deletions) (**Figure 4**).



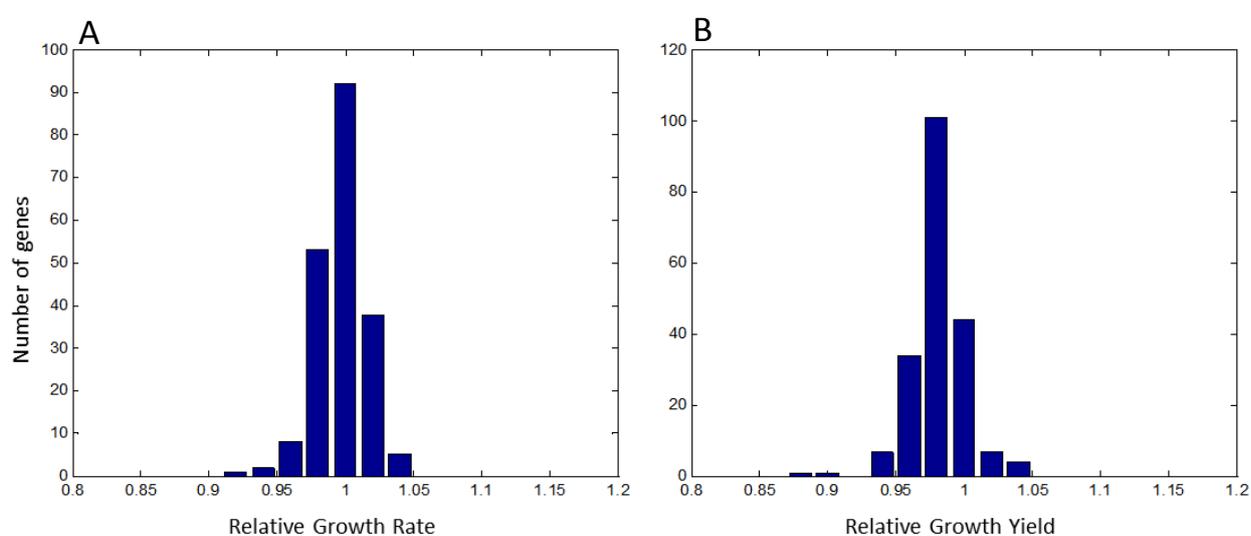
**Figure 4 –Colony size reduction for tRNA deletion strains in a set of 13 growth conditions.** Growth conditions are indicated at the top of the matrix where each row is a tRNA deletion strain and each column is a growth condition. The tRNA deletion strains are arranged in the matrix according to isoacceptors and amino acid in alphabet order. Black squares indicate a significant decrease in colony size, while white indicates no change. Lethal tRNA deletion strains are indicated by red squares.

Taken together it was expected that although rich medium exposes few growth phenotypes in this method, turning to stressful conditions exposed more condition specific phenotypes for certain tRNA deletions. In this analysis there were tRNA deletions which showed reduction in colony size across almost all the examined conditions such as *tR(CCU)J*, *tY(GUA)J2*, *tM(CAU)J1*, *tL(UAG)J* and *tG(GCC)J1*. Four of these strains also showed reduction in growth rates to various extents once examined in the OD-based method. The *tL(UAG)J* does not exhibit a growth rate defect in the OD based method but yet it exhibits a prolonged lag phase which is tightly connected to growth rate. These observations may indicate that reduced colony size may manifest the result of a defect in growth rate, rather than a defect in growth yield. In addition these five tRNA gene deletions are located on chromosome 10 (J). The connection between tRNA gene positioning on the chromosome and growth defect may be attributed to the fact that tRNA genes tend to cluster in the nucleolus<sup>95,96</sup>, consistent with this finding tRNA genes are significantly enriched for interaction with sites neighboring other tRNA genes, and can be clustered based on this tendency. Most tRNA genes on chromosome 10 (11/17) are clustered with centromeres<sup>97</sup>. Thus it is possible that the extent of phenotypes observed upon deletion of tRNA genes on this chromosome could represent cases in which features related to the centromeres' integrity and function may have been compromised.

I followed these observations by examining the similarity between all 14 conditions, in terms of their effect on each of the tRNA deletion strains (**Figure 4**). I found a high similarity between the tRNA deletions which show decreased colony size in the presence of Cycloheximide, Osmotic stress (at various NaCl concentrations) and Heat Shock (the effect was more pronounced in 40°). These conditions also exposed the largest sets of tRNA deletion. Cycloheximide which is an inhibitor of protein synthesis interferes with the peptidyl transfer step and thus blocks translation elongation. As such this drug was expected to expose tRNA deletions which are further aggravated by this drug in the translation process. The high similarity between various stress (Cycloheximide, Osmotic stress and Heat shock) and the large number of tRNA deletions exhibiting aggravation in these conditions, might manifest a similarity in the effect on translation and translation related mechanisms, such as the quality control or amino acid metabolism, in these stress conditions.

### 5.4.1. Screen of the tRNA deletion library using OD based method

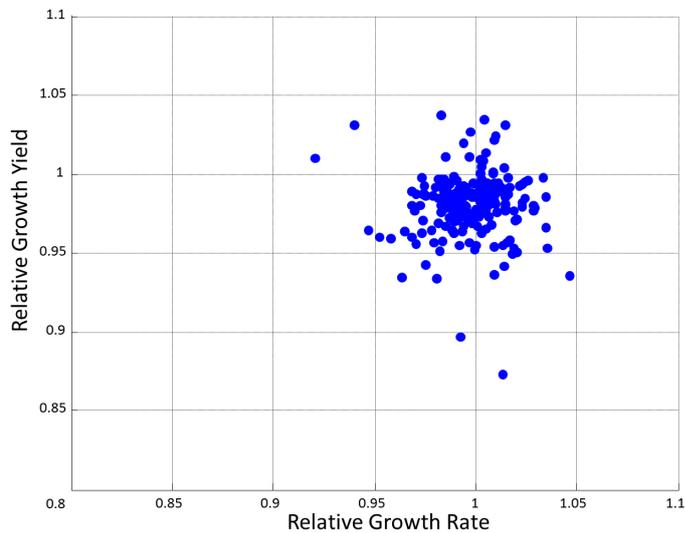
I next turned to examine the tRNA deletion library strains with a more sensitive method using automatic acquisition of growth curves for each strain following optical density measurements. This measurement method exposed a richer set of tRNA genes that show a growth defect. We characterize each deletion strain by two measures derived from its growth curve, namely the growth rate and the growth yield. The growth rate and growth yield of the Wild type strain served for normalization and they were each defined as 1. Thus each tRNA deletion strain is scored for growth rate and growth yield relative to the wild type strain; see **Figure 5** for a histogram of growth defect values.



**Figure 5 - Histogram of relative growth rate values and relative growth yield values on rich medium**

**A**-Histogram of relative growth rate values (tRNA deletions compared to wild type) for the entire tRNA deletion library on rich medium. **B**- Histogram of relative growth yield values (tRNA deletions compared to wild type) for the entire tRNA deletion library on rich medium. The wild type growth rate and growth yield values are set at 1. The X axis indicates relative growth rate values. The Y axis indicates number of tRNA deletions.

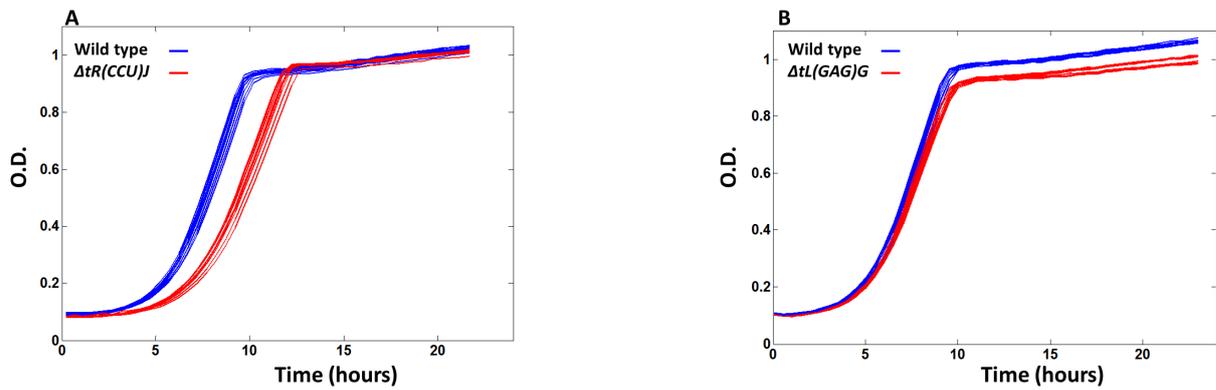
The mean growth rate of the tRNA deletion library was 0.997, representing a very minor decrease compared to the wild-type and the lowest and highest observed growth rates were 0.92 of *tR(CCU)J* and 1.04 for *tV(AAC)L* respectively. The mean growth yield for the entire library was 0.98, and the lowest and highest growth yield were 0.87 for *tW(CAA)M* and 1.03 for *tA(AGC)L* respectively. Comparing these two measures between each deletion mutant and the wild-type reveals phenotypic effects in at least one of the two measures for 20% of the library when grown in a rich medium condition (**Figure 6**). Interestingly effects on growth yield and growth rate were generally not correlated, with some distinct strains showing a defect in growth yield and others in growth rate (**Figure 6**). Further interesting is the realization that some of the deletion strains actually grow better than the wild-type, especially in terms of the growth rate (**Figure 5, Figure 6**). This situation resembles related observations made in ORF deletion strains and suggests that the cost of expressing some genes in particular condition may exceed the benefit from them <sup>75</sup>.



**Figure 6 – Dot plot of Relative Growth rate and Relative Growth Yield for the tRNA deletion library in rich medium.** Each dot in the figure represents a tRNA deletion strain, and the corresponding growth rate and growth yield values relative to the wild type. Experiment was performed in rich medium at 30°C.

### 5.5. Deletion of single-copy tRNA genes

Out of six single copy tRNA genes in the *S. cerevisiae* genome four are essential, and two are sick, though dispensable. The dispensability of these two single copy genes is intriguing and it suggests an alternative translation route upon deletion. I reasoned that the lost function is provided for by wobble interactions through other tRNA isoacceptors which are able to decode more than one triplet, by pairing with more than one type of base at the third position of the codon. Despite the wobble interaction which accounts for their dispensability the deletions of *tR(CCU)J* and *tL(GAG)G* exhibit severe growth defects which were never characterized. The *tR(CCU)J* gene that decodes the arginine AGG codon, exhibits the most severe growth rate defect in the deletion library. In addition this strain has a severely extended lag period, though interestingly with no apparent defect in growth yield (**Figure 7**). The *tL(GAG)G* that decodes the CUC leucine. To assess that the growth defects exhibited by the two single copy tRNA deletions were direct result of the tRNA deletion, and not the effect of other genomic features it was crucial that we compensated the growth defect by addition of the relevant tRNA genes.



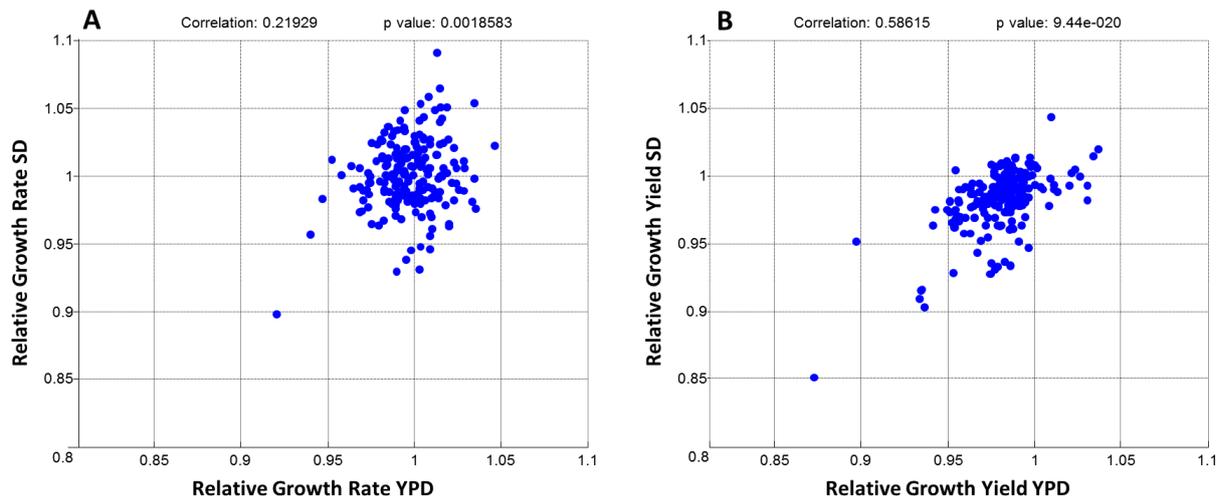
**Figure 7 - Growth curves of Wild type vs.  $\Delta tR(CCU)J$  and  $\Delta tL(GAG)G$ .**

**A-** Growth curve of  $\Delta tR(CCU)J$  (red) vs. Wild type (blue). **B-** Growth curve of  $\Delta tL(GAG)G$  (red) vs. Wild type (blue). In both figures the X axis indicates time in hours, the Y axis indicates OD values. Both growth experiments were performed using the robotic OD based method in rich medium at 30°C.

I tried to complement the growth defect using a centromeric plasmid containing each of the genes, along with flanking sequences of up to 250 base pairs to insure proper transcription of the tRNA gene. The growth of the complemented  $tR(CCU)J$  deletion strain was evaluated on several media (YPD, YP containing 0.5% glucose, minimal and minimal containing 1mM DTT) and was reassuringly found to exhibit a wild type growth rate (not shown). This complemented strain exhibited change in growth yield, a phenotype which is not characteristic of the  $tR(CCU)J$  deletion, so is most likely a result of the plasmid burden on the cell. A control using the  $tR(CCU)J$  deletion and an empty plasmid confirmed this assumption as it showed a similar inferiority in yield. The  $tL(GAG)G$  was also complemented and seemed to markedly decrease the growth yield defect but given the defect in growth yield resulting from the plasmid burden on the cell, it was difficult to conclude whether the phenotype was fully compensated. At the same time one should bear in mind that the  $tL(GAG)G$  gene is not flanked by close genomic features and thus the growth defect it exhibits is less likely to be the result of effecting surrounding genes.

## 5.6. Screening the tRNA deletion library across stress conditions

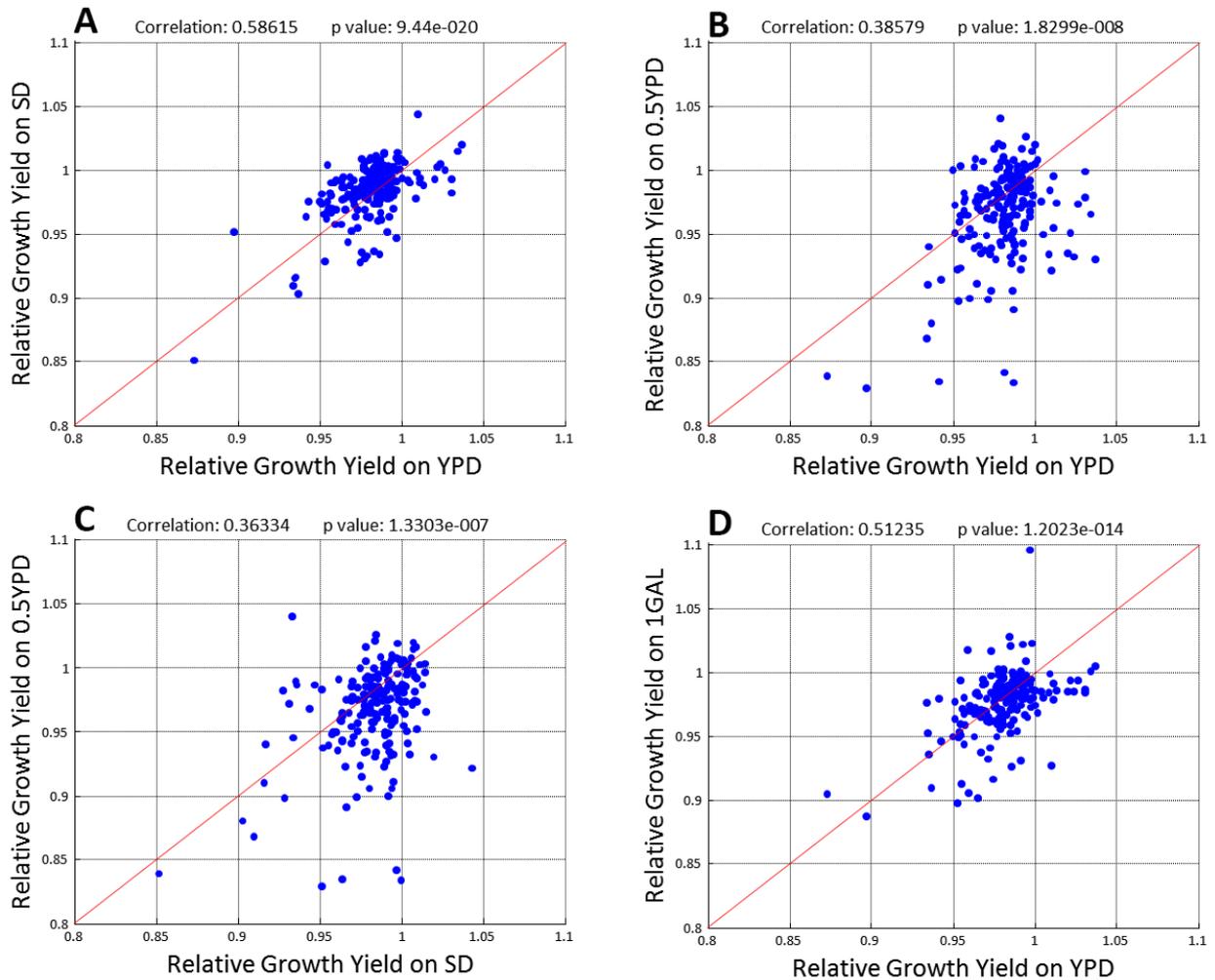
I selected four out of the 14 conditions which were examined using the colony size method (minimal medium, low glucose medium with 0.5% glucose, 1% galactose and 0.5M NaCl) for a more sensitive growth analysis using the OD based method. Using this method to screen across stress conditions exposed additional tRNA deletion strains which exhibit a growth defect. Screen on minimal medium(SD) (**Figure 8**), showed a highly similar mean growth rate of the library relative to rich medium (0.997 rich medium, 1.01 minimal medium) and yet additional tRNA deletion strains which exhibit growth rate defect were exposed (**Figure 8**).



**Figure 8 - Dot plot of Relative Growth Rate and Growth yield values in minimal vs. rich media.**

**A**-Relative growth rate values in minimal medium (SD) vs. rich medium (YPD) **B**-Relative growth yield values in minimal medium (SD) vs. rich medium (YPD). Each dots indicates a tRNA deletion strain. Correlation values and p-values are indicated above each figure.

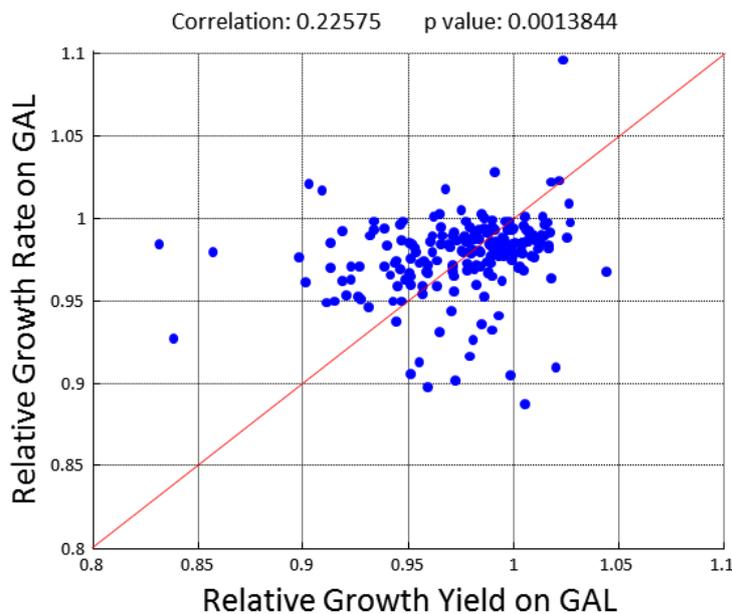
In addition there were strains which exhibit growth rate defect in both conditions. The mean growth yield is almost identical between these two conditions (0.981 rich medium, 0.983 minimal medium) and there is a relatively high correlation (0.58 p-value  $9 \times 10^{-20}$ ) between these two growth conditions in the growth yield parameter (**Figure 8**). Despite this similarity there are tRNA deletion strains which show aggravation in terms of their growth yield relative to rich medium (**Figure 8**), most of these strains show further aggravation of a growth yield defect detected in rich medium. It should be noted that the rich and minimal media, contain the same percentage of glucose (2%) which might lead to the high similarity of values. For comparison I also examined a condition of rich medium that contains only 0.5% glucose. Interestingly comparing growth defects in this medium to defects in rich medium revealed a significant number of strains that show a yield defect in rich medium containing 0.5% glucose in the growth yield parameter (**Figure 9**).



**Figure 9 - Dot plot of Relative growth Yield values in various growth conditions.**

Each figure in the above panel is a comparison of Relative growth yield values between to growth conditions. Each dot represents a strain in the tRNA deletion library. **A**-Relative growth yield values in minimal medium (SD) vs. rich medium (YPD). **B**-Relative growth yield values in low glucose (0.5YPD) vs. rich medium (YPD). **C**-Relative growth yield values in low glucose (0.5YPD) vs. minimal medium (SD). **D**-Relative growth yield values in 1% galactose (1Gal) vs. rich medium (YPD). The correlation value and the p-value are indicated above each figure.

We thus reasoned that an important factor that exposes growth yield defects among the tRNA deletion strains is the glucose content of the medium and verified that by growing a selection of strains on media containing 0.25%, 0.5%, 1%, 1.25%, and 2% glucose. We found a close to linear relationship between growth yield and glucose levels for all the examined strains (8 strains in total, data not shown). Examination of growth on rich medium containing 1% galactose, a respirator, rather than fermentative carbon source, revealed a somewhat more complex relationship between growth rate and growth yield defects (**Figure 10**).



**Figure 10 - Dot plot of Relative Growth Rate vs. Growth Yield in a medium containing 1% galactose.**

The relative growth rate values vs. the relative growth yield values on 1% galactose. Each dot represents a tRNA deletion strain. The correlation and p-value are indicated above the figure.

Interestingly there is distinct deviation between the various tRNA deletions, while some of the tRNAs show a more severe defect in the growth yield at that condition, a distinct set of strains showed greater reduction in growth rate. This condition exposed many tRNA deletion strains (90 strains) with a defect in growth rate as well as many tRNA deletions with defect in growth yield (85 strains). This effect of a medium supplemented with galactose as a carbon source is not clear, it could be the outcome of respiratory carbon sources which reveal more growth defect than fermentative ones due to a better adaption of the codons to fermentation vs. respiration<sup>94</sup>. A better adaptation of the codons to fermentation results in higher translation efficiency, thus changes in the tRNA pool which are expected to hinder translation might be better buffered. Whereas in conditions where the codons are less adapted and the translation efficiency is lower, such changes to the tRNA pool might expose more readily translation problems. It should be mentioned that using the robotic growth method does not allow good examination of respiratory conditions given that the growth medium is topped with mineral oil to prevent evaporation, so the oxygen exchange is rather low and conditions such as glycerol or ethanol could not be examined.

I looked at the results of the OD curve parameters from the screens in different stress conditions seeking for a tendency of a given strain to show a defect on a given condition, and found that tRNA deletion strains which show a defect in the growth yield in a certain condition tend to exhibit such a defect in other conditions as well. A manifestation of that is

a high correlation between the yield defects in the various conditions (**Figure 9**).

Interestingly we did not observe tight relations between the size of the isoacceptor family or the amino acid identity and the tendency for its members to show a growth defect. While the single copy tRNA genes tend to show severe phenotypes, isoacceptor families which contain relatively low gene copy number do not tend to exhibit more growth defects compared to high copy number isoacceptor families (data not shown). In addition I could not find any tendency for growth defects of tRNA deletion residing on a given chromosome, besides that of chromosome J which contains multiple tRNA deletions with severe growth defects. The results of the tRNA deletion library screens on rich medium and across condition demonstrated a high degree of backup governing the tRNA pool, while the growth defect exhibited by specific tRNA gene deletions may change between stresses, the mean growth rate and growth yield for the entire library remains relatively unchanged.

### **5.7. Wobble mediates backup between tRNA isoacceptors**

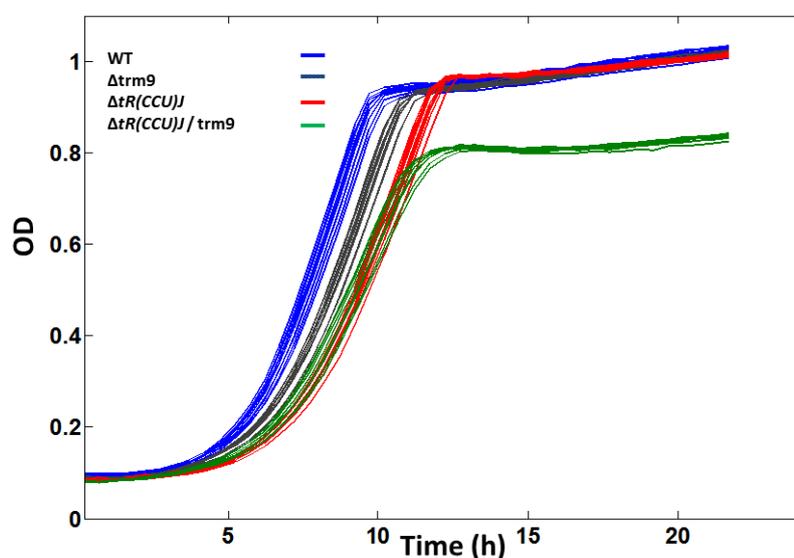
The creation of the tRNA deletion library, reestablished the essentiality of four out of six single copy tRNA genes, in addition to the lack of absolute essentiality of the remaining two. This dispensability of the *tR(CCU)J* and *tL(GAG)G* genes, was intriguing in light of the thousands of genes in the genome that contain at least one appearance of the relevant codons, and are decoded during the life-span of yeast. Such dispensability is possible due to other tRNA isoacceptors which through wobble, decode the relevant codons. The ability of certain tRNA isoacceptors to decode more than one codon triplet is known as wobble. Decoding through wobble allows the first base of the tRNA anti-codon (at position 34) to pair with more than one base in the third position (wobble nucleoside) of the codon. This phenomenon and its rules were first presented by Francis Crick in the 'wobble hypothesis'<sup>98</sup>. Since Crick presented his wobble hypothesis to explain such cases, this hypothesis has been revised multiple times and was able to explain many unexpected results regarding the decoding properties of tRNA genes. The decoding properties of tRNA molecules are further modulated by a set of modification enzymes, to allow efficient interactions with certain nucleosides. At the same time these modifications are aimed at preventing translation errors for tRNA isoacceptors which decode in split codon boxes<sup>99</sup>. Although a variety of models explaining the role of these modifications have been put forth<sup>100,101</sup>, their *in vivo* function has not been fully defined. The study of wobble modifications was based mainly on studies of the modifications' influence on structure and *in vitro* translation experiments<sup>102</sup>.

The power of analyzing genetic interactions in *S. cerevisiae* to decipher the wobble rules *in vivo* was used thus far in a single study<sup>102</sup>. However this Study focused only on a certain type of uridine modifications thus creating only a few tRNA deletions. The resulting deletion strains were evaluated merely based on their viability, while their growth parameters were not characterized. The tRNA deletion library opens a new route to systemic *in vivo* analysis of codon recognition and wobble roles among tRNA isoacceptors using genetic interactions. Studying these interactions, using the established platform of phenotypic scoring, can provide new insights concerning decoding properties and wobble mediated backup. Using the tRNA deletion library I set out to characterize the wobble mediated backup of the two dispensable tRNA gene deletions.

### **5.8. Backup between *tR(UCU)* and *tR(CCU)* tRNAs: a mechanistic basis**

The dispensability of the *tR(CCU)J* deletion strain is supported by the wobble rules, and is believed to rely on the wobble provided by the 11 copies of *tR(UCU)*<sup>103</sup>. The fact that there are 11 copies of *tR(UCU)* which can provide substantial backup, prevented thus far any direct genetic examination of this interaction. Ideally, to show such backup compensation, one would combine a knockout of the two tRNA isoacceptors and demonstrate aggravating epistasis. Yet since there are multiple copies of the *tR(UCU)* gene it is inconceivable to simultaneously delete all 12 genes. Two complementary ways were chosen to examine this interaction, separating the constituents and their contribution. In the first analysis an elegant alternative to complete deletion of the *tR(UCU)* genes, utilized a protein-coding gene, which is an important component in this backup network. It is known that the Arginine *tR(UCU)* isoacceptor, undergoes methylation of the third anti-codon position (wobble nucleoside). This methylation is believed to improve the decoding of cognate codon AGA<sup>104</sup>, and restrict the reading of other codons in this split codon box AGN. Recently it was shown that this methylation might also facilitate the wobble-based recognition of the AGG codons, normally translated by the *tR(CCU)J* tRNA<sup>103</sup>. The methyltransferase that carries out this reaction is encoded by the *TRM9* gene<sup>105</sup>, and as far as currently known is specific to the above tRNA, in addition to a tRNA for glutamic acid *tE(UUC)*<sup>106</sup>. In that respect a deletion of the *TRM9* gene, represents a minimalist perturbation of the system with focused effect on particular genes. A double deletion comprising of the *tR(CCU)J* gene and the *TRM9* methyltransferase can provide a bypass to the deletion of all 11 *tR(UCU)* copies by studying the contribution of the modification to the wobble-based backup between *tR(UCU)* and *tR(CCU)J*. In addition it had the potential to establish the methylation as the backup

mechanism. I created this double deletion  $\Delta tR(CCUG)/trm9$  along with the single deletion  $\Delta trm9$  which served as a control for growth analysis. The resulting deletion strains were evaluated for their growth in rich medium using the OD based method. Both single deletions ( $\Delta trm9$ ,  $\Delta tR(CCUG)$ ) exhibit a reduction in growth rate of 8-10%, relative to the wild type, with no apparent effect on growth yield. The double deletion  $\Delta tR(CCUG)/trm9$ , exhibited aggravation of growth rate, showing a 15% reduction relative to the wild type, and 5-7% reduction relative to each of the single deletion (**Figure 11**). In addition the double deletion  $\Delta tR(CCUG)/trm9$  exhibited substantial aggravation of growth yield (18%) relative to each of the corresponding single gene deletions and the wild type strain (**Figure 11**).



**Figure 11 - Growth curves of Wild type vs.  $\Delta tR(CCUG)$ ,  $\Delta trm9$  and  $\Delta tR(CCUG)/trm9$ .**

Growth curves of Wild type (blue),  $\Delta trm9$  (black),  $\Delta tR(CCUG)$  (red) and double deletion  $\Delta tR(CCUG)/trm9$ . The X axis indicates time in hours. The Y axis indicated OD values. The experiment was performed using the robotic OD based method in rich medium at 30°C. The growth curve of each strain in the figure is composed of 12 lines each representing a technical repetition.

To evaluate the contribution of the Trm9 protein to the wobble based backup I used the conventional non-scaled measure of epistatic interactions<sup>82</sup> (see materials and methods), to calculate the epistasis relations between the two gene deletions. Using this analysis I can evaluate whether the reductions manifest in growth rate and growth yield are either additive or synergistic effects resulting from substantial backup<sup>82</sup>. This analysis revealed a significant negative epistasis on the growth yield (-0.16) with no apparent epistasis on growth rate. The strong negative epistasis established, for the first time, a genetic interaction between this tRNA and a protein coding gene, resulting from the functional contribution of the Trm9 to the  $tR(UCU)$  molecules. This result provides evidence which strongly supports the backup through wobble provided by  $tR(UCU)$ , and defines the contribution of the modifying enzyme to the interaction. While backup activity is known to

occur between protein coding genes, especially among paralogs, this is, to the best of our knowledge, the first time in which a molecular mechanism that underlies backup activity is deciphered between genes which are not paralogs. We note that the double deletion  $\Delta tR(CC U)J/trm9$  is indeed sick yet viable, thus the codon recognition between  $tR(UCU)$  and the AGG codons is possible even without methylation (assuming no other means to methylate  $tR(UCU)$ ), however to a lesser extent. This finding further supports the previous observations that the methylation on  $tR(UCU)$ , modulates the interaction with the AGG codons<sup>103</sup>. To examine the specificity of this genetic interaction, between  $TRM9$  and  $tR(CC U)J$ , I created double deletions of the  $TRM9$  gene and random tRNA genes from various isoacceptor families. A total of 8 such double deletions were created, and their growth was evaluated on rich medium conditions. None of these double deletions exhibited growth defects relative to the single deletion  $\Delta trm9$  (data not shown). A valid concern might be that the deletion of the Trm9 modifying enzyme leads to generation of destabilized and/or inefficiently aminoacylated tRNA molecules. This concern was previously addressed by determining the steady-state and *in vivo* aminoacylation levels for  $tR(UCU)$  and  $tE(UUC)$  in  $\Delta trm9$  and wild type *S. cerevisiae* cells, and no such reduction in abundance or aminoacylation was observed<sup>102</sup>.

The second analysis aimed at establishing backup through wobble, used a more direct approach, by creating a set of double deletions of two tRNA genes, composed of various  $tR(UCU)$  genes along with the  $tR(CC U)J$  gene. Although the complete backup interactions cannot be captured, the use of these double deletions, in which only a subset of the interaction can be examined, has the potential to strengthen the previous observations by distinct examination of the components consisting these backup interaction. Using the sensitivity of the OD based growth measurement enables detection of epistasis even when substantial backup is still provided by the remaining  $tR(UCU)$  genes. A set of five such double deletions was created and their growth was evaluated in rich medium. Remarkably the growth of these double deletion strains, revealed growth aggravation for all examined strains, which exhibited a prolonged lag phase along with a substantial reduction in growth rate. At the same time most strains exhibited no aggravation of growth yield and retained the growth yield exhibited by both single deletions and the wild type strain. The growth defects ranged from 15% for the  $tR(UCU)K$  to more than 30% reduction for the most aggravated double deletion  $\Delta tR(UCU)E/tR(CC U)J$  relative to the wild type. Calculating the epistasis relations revealed strong negative epistasis in growth rate. Calculating the growth

yield epistasis for the entire set of double deletions revealed no epistasis. There were differences between the various double deletions on which I will elaborate in the next chapter concerning tRNA differential contribution.

These results further demonstrate the backup through wobble between *tR(UCU)* and *tR(CCU)J*, while providing new information regarding the contribution of each of the relevant components to the backup interaction. A striking difference was depicted in the effect each component of the backup interaction imposed on the growth. While in the  $\Delta tR(CCU)J/trm9$  the growth yield was severely aggravated, the various  $\Delta tR(UCU)/tR(CCU)J$  exhibited an extensively prolonged lag phase and severe aggravation of growth rate. It is possible that these growth differences capture different effects on the process of translation. One can speculate that while a deletion of *tR(UCU)* genes effectively reduce the tRNA quantities in the tRNA pool resulting in a prolonged lag phase and reduced growth rate, the deletion of modification enzyme only reduces the efficiency of the wobble. Both obstructing translation speed and accuracy. To validate the result obtained by both sets of double deletions, I examined the data repository of yeast genetic interactions (DRYGIN<sup>107,108</sup>) for any possible interactions between the *TRM9* and the genes flanking the *tR(UCU)* genes or *tR(CCU)J* that might lead to epistasis. Crossing all the possibilities for each gene, I found no genetic interactions between these genes that could explain my observations.

### **5.9. Wobble based complementation of $\Delta tR(CCU)J$ growth defect**

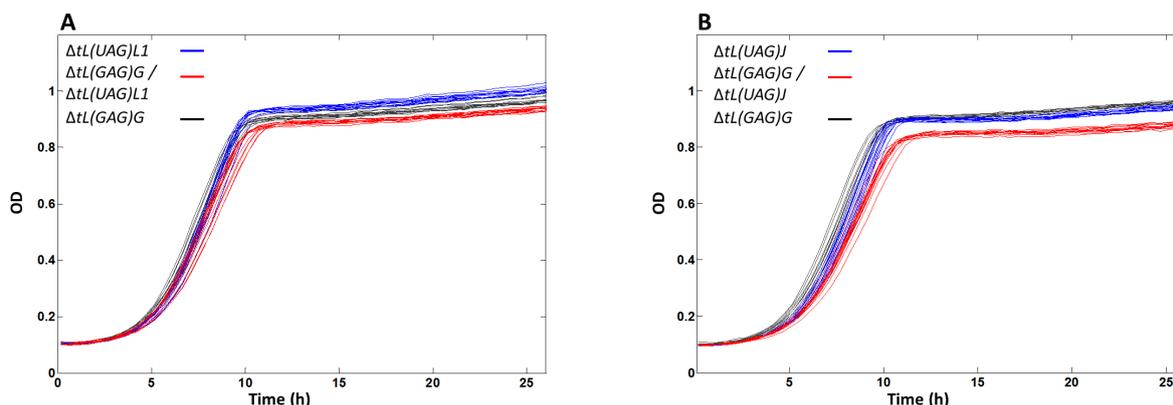
Thus far I examined the wobble based backup examining the genetic interactions through deletions and epistasis calculations. A different angle would be to study the backup through wobble by increasing the cellular levels of the various components. Given the severe growth defect of the  $\Delta tR(CCU)J$ , I reasoned that this defect can be further exploited to strengthen the evidence for backup through wobble to alleviate the observed growth defect. Introducing additional copies of *tR(UCU)* to the genetic background of the  $\Delta tR(CCU)J$  might compensate the growth rate defect to some extent. I constructed multiple centromeric plasmids containing the following *tR(UCU)* genes: *tR(UCU)M2*, *tR(UCU)E*, *tR(UCU)K*, *tR(UCU)G1* and *tR(UCU)M1*, and examined their growth. As control I compared the growth to a *tR(CCU)J* deletion strain containing an empty centromeric plasmid. Unfortunately none of the centromeric plasmids were sufficient to complement the growth defect of the *tR(CCU)J* deletion. Given that a single copy of *tR(UCU)* could not complement the growth

defect of the *tR(CCU)J* deletion, I turned to overexpress *tR(UCU)* using a high copy number plasmid. High copy number plasmids (see materials and methods) of *tR(UCU)E* and *tR(UCU)K* were introduced to both  $\Delta tR(CCU)J$  and  $\Delta tR(CCU)J/trm9$  and their growth was compared to deletion strains containing the empty vector. The growth of the resulting strains was evaluated on minimal medium to avoid plasmid loss, and as such cannot be compared to the original tRNA deletion strain. Nevertheless improved growth relative to a strain containing an empty vector can indicate that addition of multiple *tR(UCU)* copies provide stronger backup and alleviate the growth defect. The growth analysis revealed alleviation of the growth defect observed for both strains ( $\Delta tR(CCU)J$ ,  $\Delta tR(CCU)J/trm9$ ) with both *tR(UCU)* plasmids (data not shown). These results should be further validated by biological repetitions of this experiment. The growth alleviation observed by overexpression of *tR(UCU)* genes, demonstrates the dependence of this wobble based backup on *tR(UCU)* quantities, which effect translation probably by changing translation speed.

### 5.9.1. Backup between the *tL(UAG)* and *tL(GAG)* isoacceptors

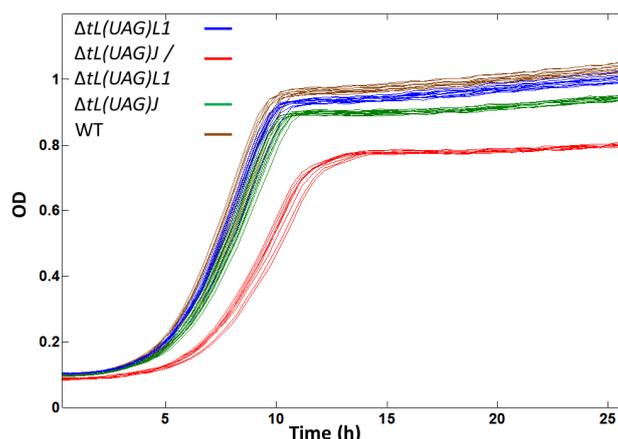
The second dispensable single-copy tRNA gene, is Leucine *tL(GAG)G*. This tRNA decodes the CUC and CUU codons in the 4-box CUN for Leucine. The *tL(GAG)G* deletion strain shows a growth defect manifested only in growth yield, without appreciable defect in growth rate (**Figure 7**). It is conceivable to assume that this strain too is compensated through wobble interactions provided by the *tL(UAG)* isoacceptor which decodes the additional codons in this Leucine 4-box. The ability of *tL(UAG)* to read the entire 4-box was shown *in vitro* for interferon treated cells<sup>109</sup>. Since there is no enzyme analogous to *TRM9* which is known to modify the *tL(UAG)* tRNAs and there are two *tL(UAG)* genes, the genetic analysis could be easily preformed, by creating multiple sets of tRNA double deletions. A third *tL(UAG)L2* gene was deleted in the creation of the Y5565 genetic background along with the *MET15* gene and thus was not examined in my study<sup>76</sup>. The first set of double deletions was of the *tL(GAG)L* gene with one of the *tL(UAG)* genes (**Figure 12**). None of the single *tL(UAG)* deletions exhibits a growth rate defect, yet both exhibit various growth yield defects of up to 9% relative to the wild type. Both double deletions, showed a reduction of growth rate, as well as growth yield relative to the wild type and single deletions (**Figure 12**). The  $\Delta tL(GAG)G/tL(UAG)J$  exhibited a more severe growth defect with 6% reduction in growth rate and 6% reduction in growth yield compared to the relevant single deletions. The  $\Delta tL(GAG)G/tL(UAG)L1$  exhibited a milder phenotype with only 4% reduction in growth rate

and 2% reduction in growth yield relative to the relevant single deletions (**Figure 12**).



**Figure 12 - Growth curves of single vs. double deletions of the *tL(UAG)* and *tL(GAG)* isoacceptors.**  
**A**-Growth curves of  $\Delta tL(UAG)L1$  (blue),  $\Delta tL(GAG)G$  (black) and the double deletion  $tL(UAG)L1/tL(GAG)G$  (red).  
**B**-Growth curves of  $\Delta tL(UAG)J$  (blue),  $\Delta tL(GAG)G$  (black) and the double deletion  $tL(UAG)J/tL(GAG)G$  (red). The experiment was performed using the robotic OD based method in rich medium at 30°C. The growth curve of each strain is composed of 12 lines each representing a technical repetition.

Calculating the epistasis relations for this set of double deletions revealed no significant genetic epistasis between *tL(GAG)G* and the *tL(UAG)L1* in terms of both growth rate (-0.02) and or growth yield (-0.02). Performing the same calculation for the double deletion  $\Delta tL(GAG)G/tL(UAG)J$  revealed aggravation in growth rate (-0.051) and a similar aggravation in growth yield (-0.05) (**Figure 12**). These growth aggravations were supporting evidence for wobble based backup between *tL(GAG)G* and the *tL(UAG)* isoacceptor. I then created the complementary double knockout that is deleted for the two *tL(UAG)* genes in a strain that contains a normal copy of *tL(GAG)* (**Figure 13**).



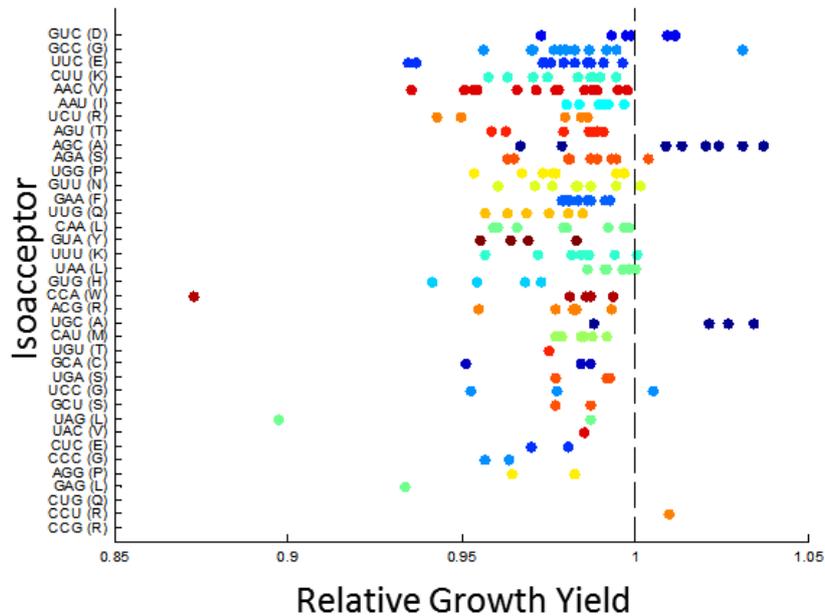
**Figure 13 - Growth curves of Wild type vs. single and double tRNA deletions of the *tL(UAG)* isoacceptor**  
 Growth curves of the wild type (brown),  $\Delta tL(UAG)J$  (blue),  $\Delta tL(UAG)L1$  (green) and the double tRNA deletion  $tL(UAG)J/tL(UAG)L1$ . The X axis indicates time in hours. The Y axis indicated OD values. The experiment was performed using the robotic OD based method in rich medium at 30°C. The growth curve of each strain is composed of 12 lines each representing a technical repetition.

This double deletion showed a prolonged lag phase and a rather mild aggravation of growth

rate (~3%) relative to the wild type and both single deletions. A severe reduction was observed in growth yield comprising a 22% reduction relative to the wild type, 19% reduction relative to  $\Delta tL(UAG)L2$  and 13% reduction relative to  $\Delta tL(UAG)J$  (**Figure 13**). Calculating the genetic epistasis for this double deletion showed, no aggravation of growth rate and a significant aggravation of growth yield ~10%. The results presented here establish for the first time genetic interactions implaying backup between the  $tL(UAG)$  and  $tL(GAG)G$  tRNAs. The observed backup is most likely through wobble interaction. More generally, these genetic analyses demonstrate an accurate measure, to reconstruct, and perhaps refine, the wobble rules by means of genetic interactions between tRNA deletion mutants. The results achieved for these two single copy tRNA deletions establishes further the backup through wobble and manifests the contribution of nucleoside modifications and modifying enzymes to this backup.

### **5.10. Differential contribution of tRNA gene copies to the tRNA pool**

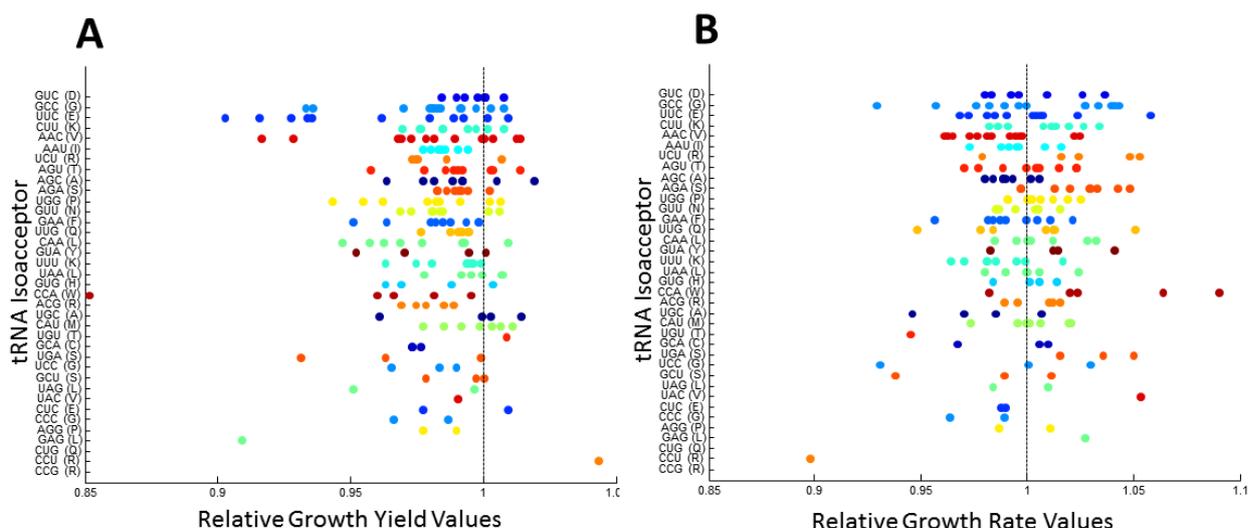
The scarce experimental data of the cellular concentrations for various tRNA isoacceptors led to the observation that the *in vivo* concentration for a given tRNA isoacceptor are highly proportional ( $r=0.91$  for *S. cerevisiae*) to the number of gene copies coding for this tRNA type<sup>71,94,110,111</sup>. In addition the detection of internal promoter elements along with the high sequence identity between tRNA gene copies in a given isoacceptor family, resulted in the assumption that all tRNA genes within an isoacceptor family are expressed to the same extent. Thus each tRNA gene copy was believed to contribute equally to the total concentration of the isoacceptor in the cell. Over the years this assumption was rarely tested due to the difficulties in measuring the actual amounts of tRNA molecules in the cell and differentiating the contribution of identical tRNA gene. Thus a fundamental question in my research concerned the contribution of each tRNA gene to the cellular pool. The contribution of each tRNA gene to the cellular fitness may serve as a proxy for the expression levels and contribution to the cellular pool. Using the platform of the tRNA deletion library and the phenotypic scoring system I aimed to decipher the contribution of different tRNA isoacceptors, and gene copies to the tRNA pool, by comparing their growth to that of a wild type strain. While screening the tRNA deletion library in rich medium as well as in a set of stressful growth conditions I observed an intriguing phenomenon, in which deletion of different tRNA genes, from the same isoacceptor family, resulted in a spectrum of phenotypes and growth defects (**Figure 14**).



**Figure 14 - Relative growth yield values in rich medium for all tRNA deletions according to isoacceptor family.** Each dot in the above figure represents the relative growth yield value of a tRNA deletion strain in rich medium, colored according to isoacceptor family and amino acid. The isoacceptors are ordered on the Y axis according to family size, starting at single copy genes at the bottom and up to 16 copies at the top; the relevant amino acid is indicated in brackets.

For instance there were tRNA deletions of genes from the *tE(UUC)* family which grow as the wild type strain, showing no apparent growth rate or growth yield phenotype, while other gene copies, from the same family, showed a significant growth defect in either growth rate or growth yield (**Figure 14**). For most *S. cerevisiae* isoacceptor families there is a variance in growth phenotypes between deletions of identical tRNA gene copies, differentially contributing to the observed fitness. These observations are not consistent with the naïve view claiming equal expression levels<sup>33</sup>, was apparent in both growth parameters. Such differential contribution of tRNA genes, was extremely surprising given the high sequence identity and presence of internal promoter elements, leading as far as we understand to identity of regulatory sequences. Conventional expression methods could not have detected this phenomenon given the high sequence identity within isoacceptor families. Using the emerging method of detecting RNA Pol-III binding to promoters by Chip-seq technology, which serves as a good proxy for tRNA transcription<sup>112,113</sup>, the Pol III occupancy of tRNA genes was measured and quantified genome-wide in various organs in a set of six mammals. In this assay it was found that the binding of the basal transcriptional machinery to individual tRNA genes of the same isoacceptor can vary considerably<sup>114</sup>. This result is consistent with our present observation and may suggest a basis for the observed differential contribution of identical copies to fitness. Examining the contribution of

identical tRNA genes in stress conditions when the expression profile of the cell changes, may further strengthen the results obtained in rich medium and increase the observed differences in fitness due to changes in the demand for various tRNA species. Screening the library in stressful conditions such as YP+1% galactose or rich medium containing only 0.5% glucose, exposed even greater differences in the growth parameters (**Figure 15**). In addition new tRNA isoacceptors were exposed which exhibit a higher contribution. The exposure of differential contribution between tRNA gene copies in multiple tRNA isoacceptor families exposed a potentially new mean to regulate translation. Implying that the backup provided within a isoacceptor family is proportional to the fitness and thus to the expression level.



**Figure 15 - Relative growth rate and growth yield values for all tRNA deletions grouped according to isoacceptor family in a rich medium containing 1% galactose.**

**A**-Relative growth yield on YP+1% galactose, **B**-Relative growth rate on YP+1% galactose. Each dot in the above figures represents a tRNA deletion strain colored according to isoacceptor family and amino acid.

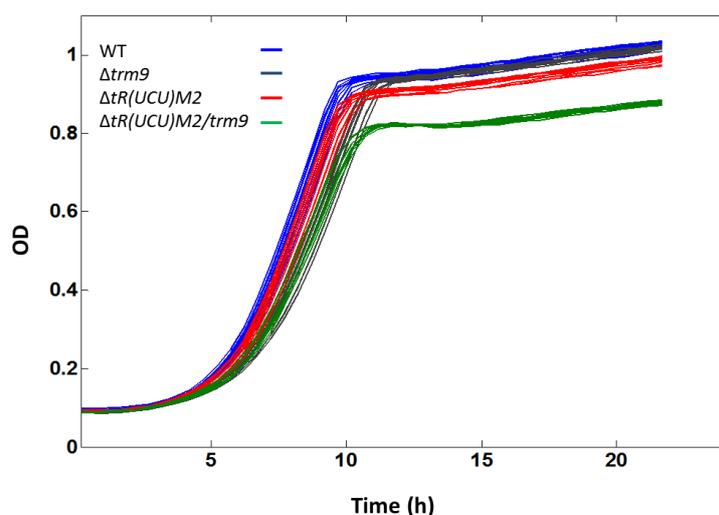
The isoacceptors are ordered on the Y axis according to family size, starting at single copy genes at the bottom and up to 16 copies at the top; the relevant amino acid is indicated in brackets.

Based on the observations of differential contribution between identical copies of a given tRNA isoacceptor, a possible explanation is the possibility that while deleting such copies near-by genome features have been affected and the difference in fitness is due to such features. To examine and potentially rule out this possibility I decided to focus on one family that manifested apparent differential contribution and investigate this phenomenon more thoroughly (see also Materials and Methods and in section 2.6 and 6). I choose the *tR(UCU)* isoacceptor which has 11 identical gene copies in the *S. cerevisiae* genome. For this isoacceptor I had five different deletion strains in the tRNA deletion library. In this family there are two tRNA copies *tR(UCU)E* and *tR(UCU)M2* which exhibit a significant defect in growth yield, when grown on rich medium (0.942787, 0.949814 respectively), in addition *tR(UCU)E* also exhibits a slight growth rate defect (0.977) in this condition. The other three

tRNA deletions strains from this family *tR(UCU)M1*, *tR(UCU)G1* and *tR(UCU)K* show no apparent growth defect in either growth rate or growth yield in rich medium conditions. I thus refer to the genes *tR(UCU)E* and *tR(UCU)M2* as "major" contributing copies to the tRNA pool, while the other three copies are "minor" contributing copies. The phenomenon of differential contribution can also be viewed in light of backup interactions between different genes belonging to the same isoacceptor. Given that the strength of backup provided by a given gene is proportional to its expression level, when the sequence is identical, this implies for differences in the strength of backup provided by the various gene copy to the other copies in the family. To further investigate the phenomenon of differential contribution I aimed to define the backup ability of these five tRNA genes. I created three complementary sets of double deletions, all designed to increase the observed differential contribution by intensifying the load on remaining *tR(UCU)* molecules in the pool. The first set exploited the fact that the *Trm9* modifying enzyme, modifies the *tR(UCU)* molecules, thus enhancing their recognition of the AGA and AGG codons, to co-delete the enzyme and the *tR(UCU)* genes. The second set decreased the effective number of tR(UCU) molecules by co-deleting two *tR(UCU)* genes. The third set exploited the backup through wobble provided by *tR(UCU)* to the *tR(CCU)J*, and co-deleted the *tR(UCU)* genes with the *tR(CCU)J* gene. This third set was used to examine two aspects of the tR(UCU) function, backup through wobble and differential contribution.

The first set of double deletions included five strains of  $\Delta tR(UCU)/trm9$  and a control strain  $\Delta trm9$ . In this set I expected a co-deletion of *tR(UCU)* genes with the modifying enzyme to aggravate the growth in a way proportional to the extent of their expression and contribution to the pool. According to this logic a loss of a "major" contributor would result in a severe growth aggravation while the loss of a "minor" contributor would in a milder growth aggravation or no aggravation of growth relative to the single deletions. Indeed upon double deletion of the tRNA genes and the modifying enzyme the double deletions containing one of the two "major" copies experienced a significant growth aggravation compare to the corresponding single deletions (**Figure 16**). At the same time there was no aggravating effect on the three "minor" copies and their growth resembled the growth of the  $\Delta trm9$  deletion strain. This backup between copies of *tR(UCU)* can also be analyzed using epistatic calculations, to assess whether the phenotypic impact of one gene is dependent on the other genes, and in this case to examine whether different genes result in different intensities of epistasis relations<sup>115</sup>. This analysis may help uncover the functional

organization and backup within this biological network. I calculated the epistasis relations for this set of double deletions using the non-scaled epistasis equation<sup>32</sup>. Once again calculations for the two growth parameters were separated. I observed a significant aggravation for the double deletion of the two "major" *tR(UCU)* copies, while the "minor" copies showed no epistasis. The  $\Delta tR(UCU)M2/\Delta trm9$  experienced aggravation in growth yield (-0.08) and no aggravation of growth rate (**Figure 16**). The double deletion  $\Delta tR(UCU)E/\Delta trm9$  experienced a (-0.04) aggravation in growth rate and no aggravation of growth yield.

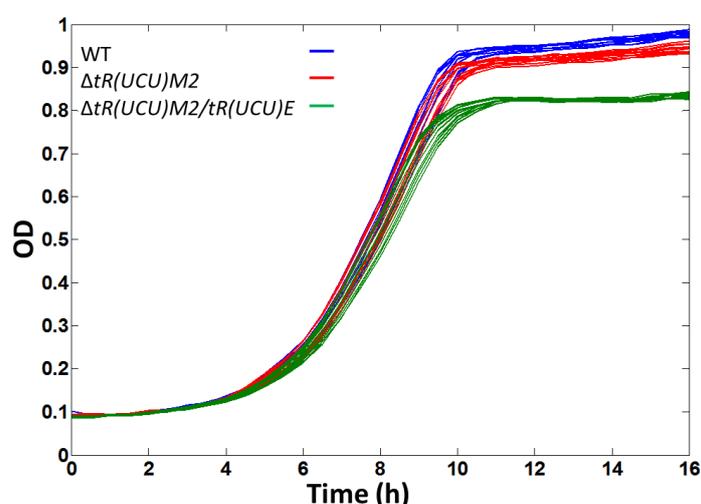


**Figure 16 - Growth curves of wild type vs.  $\Delta tR(UCU)M2$ ,  $\Delta trm9$  and  $\Delta tR(UCU)M2/trm9$ .**

Growth curves in rich medium (YPD) of wild type,  $\Delta tR(UCU)M2$ ,  $\Delta trm9$  and double deletion  $\Delta tR(UCU)M2/trm9$ . The experiment was performed using the robotic OD based method in rich medium at 30°C. The growth curve of each strain is composed of 12 lines each representing a technical repetition.

The three other double deletions  $\Delta tR(UCU)M1/\Delta trm9$ ,  $\Delta tR(UCU)K/\Delta trm9$  and  $\Delta tR(UCU)G1/\Delta trm9$  showed no epistasis of either growth rate or growth yield. The negative epistasis observed between the deletion of the two "major" tRNA copies and the *Trm9* enzyme indicate that despite the fact that all tRNA gene products are identical and the enzyme modifies all of them to the same extent, the effect of its deletion is aggravating the *tR(UCU)* genes in a differential manner. There is a perfect correlation between the effect the single tR(UCU) deletion impose on the cells and the negative epistasis observed for the relevant double deletions with *Trm9*. The epistasis calculations indicate that there is a difference in the strength of backup provided by the two "major" copies relative to the "minor" copies. This might indicate that these "major" copies are highly expressed thus providing substantial backup to the cell upon deletion while the "minor" copies are transcribed to a lesser extent. Along the long process of tRNA maturation changes in expression levels between the tRNAs

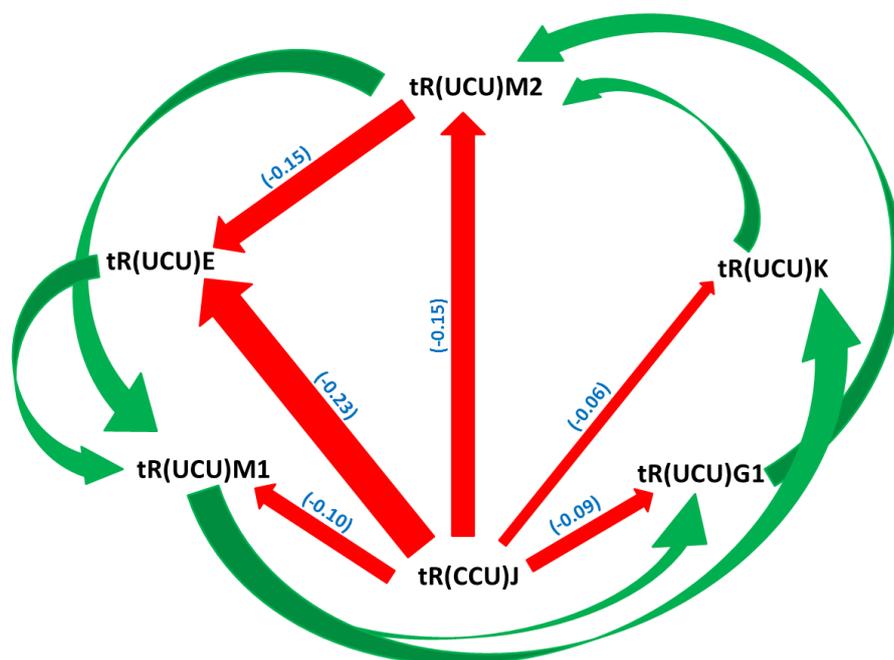
represent the step where there is still differences between them, in terms of sequence. In all the following steps tRNAs from the same family are undistinguishable. The second set of double deletions contained a total of six double deletions of the five *tR(UCU)* genes deletions present in the library. If indeed differential expression lays at the basis of differential contribution, one would expect that the double deletion of the two "major" copies *tR(UCU)M2* and *tR(UCU)E* would exhibit a saver severe growth aggravation, while the double deletions of other copies would show no aggravation of growth, or a very mild one. These double deletions were evaluated for their growth phenotype using the OD based measurement on rich medium. The double deletion of the two major copies showed a growth aggravation of the growth yield, but not the growth rate. Double deletions containing only one of the "major" gene copies resulted in a minor aggravation of the phenotype, while deletions of the two "minor" gene copies showed no aggravation of growth, and resembled the growth parameters of the single gene deletions (**Figure 17**). Calculating the epistasis interactions of this set of double deletions revealed no epistasis effect on the growth rate, and a significant aggravation (-0.15) of the growth yield (**Figure 18**). As expected no epistatic interactions were exposed for any other double deletions.



**Figure 17 - Growth curves of  $\Delta tR(UCU)M2/tR(UCU)E$  relative to wild type and  $\Delta tR(UCU)M2$  in rich medium.** Growth curve in rich medium of wild type,  $\Delta tR(UCU)M2$  and  $\Delta tR(UCU)M2/tR(UCU)E$ . The experiment was performed using the robotic OD based method in rich medium at 30°C. The growth curve of each strain is composed of 12 lines each representing a technical repetition.

Given that the isoacceptor *tR(UCU)* provides back up by wobble to the *tR(CCU)J* isoacceptor, an interesting analysis would be to examine the differential contribution in light of the wobble. To examine the effect of *tR(UCU)* gene deletions on the deletion of *tR(CCU)J*, I created the third set of double deletions, and examined their growth on rich medium. Upon

double deletions the "major" copies of *tR(CCU)J* are expected to further aggravate the *tR(CCU)J* growth. The growth aggravation of the "minor" copies is expected to be milder. In this analysis I found that the growth rate of all double deletions was aggravated to various extents (data not shown). The most severely aggravated double deletions were  $\Delta tR(CCU)J/\Delta tR(UCU)E$  and  $\Delta tR(CCU)J/\Delta tR(UCU)M2$ , which include the two "major" copies. Calculating the epistasis interactions in this set of double deletions revealed no epistasis in growth yield, and negative epistasis in growth rate (**Figure 18**). As expected the two "major" copies resulted in strong negative epistasis values compared to double deletions including the "minor" genes (**Figure 18**).



**Figure 18 - Schematic representation of epistasis interactions between the *tR(UCU)* and *tR(CCU)J* genes.** In the above the epistasis interactions between various members of the *tR(UCU)* family and *tR(CCU)J* are represented. Negative epistasis is indicated by red arrows proportional to the epistasis value. No epistasis is indicated by green arrows. The calculated epistasis values<sup>82</sup> are indication on the relevant arrows.

The results presented here from the growth conditions screens of the tRNA deletion library in addition to the three specific sets of double deletions provide a substantial basis for the existence of differential contribution within tRNA isoacceptor families. Further analysis performed on the *tR(UCU)* gene copies revealed that the differential essentiality observed among identical genes in rich medium, seems to be proportional to the backup they provide within the isoacceptor family and through wobble to the *tR(CCU)J*.

### 5.11. Molecular signature of tRNA deletions: effects on the transcriptome

Translation is a key process in the cell and as such, it is highly regulated to adjust protein expression in order to accommodate the requirements of the cell. The tRNA pool is a major component in this process and thus it is only reasonable to speculate that it too might be subjected to various levels of regulation. A tRNA gene deletion might produce a perturbation in the cell, switching on or off several pathways as a response to the change in the tRNA pool. To characterize the tRNA deletion strains at the molecular level, the transcription profiles of multiple tRNA deletion strains (13 different tRNA deletions and one double deletion  $\Delta tR(CCU)/trm9$ ), were evaluated using RNA microarray. The analysis of the data was performed in collaboration with Sivan Navon a Ph.D. student in my lab. The tRNA deletion strains chosen for analysis were of different isoacceptors and mostly different amino acids. They represent cases of single copy tRNA genes, isoacceptor families containing few gene copies, and representatives of multi-copy isoacceptor families. Most deletion strains show a growth defect, in either growth rate or growth yield, however to different extent (**Table 2** first column). The experiment was performed on cultures grown at optimal conditions (30°C on rich medium), to mid log phase ( $\sim 1.5 \times 10^7$  cells per ml culture) or early stationary phase ( $2 \times 10^8$  cells per ml culture), the phase at which the growth yield parameter is extracted. The expression profiles for all 13 tRNA deletion strains were examined for potential effects arising from flanking genes. This analysis revealed that apart from one gene which exhibited a significant down regulation of the flanking gene, the remaining tRNAs exhibited no such change in the expression of the flanking genes (see materials and methods). Following this reassuring analysis we turned to examine the molecular signature of each tRNA deletion.

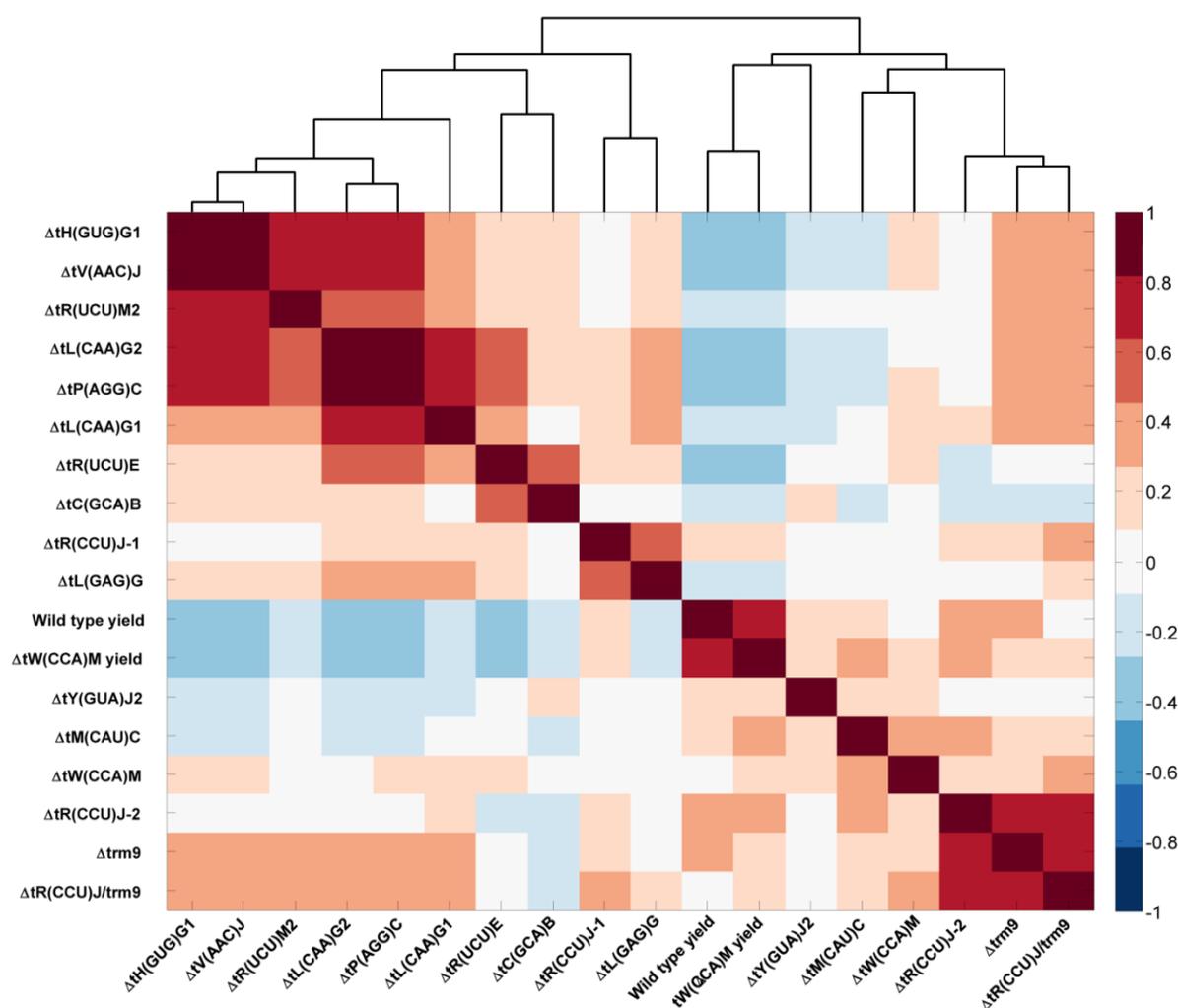
Strain	Amino acid	No of gene copies	Growth rate on YPD	Growth yield on YPD	Up-regulated genes	Down-regulated genes	Total
WT yield					379	134	513
$\Delta tR(CC U)J-1$	Arginine	1	0.921	1.010	218	115	333
$\Delta tR(CC U)J-2$	Arginine	1	0.921	1.010	195	136	331
$\Delta tL(GAG)G$	Leucine	1	0.981	0.934	27	65	92
$\Delta tW(CCA)M$	Tryptophan	6	1.013	0.873	7	1	8
$\Delta tW(CCA)M$ yield	Tryptophan	6	1.013	0.873	500	526	1026
$\Delta tM(CAU)C$	Methionine	5	0.974	0.988	13	3	16
$\Delta tR(UCU)E$	Arginine	11	0.975	0.943	5	5	10
$\Delta tC(GCA)B$	Cysteine	4	0.982	0.951	2	13	15
$\Delta tY(GUA)J2$	Tyrosine	8	0.947	0.964	12	2	14
$\Delta tL(CAA)G1$	Leucine	9	0.983	0.980	14	4	18
$\Delta tL(CAA)G2$	Leucine	9	0.958	0.959	39	18	57
$\Delta tP(AGG)C$	Proline	2	0.978	0.965	10	28	38
$\Delta tR(UCU)M2$	Arginine	11	1.018	0.950	10	1	11
$\Delta tH(GUG)G1$	Histidine	7	1.014	0.941	16	19	35
$\Delta tV(AAC)J$	Valine	14	1.036	0.953	13	10	23
$\Delta tR(CC U)J/trm9$	Arginine	1	0.849	0.826	148	98	246
$\Delta trm9$			0.897	0.995	99	63	162

**Table 3 – Change in expression for tRNA deletions examined in the microarrays**

For each tRNA deletion strain examined in the microarrays the above table indicates the amino acid, the copy number for the relevant isoacceptor family, growth rate and growth yield values. In addition the total number of significantly changed genes (above two fold), dividing them to up and down regulated genes, relative to the wild type strain measured in exponential phase.

For each deletion Table 3 indicates the number of genes that changed significantly (more than 2 fold) in response to the deletion, distinguishing between up-regulated and down-regulated genes. Both repetitions of the single copy tRNA gene *tR(CC U)J* deletion resulted in a substantial response, about 6% of the genes in the array showed significant change. The deletion of the second single copy tRNA gene, *tL(GAG)G*, resulted in a weaker response, about 2% of the genes on the array. Much to our surprise, given the cellular response to each of the single deletions,  $\Delta tR(CC U)J$  (6%), and  $\Delta trm9$  (2%), the change in the expression profile of the double deletion  $\Delta tR(CC U)J/trm9$  involved only about 4% of the genes on the array, displaying a significant overlap with both single deletions, 133 genes with  $\Delta tR(CC U)J$  and 94 with  $\Delta trm9$ . The remaining tRNA deletions, all part of multi-copy families, showed relatively weak effects on gene expression, involving a similar amount of affected genes. Substantial changes were observed in the expression profile of the wild type strain in the yield phase compared to the profile obtained in the logarithmic phase. In addition substantial changes in expression were observed for the tRNA deletion *tW(CCA)M* sampled in the yield phase compared to the wild type in this phase, with 500 genes up-regulated, and 526 genes down regulated, defining a unique molecular signature for a tRNA deletion in this phase of the growth curves. Considering the different strains as biological repetitions for the event of a tRNA gene deletion, given that the preformed function during translation is the

same, leads to the conclusion that such a deletion perturbs the cell, causing the relevant changes in the tRNA pool for each gene. The cellular response to this perturbation leads to changes in gene expression. Trying to understand what are the characteristics of such a cellular response, we next turned to analyze the expression correlation between the various deletions. We computed correlations for all pairs of the examined deletions using both Pearson and Spearman correlation calculation and we found substantial correlations between several tRNA deletions. Figure 19 demonstrates the Pearson correlation observed between the different tRNA deletion strains (after two outlier genes were excluded from the calculations).

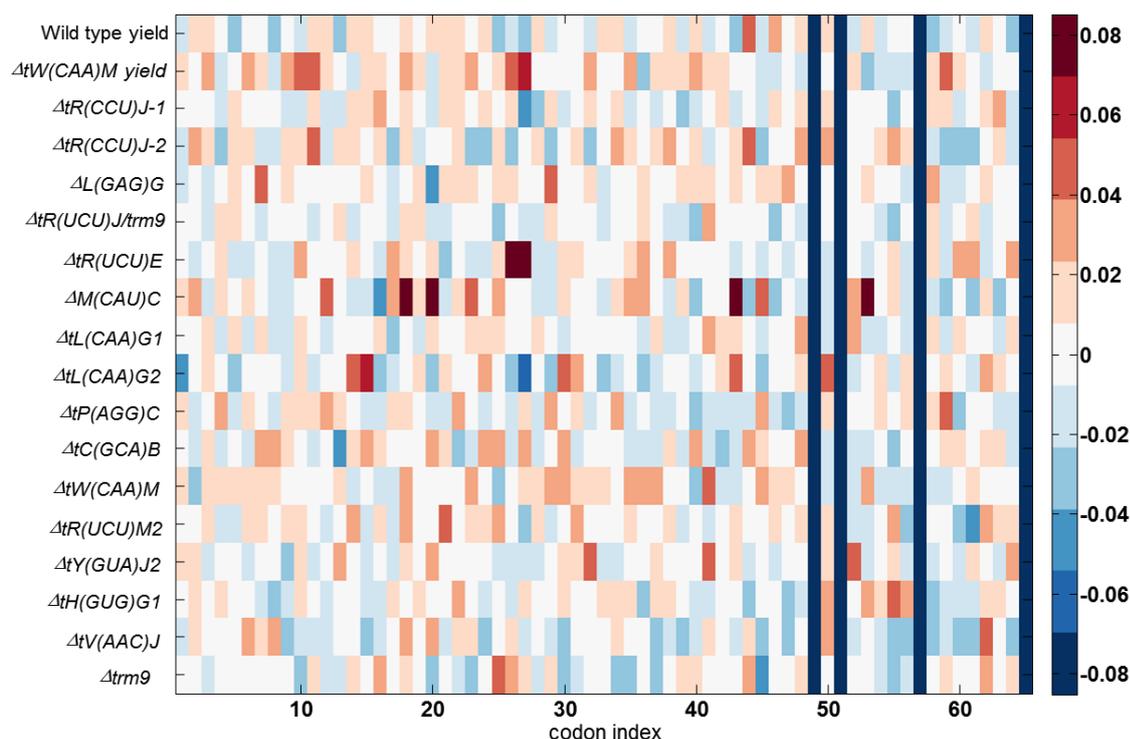


**Figure 19 – Pearson's correlation of transcriptom changes between the various tRNA deletion strains.** The x and Y axis indicate the various strains examined in the microarrays. Samples taken at the yield phase are indicated as yield in the strain name. The color bar indicates the correlation value between changes in the transcriptom for the various tRNA deletion strains, red indicates positive correlation, blue indicates negative correlation. The correlation was calculated based on all changes detected in the transcriptom for each strain.

This analysis revealed that the tRNA deletions can be roughly divided into two groups, which exhibit a relatively high level of expression similarity among them. The largest group consists

of the following eight tRNA deletions: *tH(GUG)G1*, *tV(AAC)J*, *tR(UCU)M2*, *tL(CAA)G2*, *tP(AGG)C*, *tL(CAA)G1*, *tR(UCU)E* and *tC(GCA)B*. This group corresponds to tRNA deletions that are part of multi-copy families, in which the remaining genes can provide backup, or in which backup is provided through wobble. The strains in this group, also exhibit a negative correlation with the two samples taken from the yield phase, which further strengthens the physiological differences between the two growth phases. The second group exhibits overall weaker correlations and can be further divided into smaller groups. This group contains the two single copy tRNA deletions *tR(CCU)J* and *tL(GAG)G*, along with the deletions of *tM(CAU)C*, *tW(CCA)M*, *tY(GAU)J2*, *trm9* and the double deletion *tR(CCU)J/trm9*. Within this group a weak correlation was observed between the two biological repetitions of  $\Delta tR(CCU)J$  (0.28) which can correspond to batch effects, and technical problems in the arrays. Reassuringly a stronger correlation of 0.7 was observed between the *tR(CCU)J-2* deletion and the double deletion *tR(CCU)J/trm9* which were hybridized to on the same batch. The second group of tRNA deletions corresponds either to single copy tRNA gene deletions or to amino acids represented by a single tRNA isoacceptor. The two samples taken from the yield phase are also included in this group, and interestingly, as opposed to the negative correlations observed between these samples and the first group of tRNA deletions, this group exhibits low positive correlations with the yield phase samples. The correlations observed between the various tRNA deletions further reassure our finding that tRNA deletions create a specific and unique signature on the transcriptome. We next turned to examine the possibility that the differences in expression were a direct outcome of cellular coordination between tRNA supply and the demand imposed by the translated mRNAs for certain tRNA isoacceptors. If such coordination exists it would lead to a simple negative feedback loop in which deletion of a tRNA results in up-regulation of transcripts containing the relevant codon in order to compensate for the reduction in translation. To examine such a possibility we calculated the correlations between codon usage of the deregulated genes for each tRNA deletion strain and the extent to which they use the corresponding codon (**Figure 20**). In this analysis the initiator methionine and three stop codons were not included as they are obviously present in all genes regardless of codon bias. For purpose of normalization we used codon frequency which normalizes out the gene length. Most of the tRNA deletion strains showed no significant correlation between expression change and the frequency of the corresponding codon. This analysis refuted the possibility that the expression changes observed in the tRNA deletions were the result of a simple feedback

loop, exposing a more complex molecular mechanism for all the examined tRNA deletions.



**Figure 20 - Pearson's correlation between Codon frequency in transcriptom for each tRNA deletion and the 65 codons.** The matrix indicates the correlation values between codon frequency (codon usage divided by gene length) in the changed transcriptom of each tRNA deletion strain and the 63 codons in the genome. The four dark blue columns indicate stop codons and the initiator methionine, which were excluded from the analysis.

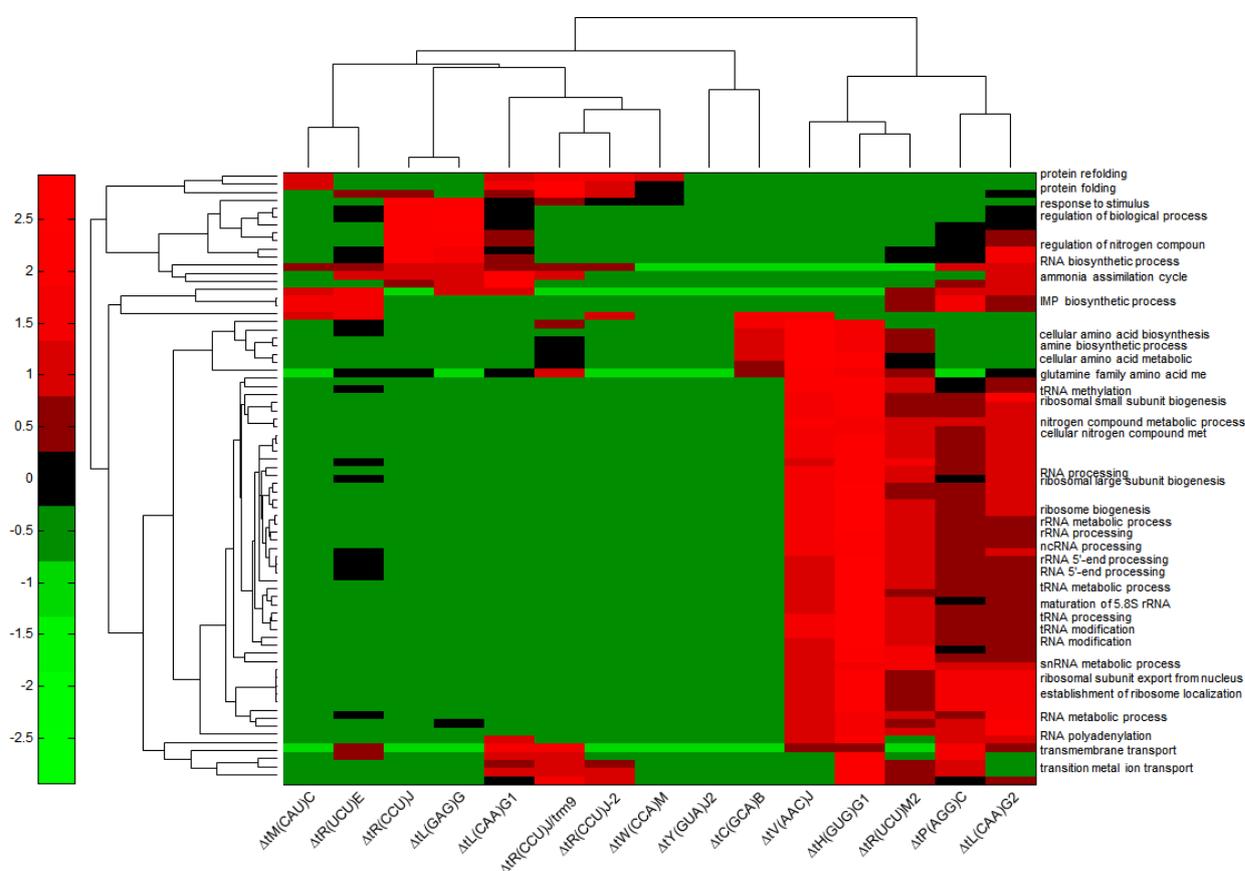
### 5.11.1. Cellular pathways possibly coupled to tRNA deletions

To get a better understating of the cellular pathways which might be affected by deletions of tRNA genes we used two different analyses. The first was examination of enriched GO terms using the Gene Ontology enrichment analysis and a corresponding visualization tool<sup>83</sup>. The second analysis correlated the expression changes observed for the tRNA deletions to a database containing expression data from about 300 ORF deletions, known as the “Compendium”<sup>84</sup>, and various growth and stress conditions<sup>85</sup>. These analyses have the potential to expose cellular pathways which are difficult to identify at single gene resolution by exposing enrichment in elevated or repressed pathways or by exposing similarities to expression profiles of ORFs which belong to well defined pathways. Combining these analyses with the significantly regulated genes defined for each tRNA deletion, revealed several pathways which might be coupled with the cellular response to a tRNA deletion.

### 5.11.2. Coupling of tRNA deletion to ribosome biogenesis and rRNA processing

Several translation related pathways changes upon tRNA deletion, including ribosome

biogenesis, rRNA processing, and tRNA processing. Interestingly the tRNA deletion strains could be grouped into two groups with opposite molecular signature according to the change in expression pattern. tRNA deletion strains corresponding to multi-copy families such as *tR(UCU)M2*, *tH(GUG)G1*, *tV(AAC)J* *tL(CAA)G2*, and *tP(AGG)C* were enriched in up-regulated genes responsible for "ribosome biogenesis" (*tL(CAA)G2* p-value  $6 \times 10^{-31}$ , *tH(GUG)G1* p-value  $7 \times 10^{-63}$ ) as seen in **Figure 21**. In addition processes which are coupled to ribosome biogenesis such as "rRNA processing" and related terms were up regulated (*tL(CAA)G2* p-value  $9 \times 10^{-27}$ , *tH(GUG)G1* p-value  $3 \times 10^{-55}$ ). The elevation in ribosome biogenesis and rRNA processing was accompanied by up-regulation of "tRNA processing" and "tRNA methylation" (*tL(CAA)G1* p-value  $7 \times 10^{-9}$  *tH(GUG)G1* p-value  $2 \times 10^{-17}$ ) (**Figure 21**).



**Figure 21 - Clustergram of normalised p-values for GO terms enriched in up-regulated genes in various tRNA deletion strains.** Presented are normalised p-values for GO terms which were enriched for at least 5 different tRNA deletions.

An interesting example for the elevation in all translation related processes was observed in the deletion of *tR(UCU)M2* which was enriched in up-regulated genes for "tRNA wobble base modifications" (p-value  $6 \times 10^{-7}$ ). These included the *TRM9* gene, encoding the enzyme responsible for modifying the anti-codon of this tRNA to modulate codon recognition<sup>103</sup>. In contrast deletions of tRNA genes from isoacceptors responsible for decoding all codons of a

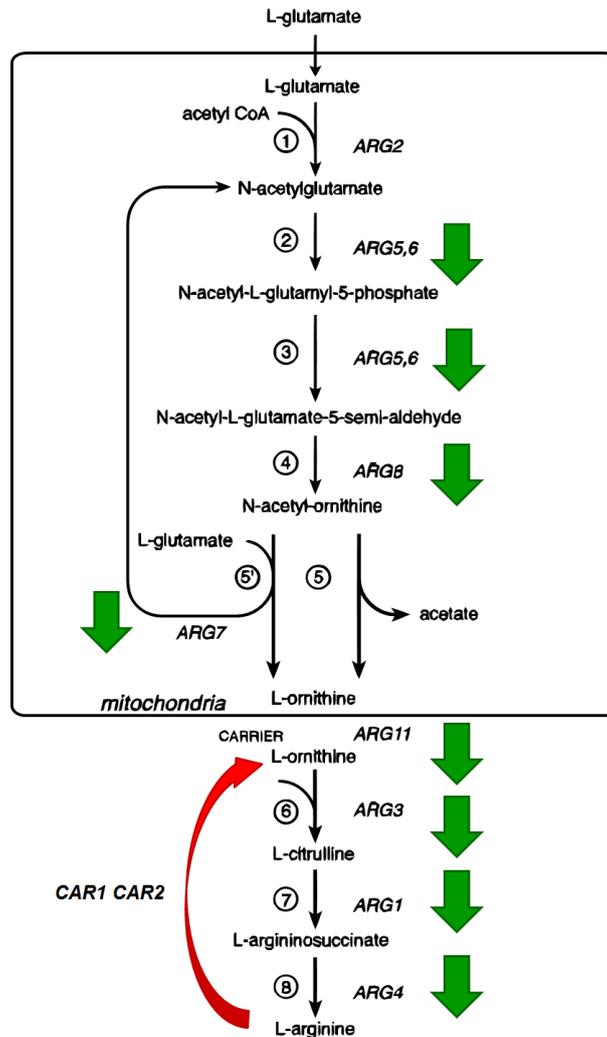
given amino acid, as *tM(CAU)C* and *tY(GUA)J2* resulted in down regulation of genes which belong to the "preribosome" complex (*tM(CAU)C* p-value  $6 \times 10^{-07}$ , *tY(GUA)J2* p-value  $2 \times 10^{-10}$ ), in addition to down-regulation of genes responsible for "rRNA processing" (*tM(CAU)C* p-value  $7 \times 10^{-6}$ , and *tY(GUA)J2* p-value  $9 \times 10^{-12}$ ).

This division of tRNA deletions, to those which result in up-regulation of translation-related process versus those which result in down-regulation, was also depicted in correlations with the relevant genes in the "compendium". Positive correlations were observed between the tRNA deletions which up-regulate ribosome biogenesis and rRNA processing to deletions of genes in these pathways. At the same time significant negative correlations were observed for the same genes and tRNA deletions which result in down-regulation of these processes. For example the down regulation of the *ECM16* essential gene, required for 18S rRNA processing resulted in positive correlations with tRNA deletions that belong to multi-copy families (*tL(CAA)G2*-0.44 p-value  $1 \times 10^{-195}$ , *tH(GUG)G1*-0.42 p-value  $4 \times 10^{-188}$ ) and negative correlations with tRNA deletions corresponding to families with few copies which represented by a single tRNA type (*tM(CAU)C*-0.25 p-value  $4 \times 10^{-64}$ , *tY(GUA)J2*-0.26 p-value  $1 \times 10^{-68}$ ).

The expression changes in translation related process may indicate that the translation process is affected once a tRNA gene is deleted. Elevation of these processes in tRNA deletions, which are part of a multi-gene family, may indicate a cellular attempt to increase translation by raising the number of ribosomes and tRNA molecules thus compensating for a tRNA reduction. In contrast deletion of tRNA genes for which substantial backup cannot be provided, results in a decrease in these very same processes. The coupling observed between these pathways is in line with the fact that in *S. cerevisiae*, as well as in other eukaryotes, the early processing of tRNA and rRNA are localized to the nucleolus, the site of ribosome biosynthesis<sup>95</sup>. The 5S rRNA, which is a part of the ribosome, is transcribed and processed by the same machinery as tRNA genes. Several indirect evidence for coordinating of rRNA and tRNA synthesis were put forth<sup>116,117</sup>, yet never was such a tight coupling observed in the synthesis of the two molecules involved in the translation process.

### **5.11.3. Coupling of tRNA deletion to amino acid biosynthesis**

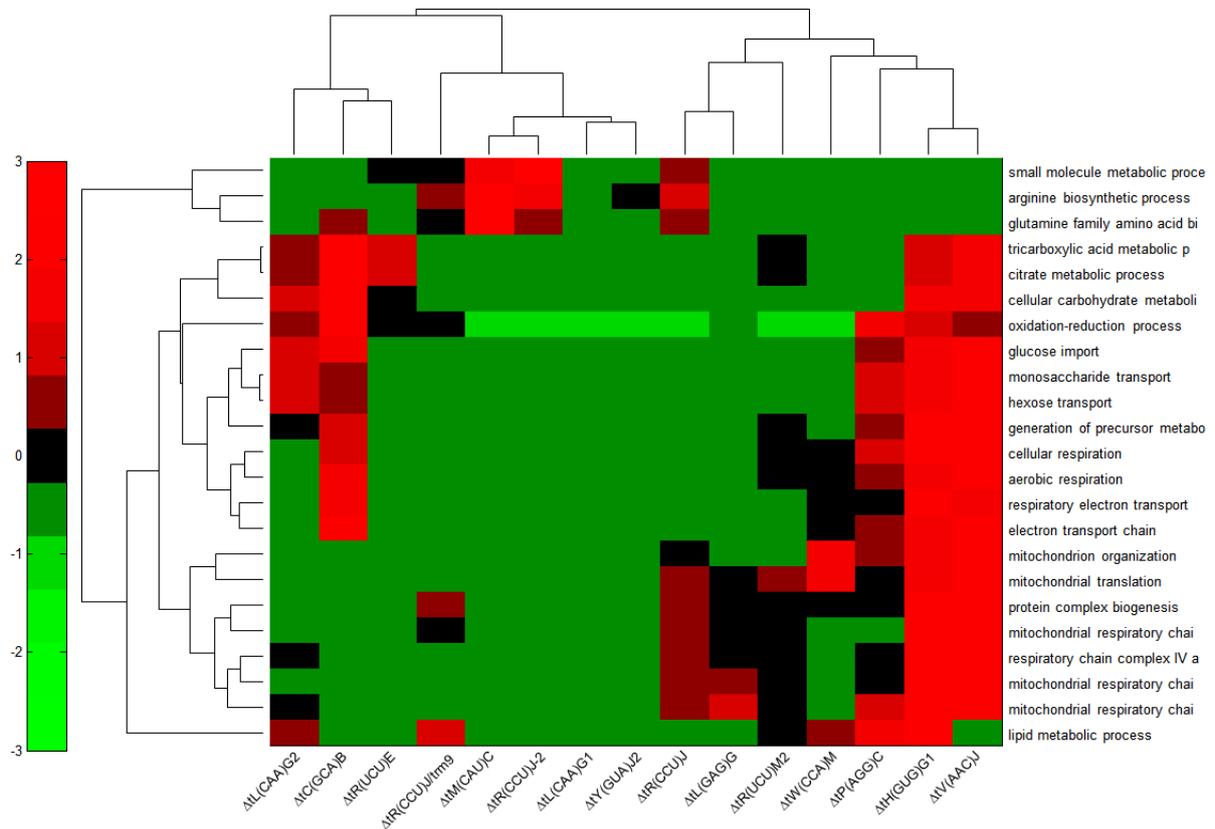
Amino acid metabolism was an additional cellular pathway that showed molecular response in multiple tRNA deletions. A striking effect on an amino acid biosynthetic pathway was observed for the *tR(CCU)J* deletion, in which the entire biosynthetic pathway of arginine was significantly down regulated (**Figure 22**).



**Figure 22 - Arginine biosynthetic pathways in *S. cerevisiae*.**

The yeast genes composing the Arginine biosynthetic pathway are designated by their historical nonsequential name. The *CAR1* and *CAR2* are involved in Arginine degradation and were added to the figure. Green arrows indicate significantly down-regulated genes, red arrows indicate significantly up-regulated genes<sup>118</sup>.

The biosynthetic pathway of Arginine is composed of 8 different enzymes in *S. cerevisiae*<sup>118</sup>. The transcription of this pathway is regulated by the general amino acid control, while most genes in the pathway are transcriptionally repressed by increased levels of arginine. In addition to the enzymes of the biosynthetic pathway which are down regulated in this tRNA deletion strain, the enzymes which are responsible for arginine degradation *CAR2* and to a lesser extent *CAR1* are significantly up-regulated. The down regulation of the arginine biosynthesis pathway was also depicted in enrichment of the relevant GO terms "arginine biosynthetic process" (p-value  $6 \times 10^{-10}$ ) "arginine metabolic process" (p-value  $5 \times 10^{-9}$ ) in down-regulated genes (**Figure 23**).



**Figure 23 - Clustergram of normalised p-values for GO terms enriched in down-regulated genes in the various tRNA deletion strains.**

Presented are normalised p-values for GO terms enriched in down-regulated genes in at least 5 tRNA deletions.

The deregulation of this pathway indicates that a single tRNA deletion leads to accumulation of arginine in the cell; to which the cell responds by reducing activity in the pathway and increasing degradation to accommodate cellular needs. Further support for the down-regulation of this process was found in the double deletion of  $\Delta tR(CCU)J/trm9$ , this strain also shows a significant (p-value  $3 \times 10^{-9}$ ) enrichment in down-regulated genes with the GO term "arginine metabolic process".

The down regulated genes in this group are identical to those found for the  $\Delta tR(CCU)J$ , and may serve as a biological repetition. While in the  $\Delta trm9$  strain these genes are not down regulated. An additional interesting case is that of the initiator Methionine tRNA deletion. The GO enriched terms for this deletion strain showed a down regulation of several general terms of amino acid biosynthesis such as "cellular amino acid biosynthetic process" (p-value  $2 \times 10^{-16}$ ) in addition to "amine biosynthetic process" (p-value  $5 \times 10^{-16}$ ), "cellular amino acid metabolic process" (p-value  $9 \times 10^{-14}$ ). In addition, in down-regulated genes, GO terms were enriched for specific amino acid process, "arginine metabolic process", "lysine biosynthetic process", and "aspartate family amino acid metabolic process". For this tRNA deletion the entire GO tree concerning amino acid metabolic process was significantly enriched in down-

regulated genes, which correlates with reduction of translation related processes. Once again an opposite response was observed for tRNA deletions which up-regulate translation related processes, and are also enriched in up-regulated genes for "cellular amino acid metabolic process" (*tH(GUG)G1* p-value of  $9 \times 10^{-17}$ , *tP(AGG)C* p-value  $6 \times 10^{-6}$ ).

Examining the expression correlations with the "compendium" database, revealed interesting correlations to several amino acid synthetases, which are essential genes. For instance we observed correlations with down regulation of *GUS1*, an essential gene which encodes glutamic acid synthase ( $\Delta tR(CC U)J$ -0.264 p-value 0,  $\Delta tL(GAG)G$ -0.313 p-value 0). Down regulation of an amino acid synthase would lead to accumulation of the relevant amino acid, and thus to a cellular response similar to the deletion of tRNA genes. This coupling between changes in the tRNA pool and amino acid biosynthesis presents a possible cellular route to sense the deletion of a tRNA and regulate the amounts by production and degradation.

#### **5.11.4. Coupling of tRNA deletion to protein misfolding**

A different angle of translation obstruction may be manifested by production of misfolded proteins, and the consequent cellular handling and response to unfolding stress. Multiple tRNA deletions (*tR(CC U)J*, *tM(CAU)C*, *tW(CCA)M*, *tL(CAA)G2*, *tR(CC U)J/trm9*) exhibited significant up-regulation (more than two fold) of various heat shock proteins and chaperons such as *HSP82*, *HSP78*, *HSP12*, *HSP26*, *HSP42*, and *HSP104*. The up-regulation of these genes resonates with their role in refolding and disaggregating misfolded proteins and their function in the quality control machinery<sup>119-121</sup>. The up-regulation in genes encoding heat shock proteins was accompanied in several strains by autophagy components such as the *ATG7*, *ATG8*, and *ATG9* genes. Autophagy has been implicated in the clearance of protein aggregates and possibly also ubiquitinated misfolded proteins, and may thus be coupled to protein misfolding<sup>86,122</sup>. Examination of enriched GO terms added further evidence for potential unfolding stress, for example 5 tRNA deletions were enriched in up-regulated genes for GO terms "protein refolding" (*tM(CAU)C* p-value  $9 \times 10^{-7}$ ), "protein unfolding" (*tM(CAU)* p-value  $5 \times 10^{-6}$ ) and "protein folding" (*tM(CAU)C* p-value  $9 \times 10^{-6}$ ) (**Figure 21**). In addition several of these tRNA deletions were also enriched in up-regulated genes of the terms "proteolysis" (*tR(CC U)J* p-value  $3 \times 10^{-11}$ ), "ubiquitin-dependent protein catabolic process" (*tR(CC U)J* p-value  $7 \times 10^{-9}$ ) and in relevant complexes "proteasome complex" and "proteasome storage granule" (*tR(CC U)J* p-value  $2 \times 10^{-16}$ , and  $1 \times 10^{-12}$  respectively). Most

soluble misfolded proteins are cleared through the ubiquitin-proteasome system, which is the major proteolytic pathway<sup>123</sup>.

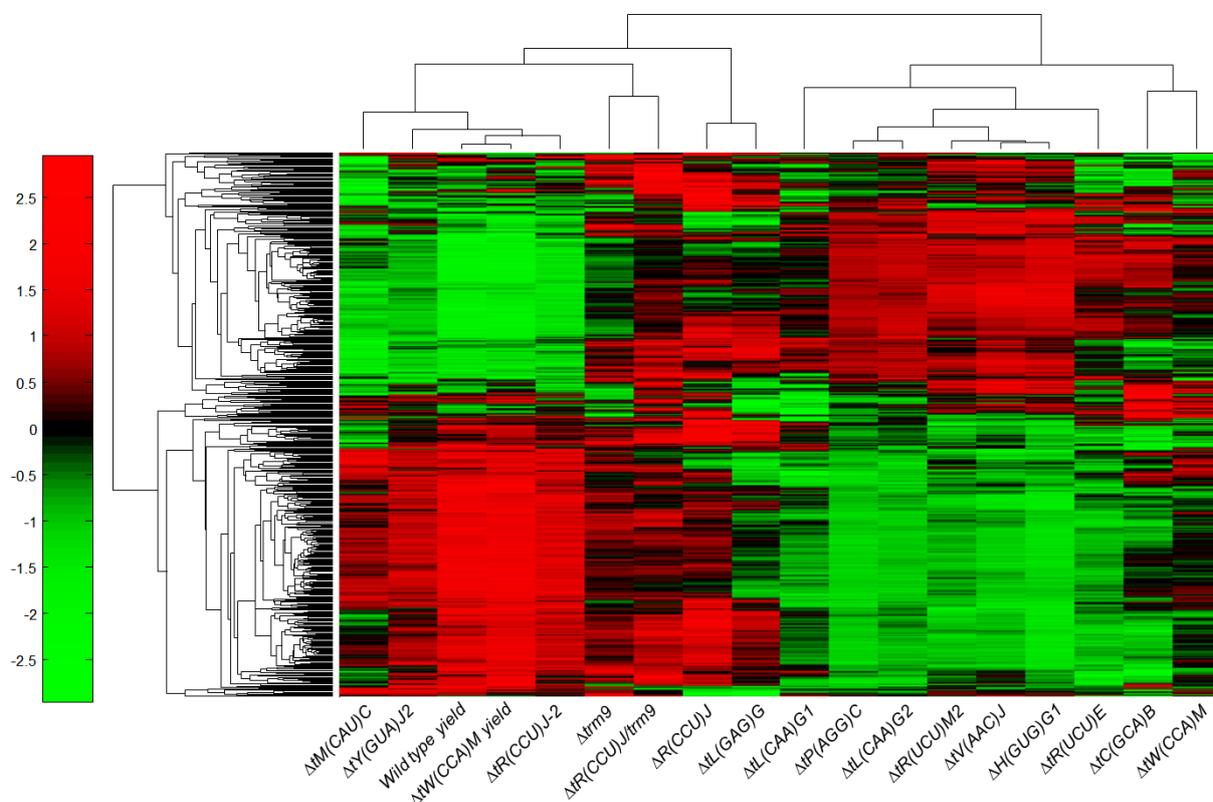
Additional indications for possible unfolding stress were found once examining the correlations with the "compendium" database. Significant correlations were observed with the deletion of *HSF1* a transcription factor that regulates transcription in response to stress (*tR(CCU)J*-0.32, *tL(GAG)G*-0.28; p-value 0). The activation of *HSF1* is dependent on accumulation of denatured or misfolded proteins<sup>124</sup>, further strengthening the connection to protein unfolding. Interestingly correlations were also observed for several tRNA deletions with the expression profiles retrieved by Travers et al. 2000, in which the transcriptional scope of the unfolded protein response (UPR) was determined using DTT, Tunicamycin (two unfolding drugs), and deletions of key components in the process<sup>125</sup>. High correlations were observed upon deletion of *IRE1* followed with DTT treatment (*tR(CCU)J*-0.4 p-value  $4 \times 10^{-159}$ ) or Tunicamycin treatment (*tR(CCU)J*-0.37 p-value  $7 \times 10^{-142}$ ). Correlations were also observed with additional experiments in which cells were treated with DTT and Tunicamycin as well as treatment with Diamide or heat shock. Taken together, the potential outcome of changes to the tRNA pool following a tRNA deletion, appears to be protein misfolding leading to folding stress in the cells.

#### **5.11.5. Specific vs. general effect of tRNA deletions on the cell**

An interesting pattern that emerged from the expression profile and all consequent analysis, the molecular signature seemed to varied between tRNA deletions from specific responses including few changed genes and specific functions and up to wide changes in both the number of genes that changed and the functions they effect resulting in a general effect on the cell. The group of "multi-copy gene" tRNA families exhibited changes in specific functions mostly translation related, such as ribosome biogenesis rRNA processing and tRNA processing. In addition to genes related to amino acid biosynthesis. The second group of "few gene copies" tRNA families which includes the two single copy gene tRNAs, which exhibit a general effect on the cell indicated by their effect on multiple cellular process resulting in enrichment in up-regulated genes of GO terms such as "response to stimulus" (*tR(CCU)J* p-value  $1 \times 10^{-10}$ ; *tL(GAG)G* p-value  $1 \times 10^{-6}$ ) as well as terms such as "regulation of biological process", "cell cycle process" and "signal transduction". In addition this group seems to exist in an inherent state of stress, the severity of which varies between them.

To summarize we identified two distinct molecular signatures, dividing the tRNA deletion

strains according to their expression profile into two types of responses. This division could be depicted in the correlations between tRNA deletion strains (**Figure 19**), and is also clearly seen in the correlations to the "compendium" database (**Figure 24**). Which demonstrates a clear division to two groups and correlations to different genes and conditions.



**Figure 24 - Clustergram of correlations to the "compendium" database for the various tRNA deletion strains.** Spearman correlations normalised per row for each of the tRNA deletion strain with the "compendium" database.

The response to a tRNA deletion seems to depend on the copy number and on the presence and decoding properties of additional isoacceptors for that amino acid, as was observed for the 13 examined tRNA deletions. The group of "multi-copy gene" tRNA deletions demonstrate a cellular response that involves up-regulation of translation related processes such as ribosome biogenesis, rRNA and tRNA processing. The group of "few gene copies" tRNA deletions seems to exist in an inherent state of stress, in which the two single copies tRNA deletions exhibit the most severe state of stress. The cellular response to this group effects ribosome biogenesis and amino-acid biosynthesis, which are down regulated in addition to up regulating functions of protein folding and refolding. In contrast the deletion of tRNAs from "multi-copy gene" families did not show such hallmarks of stress response. The molecular signatures obtained for the various tRNA deletions, provided the first evidence that a tRNA deletion results in specific effects on molecular pathways, and points to two different cellular responses.

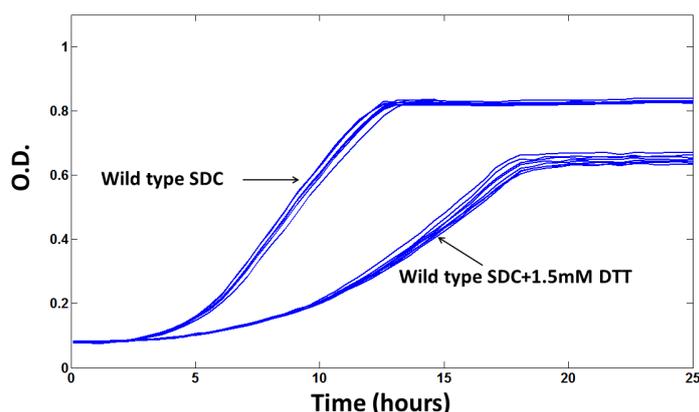
## 5.12. Deletion of tRNA genes inflicts folding stress in the cells

A first clue for the potential impact of tRNA deletions on protein folding in the cell comes from the expression profile analysis of the various tRNA deletion strains. The observations that among the most highly induced functional categories in several tRNA deletions were functions related to folding, unfolding and re-folding of proteins accompanied by functions related to proteasomal degradation and proteolysis. This suggested that in these tRNA deletion strains hampering the flow of ribosomes by changes in the tRNA pool may have affected proper protein folding, thus increasing the load on the quality control machinery and causing folding stress in the cells. Maintaining proper protein folding is essential for cell viability, while failure to eliminate misfolded proteins can result in toxic aggregates, driving the cells to develop elaborate machinery that monitors and maintains protein folding<sup>126</sup>. Preserving protein homeostasis involves several parallel strategies that aim at refolding, degrading and/or sequestering misfolded polypeptides<sup>127</sup>. Central to these pathways, is a network of molecular chaperons that recognizes misfolded proteins and can actively promote either refolding or degradation of the misfolded proteins<sup>128</sup>. The connection between protein folding and translation was examined in multiple studies over the years, mostly focused on the effects of codon usage on protein folding<sup>129-131</sup>. These studies found that local translation rates influence *in vivo* protein-folding, while changes in codon usage may lead to increased levels of protein misfolding<sup>130</sup>. Changes in codon usage also effect translation error rate, optimal codons may be required to reduce the frequency of translation errors at aggregation-prone sites that coincide with certain functional sites of the protein, such as protein-protein interfaces. Alternatively optimal codons may be required for rapid translation of aggregation-prone regions<sup>131</sup>. The effect of tRNA concentrations on translation speed and accuracy was not thoroughly examined, yet was found to contribute substantially to translation speed, which is tightly connected to protein folding<sup>132,133</sup>. Thus far attempts to study the effect of codon usage or tRNA concentrations on protein folding, had two major shortcomings, studies were performed on single genes and changed synonymous codons, thus changing intrinsic mRNA properties such as secondary structure. Using tRNA deletions enables delicate perturbations of the tRNA pool and elegantly overcomes these weaknesses. The effects can be studied on a genome wide scale without effecting the mRNA inherent properties. To examine the connection between changes in the tRNA pool and protein misfolding, I chose two complementary routes. The first was an indirect approach, to assay the growth of the tRNA deletion mutants in the

presence of drugs that inflict unfolding stress and examine whether they are differentially affected compared to the wild type strain. The second route was direct monitoring of the load on the quality control machinery using an indicator for unfolding stress.

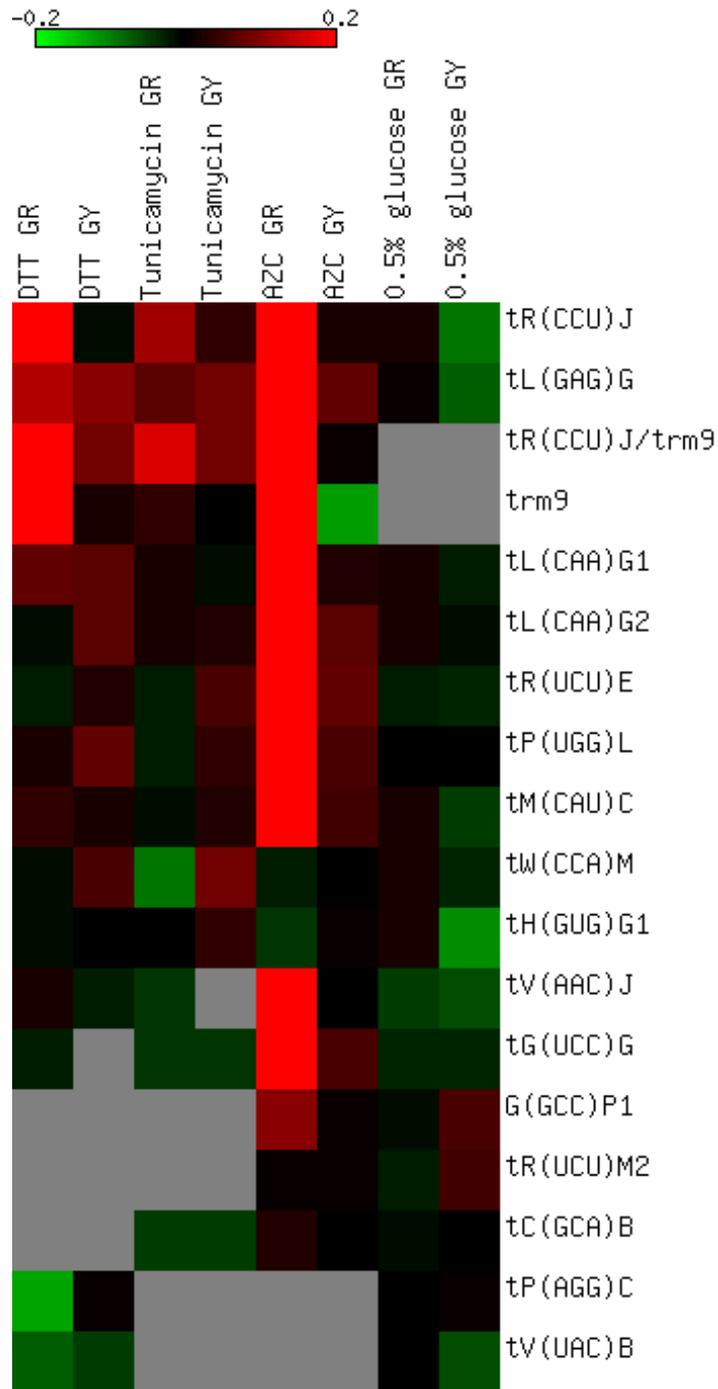
### 5.13. Deletion of tRNA genes confers auto-protection against unfolding stress

To analyse the growth of tRNA deletions in unfolding environment I selected three different unfolding agents, Dithiothreitol (DTT), a reducing agent; Tunicamycin, a blocker of all N-linked glycoproteins synthesis and Azetidine-2-carboxylic acid (AZC), a toxic analog of proline. The concentration of each unfolding agent was calibrated using both the wild type strain and 7 tRNA deletion strains, which exhibit various growth defects. The desired concentration was one in which the growth was aggravated considerably, yet strains were able to reach stationary phase within a reasonable time frame, avoiding massive cell cycle arrest and cell death. **Figure 25** shows a representative growth curve of the wild type on minimal medium vs. minimal medium supplemented with 1.5mM DTT.



**Figure 25 - Growth curves of wild type in minimal medium vs. medium supplemented with 1.5mM DTT.** Growth curve of wild type strain in minimal medium and minimal medium supplemented with 1.5mM DTT. Each growth curve is composed of 12 lines each representing a technical repetition.

The growth in the presence of DTT resulted in a longer lag phase accompanied by reduction in growth rate and yield. Aggravations of growth were also observed when examining the growth on media supplemented with 1.5 $\mu$ g Tunicamycin or 5mM AZC (data not shown). The growth experiments were conducted as previously described (see Materials and Methods), and relative growth measurements in unfolding stress were compared to growth values in media without unfolding drugs (YPD, YPD+DMAO, SDC see Material and Methods for details). The strains selected for these analyses were tRNA deletions exhibiting a range of growth phenotypes, from no growth defect to strains exhibiting the most severe growth defects on rich or/and minimal media in either growth parameter. Emphasis was put on selecting deletions that would represent a wide range of isoacceptors and amino acids enabling a broad examination of the effect of different deletions on the tRNA pool.



**Figure 26 - Differences in relative growth parameters across stress conditions.**

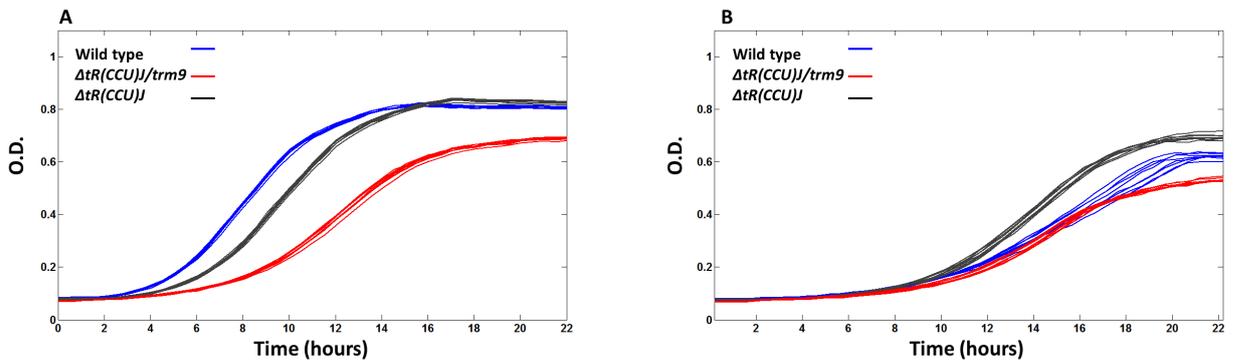
Change in relative growth parameters, growth rate (GR) and growth yield (GY) for each strain were calculated by subtracting the values obtained without stress for each strain to the values obtained in stress conditions. The differences were scaled between strains. The color intensity is proportional to the change in growth parameters. Red denotes increase and green denotes decreases in growth parameters.

A summary of the growth differences for a subset of 17 tRNA deletions, in various stress conditions including the three unfolding agents and a metabolic stress is presented in **Figure 26**. The color map indicates the change in a given condition separating growth rate and growth yield, relative to the values obtained without stress for these tRNA deletion. The differences in growth values are indicated in the heat map by growth alleviation (red) or

aggravation (green), no change and retention of growth relative to the wild type is indicated in black. A missing measurement for a given strain is indicated in grey. As a whole the various tRNA deletion screened in these assays can be divided into three major groups according to their growth in the presence of the various drugs. The first group of tRNA deletions demonstrated no growth defects once grown in the presence of unfolding agents (upper part of **Figure 26**), relative to their severe growth defects in conditions of rich or minimal media. Some of the tRNA deletions in this group even grow better than the wild type, in several unfolding stress conditions,  $\Delta tR(CC U)J$  and  $\Delta tL(GAG)G$  are the most dominant in this group. It should be noted that the tRNA deletions which seem to perform better in unfolding stress conditions, often exhibit growth aggravation once examined on other types of stress such as metabolic or salinity. A second group was composed of tRNA deletions which retained their growth relative to the wild type strain in most of the unfolding stress (middle part of **Figure 26**) thus the inflicted folding stress effects both tRNA deletions and wild type strain to the same extent ( $tR(UCU)M2$ ,  $tH(GUG)G$ ). The third group contained tRNA deletion strains which exhibited aggravation of growth in the presence of unfolding agents in either growth rate or yield (lower part of **Figure 26**). These tRNA deletion strains exhibit growth aggravation once unfolding stress is inflicted while exhibiting a mild or no defect in metabolic stress ( $tC(GCA)B$ ,  $tP(AGG)C$ ,  $tV(UAC)B$ ). Some tRNA deletions demonstrated complex behavior in which their growth was effected differently by each unfolding agent ( $tW(CCA)M$ ). It should be mentioned that although all three agents inflict unfolding stress in the cells the severity of the inflicted stress may vary between agents.

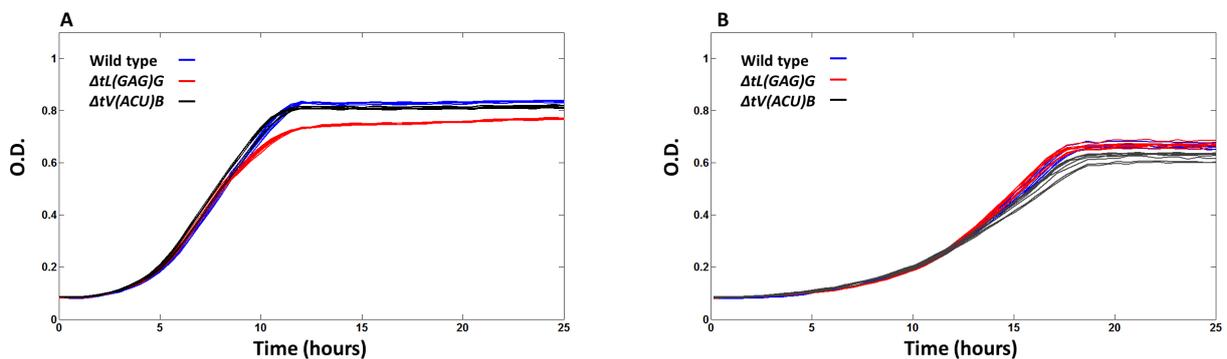
### 5.13.1. Deletion of tRNA genes results in auto-protection against DTT

The growth of 20 tRNA deletion strains including the double deletion  $\Delta tR(CC U)J/trm9$  were examined in the presence of 1.5mM DTT. DTT is a strong reducing agent that is often used to promote unfolding stress, by preventing disulfide bond formation, which in turn leads to accumulation of misfolded proteins in the cell<sup>134</sup>. DTT inflicted stress in *S. cerevisiae* was found to effect translation by inhibiting translation initiation<sup>135</sup>, in addition treatment with DTT causes aggregation of ribosomal proteins<sup>136</sup>. Based on these findings it was suggested that the production of new ribosomes is sensitive to DTT<sup>136</sup>. In light of the above it is clear that DTT treatment leads to a strong unfolding stress effecting protein folding and possibly ribosome biogenesis. **Figure 27** and **Figure 28** show growth curves of  $\Delta tR(CC U)J$ ,  $tL(GAG)G$  and  $\Delta tR(CC U)J/trm9$  which exhibit a severe growth defect on minimal medium (**Figure 27-A**, **Figure 28-A**) and demonstrate a remarkable alleviation of growth defects once unfolding stress is inflicted by DTT (**Figure 27-B**, **Figure 28-B**).



**Figure 27 - Growth curves of  $\Delta tR(CCU)J$  and  $\Delta tR(CCU)J/trm9$  vs. wild type in minimal medium with 1.5mM DDT.** A-Growth curves of  $\Delta tR(CCU)J$  and  $\Delta tR(CCU)J/trm9$  vs. wild type in minimal medium. B-Growth curves of  $\Delta tR(CCU)J$  and  $\Delta tR(CCU)J/trm9$  vs. wild type in minimal medium supplemented with 1.5mM DTT. Each curve is composed of 12 technical repetitions of the experiment.

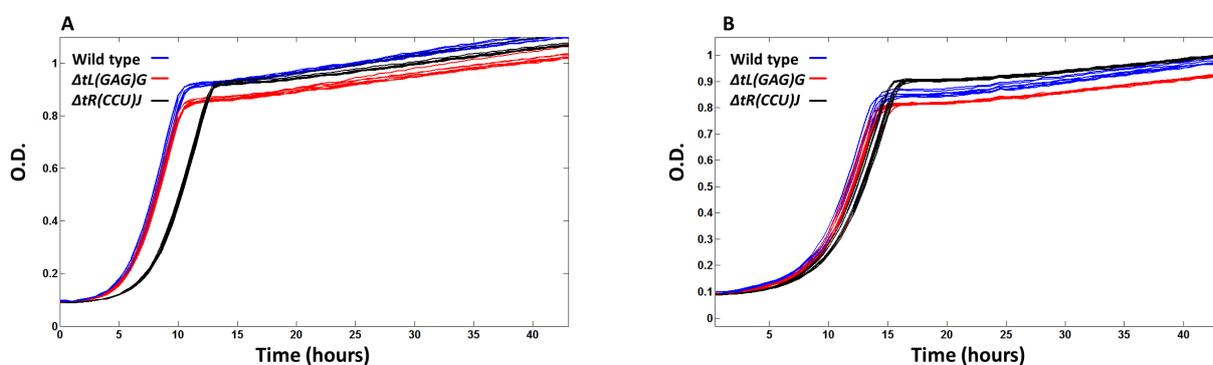
Half of the 20 examined tRNA deletions demonstrated alleviation to various extent of either growth rate or growth yield once unfolding stress was inflicted by DTT. As opposed to the growth in unfolding conditions, growth of the same strains in indifferent stress conditions (such as metabolic or salinity) resulted in substantial growth defects (Figure 26 right columns). The fact that these tRNA deletion strains are less effected by the inflicted unfolding stress compared to the wild type strain raises the possibility that the molecular response to a tRNA deletion confers auto-protection against DTT inflicted unfolding stress. Further support for this was revealed when the concentrations of unfolding agent were increased, to a level which inhibit the growth of the wild type strain (2mM DTT), yet  $\Delta tR(CCU)J$  and  $\Delta tL(GAG)G$  were able to grow. The remaining 10 tRNA deletion strains were either effected to the same extent as the wild type thus retaining their relative growth, such as  $tH(GUG)G1$ , or exhibited growth aggravation such as  $tV(UAC)B$  (Figure 26).



**Figure 28 - Growth curves of  $\Delta tL(GAG)G$  and  $\Delta tV(UAC)B$  vs. wild type in minimal medium with 1.5mM DDT.** A-Growth curves of  $\Delta tL(GAG)G$  and  $\Delta tV(UAC)B$  vs. wild type in minimal medium. B- Growth curves of  $\Delta tL(GAG)G$  and  $\Delta tV(UAC)B$  vs. wild type in minimal medium supplemented with 1.5mM DTT. Each curve is composed of 12 technical repetitions of the experiment.

### 5.13.2. Deletion of tRNA genes results in auto-protection against Tunicamycin

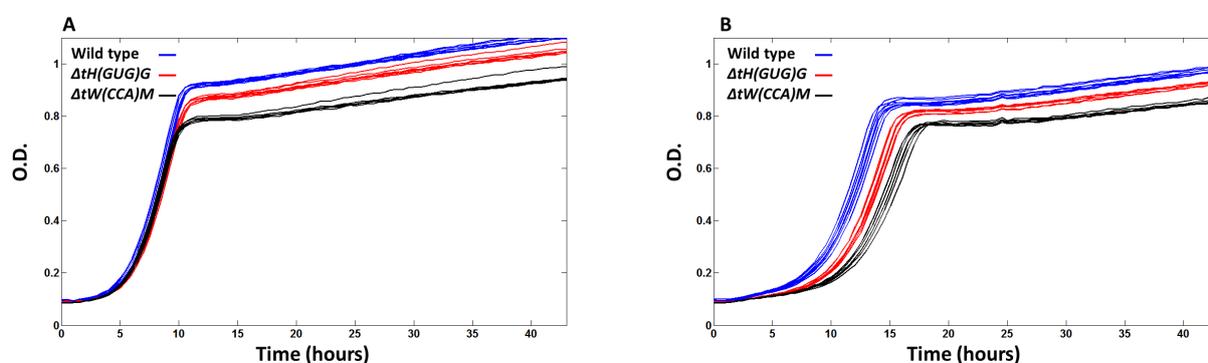
An unfolding stress induced by DTT treatment leads to accumulation of misfolded proteins in the cell followed by activation of the unfolded protein response (UPR). The unfolded protein response (UPR) regulates gene expression in response to stress in the endoplasmic reticulum (ER). Yet there are many genes which are up-regulated in response to DTT treatment whose functions seem unrelated to the ER and do not form part of the UPR response. Tunicamycin is an ER specific drug that blocks the synthesis of all N-linked glycoproteins (N-glycans) N-linked glycoproteins (N-glycans) by inhibiting the enzyme which performs the first step in glycoprotein synthesis. Due to this specific inhibition Tunicamycin is regularly used to induce the unfolded protein response (UPR) in the ER<sup>125</sup>. To examine directly the effect of misfolding in the ER and possible activation of the unfolded protein response, I examined the growth of the tRNA deletion strains in the presence of Tunicamycin. The growth of 13 tRNA deletions was examined in rich medium supplemented with 1.5µg/ml Tunicamycin. As was observed in the DTT treatment, 7 tRNA deletions exhibited alleviation of the growth defects observed on rich medium. **Figure 29** shows representative growth curves of  $\Delta tL(GAG)G$  and  $\Delta tR(CCU)J$  which demonstrate such alleviation of growth.



**Figure 29 - Growth curves of  $\Delta tL(GAG)G$  and  $\Delta tR(CCU)J$  vs. wild type in rich medium with 1.5µg Tunicamycin. A-Growth curves of  $\Delta tL(GAG)G$  and  $\Delta tR(CCU)J$  vs. the wild type in rich medium supplemented with DMSO. B-Growth curves of  $\Delta tL(GAG)G$  and  $\Delta tR(CCU)J$  vs. the wild in rich medium supplemented with 1.5µg/ml Tunicamycin Each curve is composed of 12 technical repetitions of the experiment.**

These tRNA deletion strains seem to handle the inflicted unfolding stress better than the wild type, which was further established once elevating the Tunicamycin concentrations. Eventually in a concentration of 10µg/ml Tunicamycin growth was not feasible for the wild type strain, yet once again, the two single copy tRNA deletions  $tR(CCU)J$  and  $tL(GAG)G$  were able to grow. There were 5 tRNA deletion strains in which the effect of Tunicamycin was similar in both tRNA deletions and wild type strain, thus relative growth values were

unchanged. Some of these strains while retaining the relative growth in terms of growth rate and yield, exhibited an extended lag phase relative to the wild type such as  $\Delta tH(GUG)G1$  (Figure 30). Only a single tRNA deletion strain  $tW(CCA)M$  suffered from growth aggravation in this condition displaying a severe reduction of growth rate in addition to an extended lag phase (Figure 30).



**Figure 30 - Growth curves of  $\Delta tH(GUG)G$  and  $\Delta tW(CCA)M$  vs. wild type in rich medium with 1.5  $\mu$ g Tunicamycin.**

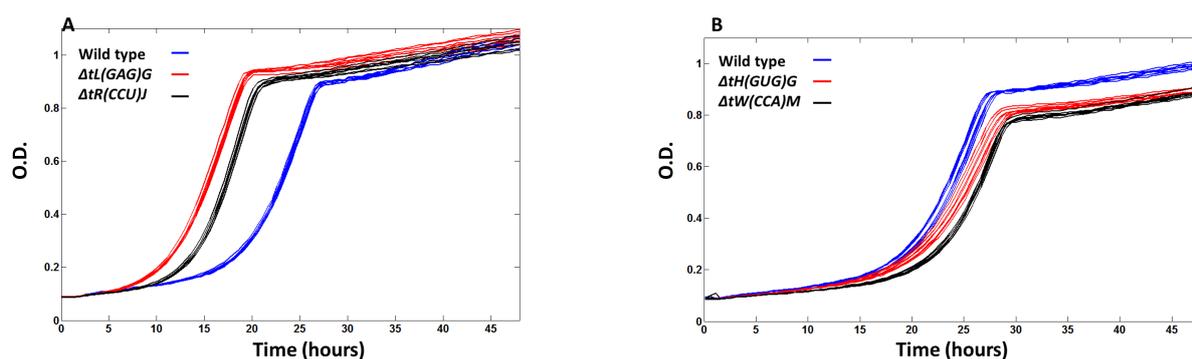
**A**-Growth curves of  $\Delta tH(GUG)G$  and  $\Delta tW(CCA)M$  vs. wild type in rich medium supplemented with DMSO. **B**-Growth curves of  $\Delta tH(GUG)G$  and  $\Delta tW(CCA)M$  vs. wild type in rich medium supplemented with 1.5  $\mu$ g/ml Tunicamycin. Each curve is composed of 12 technical repetitions of the experiment.

The growth experiments performed using Tunicamycin as an unfolding agent reestablished the observations obtained using DTT as unfolding agent. Growth defects exhibited by a subset of tRNA deletions in rich medium were alleviated, once again indicating the ability of the tRNA deletions to cope with unfolding stress. High similarity in the identity of tRNA deletions exhibiting alleviation can be clearly observed in Figure 26. The extent to which Tunicamycin alleviated the growth was milder relative to the differences observed for DTT treatment, this might be the outcome of a more focused response in which only specific pathways related to the ER are affected and misfolded proteins accumulate only in the ER as opposed to the effect of DTT on the cell.

### 5.13.3. Deletions of tRNA genes results in auto-protection against AZC

To further examine the effect of unfolding on newly synthesized proteins in tRNA deletion strains, I supplemented the growth medium with Azetidine-2-carboxylic acid (AZC). AZC is a toxic analog of proline which was shown to generate folding stress in the cell, by competing with proline for incorporation into newly synthesized cellular proteins<sup>128</sup>. Incorporation of the analog into proteins is known to cause reduced thermal stability or misfolding<sup>137,138</sup>. Microarray expression data of cells treated with AZC suggest that the observed effects on gene expression are most likely due to the misfolding of proteins. In addition AZC treatment

selectively causes the majority of the gene expression changes characteristic of temperature upshift (25°C to 37°C) which further strengthen the connection to protein folding. The relative resemblance in up-regulated heat shock proteins between cells treated with AZC and tRNA deletion strains further motivated the use of this unfolding agent in growth experiments. The growth of 15 tRNA deletion strains including the double deletion  $\Delta tR(CC U)J/trm9$ , was analyzed using 5mM AZC to inflict unfolding stress. The results of these growth experiments were rather striking, as most tRNA deletion strains (12 strains) exhibited higher growth rate values relative to the wild type. While 6 tRNA deletion strains exhibit a growth rate defect in rich medium, for which they show complete alleviation upon treatment with AZC, no such defect was exposed for the remaining strains which grow essentially as the wild type in rich medium. Given the severe growth defect experienced by certain tRNA deletions of up to 15% growth reduction for the double deletion  $\Delta tR(CC U)J/trm9$  and 8% growth reduction for  $\Delta tR(CC U)J$  the alleviating effect was dramatic leading to a more than 40% change in growth rate for the  $\Delta tR(CC U)J$  and 30% for the  $\Delta tR(CC U)J/trm9$  (**Figure 26, Figure 31-A**). All 12 strains, which display increased growth rate also demonstrated a shorter lag phase, which further emphasizes their ability to cope better with the stress (**Figure 31-A**). Alleviation of the growth defects observed in rich medium, was also apparent in growth yield values yet to a smaller extent. Out of 12 tRNA deletion strains, which exhibit a growth yield defect in rich medium, 6 showed a complete alleviation of the defect exhibiting growth yield values similar to thus of the wild type (**Figure 31-A**). The remaining strains retained their growth relative to the wild type.



**Figure 31 - Growth curves of tRNA deletions vs. wild type in rich medium with 5mM AZC.**

**A**-Growth curves of  $\Delta tL(GAG)G$  and  $\Delta tR(CC U)J$  vs. wild type in rich medium supplemented with 5mM AZC.

**B**-Growth curves of  $\Delta tH(GUG)G$  and  $\Delta tW(CCA)M$  vs. the wild type in rich medium supplemented with 5mM AZC. Each curve is composed of 12 technical repetitions of the experiment.

Despite the dramatic effect on growth for most tRNA deletions, there were two tRNA deletions (*tR(UCU)M2* and *tW(CCA)M*) for which AZC inflicted unfolding stress, resulted in no change in relative growth values, in both growth rate and growth yield. Moreover the deletion of *tH(GUG)G1* exhibited a slight aggravation of growth rate relative to the wild type and no change in growth yield values. All three tRNA deletion strains experienced an extended lag phase (**Figure 31-B**). Thus it appears that the effect of AZC was mainly manifested in the growth rate parameter and in the length of the lag phase which is tightly linked to growth rate. Most tRNA deletion strains demonstrated a better ability to cope with the inflicted unfolding stress relative to the wild type reinforcing the observation of auto-protection upon unfolding stress.

The examination of tRNA deletions across unfolding stress demonstrated a strong tendency for certain tRNA deletions to exhibit alleviation and auto-protection against unfolding stress for all the examined unfolding agents. These is in line with prior indications for a cellular response to unfolding stress observed for these tRNA deletions in the expression profile. A good agreement was observed between the severities of the growth defect experienced by the various tRNA deletions in default conditions and the level of protection. Growth defects manifested in both growth rate and growth yield granted protection against the various misfolding agents. This observed protection is not likely to be the result of a reduced growth defect as most of the strains selected for the growth analysis suffered from growth yield defects.

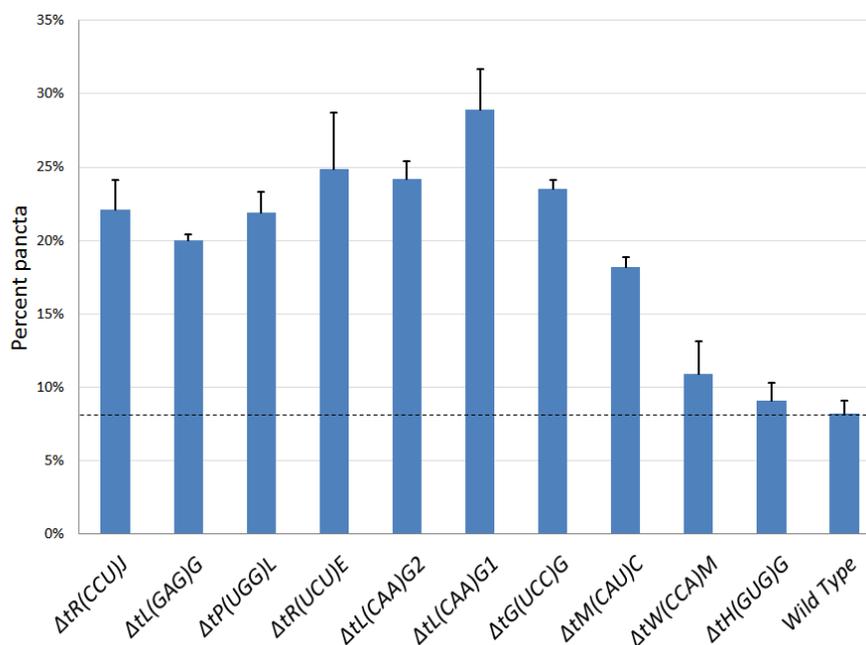
#### **5.14. Deletion of tRNA genes intensifies the load of misfolded proteins**

The quality control machinery partitions misfolded proteins, on the basis of their ubiquitination state and solubility, among two distinct quality control compartments the JUNQ ('Juxta Nuclear Quality Control') and IPOD ('Insoluble Protein Deposit'). Following misfolding most proteins are recognized by the quality control machinery, which directs them to the JUNQ, were they are disaggregated by chaperones and the 26S proteasome complex<sup>86</sup>. Sending misfolded proteins to the JUNQ is more prevalent under normal cellular conditions, yet when the load increases, and the quality control machinery needed for sorting in the 'JUNQ' is saturated, misfolded proteins are directed to the 'IPOD', which seems to terminally sequester and degrade protein aggregates<sup>86</sup>. Motivated by the expression-based indications that tRNA deletions might inflict an unfolding stress on the cell, and in light of the growth experiments preformed in the presence of protein unfolding

agents, I attempted to directly examine protein homeostasis in the tRNA deletions strains. Direct examination of protein homeostasis was achieved by adopting the unfolding assay developed by Kaganovich et al., 2008. This assay uses an unassembled proteins, such as the von Hippel-Lindau (VHL) tumor suppressor protein, as indicators for the load on the quality control machinery. The folding of the VHL protein is tightly coupled to its assembly into a ternary complex with its partner proteins Elongin B and C<sup>126</sup>, correct folding is possible only after binding to its cofactors. Impairment of binding or expression in cells lacking the relevant cofactors (such as *S. cerevisiae*) leads to misfolding of the protein and consequent degradation<sup>139</sup>. The misfolded VHL protein is handled by the cell's quality control machinery according to the general load of misfolded proteins in the cell and can thus serve as an indicator for increased load on the quality control machinery and cellular unfolding stress. Upon saturation of the quality control machinery unfolded VHL is send to the JUNQ and IPOD, and the fluorescently tagged protein is no longer soluble and accumulates in inclusions in the cell which correspond to JUNQ, IPOD or random aggregates. Tagging the VHL gene with a fluorescent marker allows easy monitoring of the unfolded protein accumulation in the cell. Accumulation of unfolded VHL in inclusions forms punctum structures which can be viewed due to the fluorescent marker, and counted per strain. The number of puncta per cell in the population (number of puncta divided by number of cells) can serve as a relative measure for unfolding stress in a given strain.

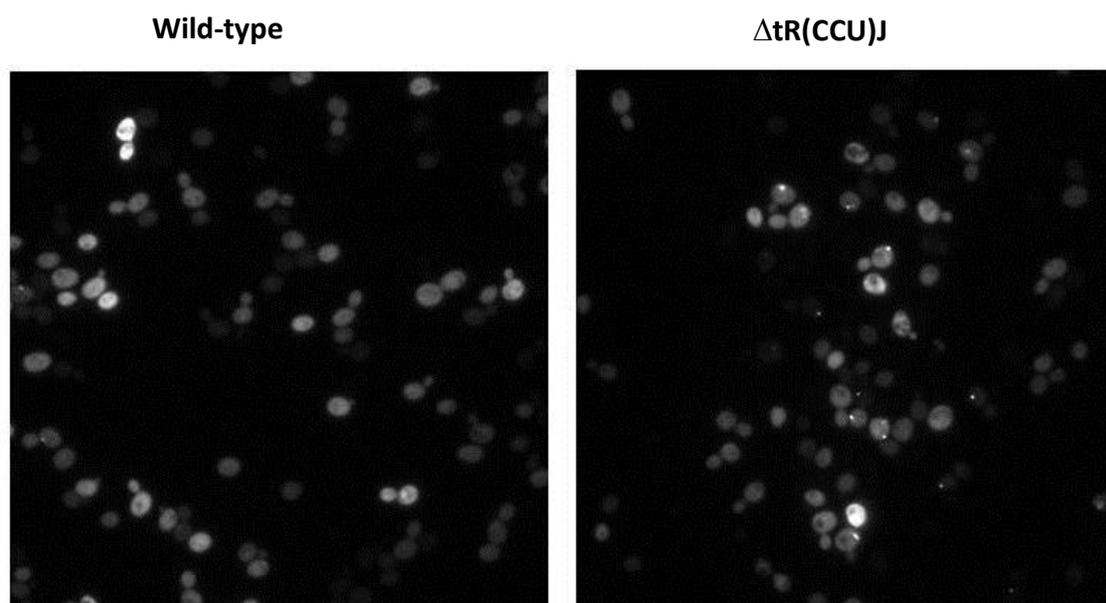
To examine whether tRNA deletion strains suffer unfolding stress I selected multiple tRNA deletions and transformed them with a high copy number plasmid carrying the VHL gene tagged with mCherry (CHFP-VHL). The strains for this assay were selected based on the growth experiments performed in the presence of unfolding agents, and included the two single copy tRNAs  $\Delta tR(CC U)J$  and  $\Delta tL(GAG)G$  as well as 8 additional tRNA deletion strains. A three-dimensional fluorescence deconvolution microscopy was used to view the VHL accumulation in puncta in the cells. Each strain was scored for the percent of puncta appearances in the population by counting the number of puncta per cell and dividing by the number of cells in all counted fields. Three biological repetitions of each tRNA deletion were scored, and about 500 cells were counted in each repetition; in addition at least two genetically identical clones for each strain. The wild type strain scored 7% percent puncta with a standard deviation of 1% between repetitions (**Figure 33**). The tRNA deletion strains were normalized to the wild type strain by dividing the percent puncta for each strain with that of the wild type. The various tRNA deletion strains showed a value either significantly

above or similar to that reference point. Strikingly the mutants that exhibit elevated resistance to the unfolding drugs showed increased levels of misfolded VHL which resulted in relatively high percentage of puncta more than two fold that of the wild type (**Figure 32**, **Figure 33**). Certain tRNA deletion strains which show no aggravation and retain their growth relative to the wild type in the presence of unfolding agents, show similar percent of puncta as the wild-type (**Figure 32**, **Figure 34**).



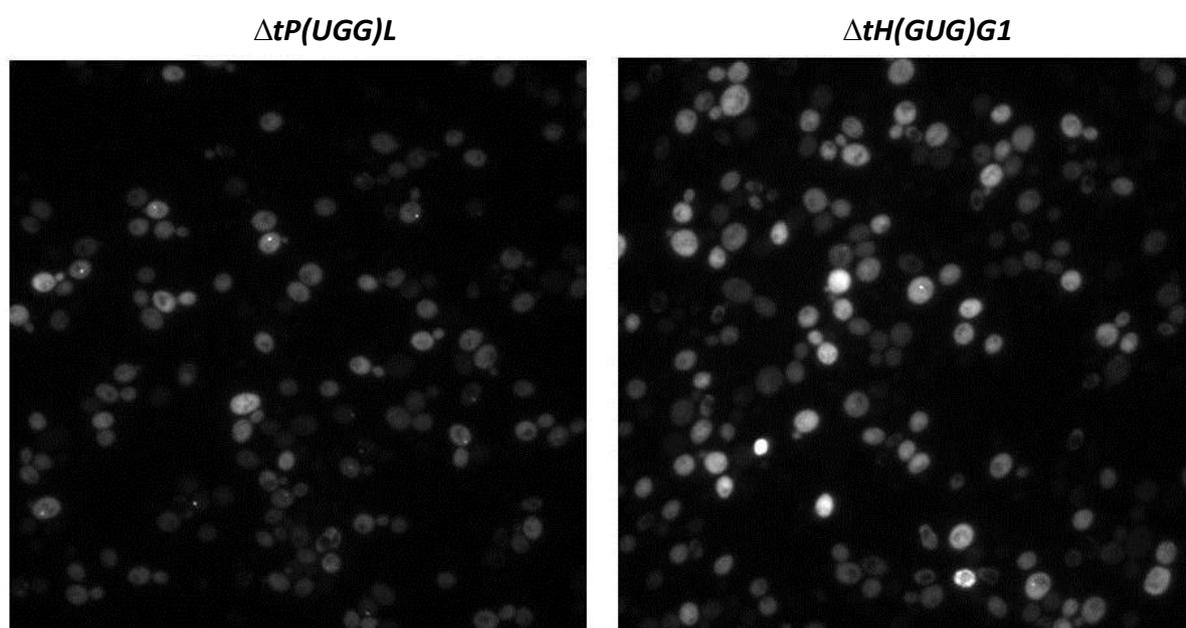
**Figure 32 – Percent of puncta appearances in the various tRNA deletion strains.**

The percent of puncta appearances in the population of each tRNA deletion strain was calculated by dividing the puncta appearances by the number of CHFP-VHL expressing cells. The represented values are average of at least 3 biological repetitions in which 500 cells were scored, standard deviation between biological repetitions are indicated for each strain.



**Figure 33 - Sorting of CHFP-VHL by the quality control machinery in wild type and ΔtR(CCU)J cells.** Representative fields of CHFP-VHL unfolded protein sorting in wild type cells (left) and ΔtR(CCU)J cells (Right)

Overall the two tests of puncta formation and resistance to the various unfolding drugs are congruent: strains that show resistance to the unfolding drugs appear to be in a chronic state of unfolding in the cell even when not treated with the drugs, and drug-sensitive strains are not experiencing an unfolding stress when no such stress is applied. It is thus tempting to draw a causal link between the two observations and suggest that the drug-tolerating strains own their tolerance to their being in a constant, pre-exposure, unfolding state, which appears to protect them against the unfolding drug when it arrives. In contrast the other strains which do not experience unfolding stress prior to exposure to the drug are more sensitive to the drug when they encounter it.

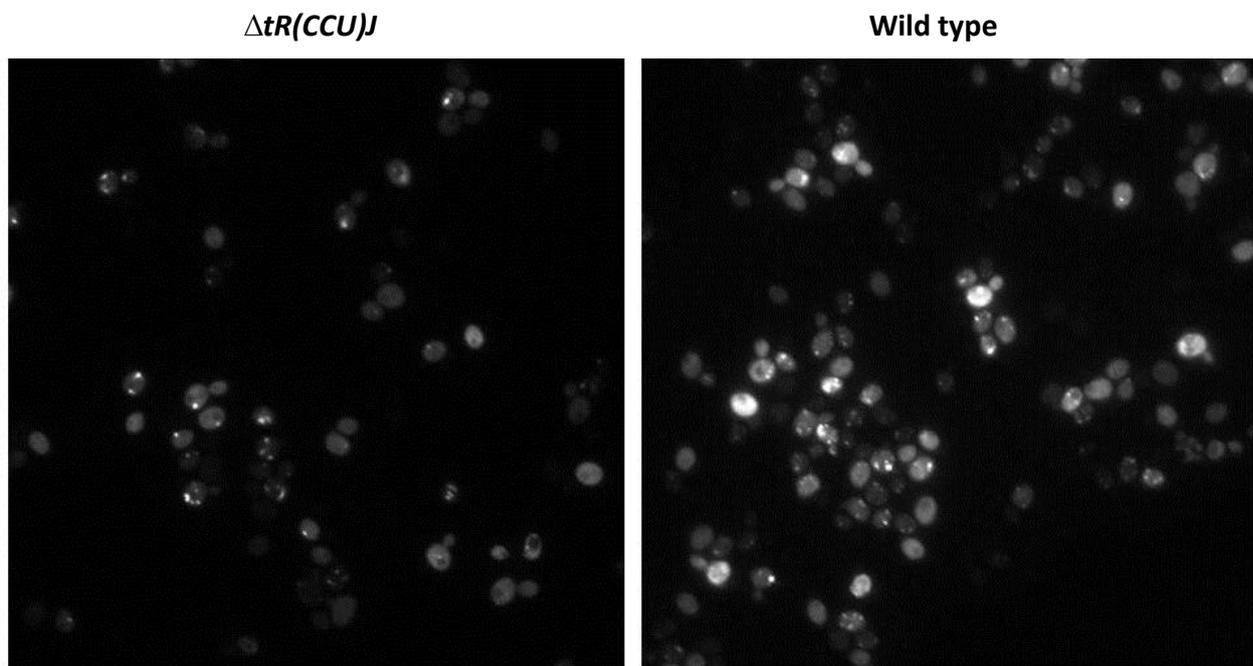


**Figure 34 – Sorting of CHFP-VHL by the quality control machinery in  $\Delta tP(UGG)L$  and  $\Delta tH(GUG)G1$  cells.** Representative fields of CHFP-VHL unfolded protein sorting in  $\Delta tP(UGG)L$  cells (left) and  $\Delta tH(GUG)G1$  cells (Right).

#### **5.14.1. Deletion of tRNA genes confers auto-protection against unfolding stress**

In light of the results achieved in the growth experiments using various folding agents and the CHFP-VHL localization experiments I hypothesized that some tRNA deletion strains experience translation hindering due to changes in the tRNA pool, which creates a burden on the translation machinery, and eventually result in protein misfolding. Saturation of the quality control machinery due to extensive misfolding is manifested by the increased number of puncta in multiple tRNA deletions. The deletion of tRNA genes in these strains confers a chronic state of translation disorders and unfolding stress in the cells. As a response various components of the quality control machinery are elevated enabling these

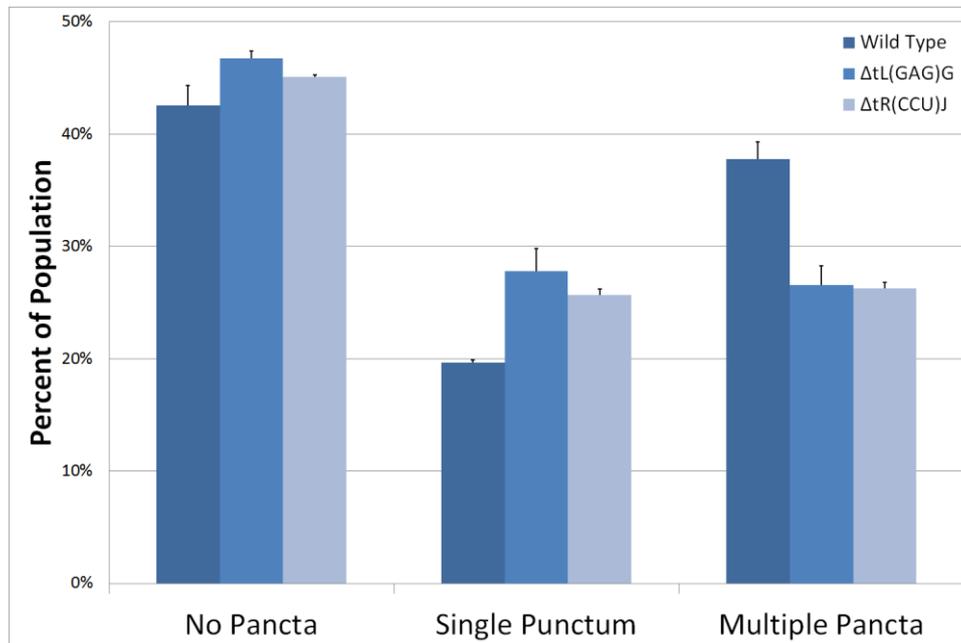
tRNA deletion strains to cope better with the externally inflicted unfolding stress (growth experiments) and conferring auto-protection against unfolding stress. To further the ability of tRNA deletion strains to result in cellular auto-protection against externally inflicted unfolding stress; I set to examine the load on the quality control machinery upon AZC treatment. Using the unfolding assay (percent puncta appearances)  $\Delta tR(CCUG)$ ,  $\Delta tL(GAG)G$  and the wild type strain were treated with AZC and scored for relative puncta presents. The concentration and exposer time of AZC treatment had to be carefully calibrated, such that the stress was not too harsh and that the cells were not saturated with aggregates. The AZC concentration used in the growth experiments (5mM) was found to be too high even when applied for a very short time period (of 15 minutes), and thus a lower concentration of 2.5mM was used in this experiment. Following the AZC treatment cells were visualized using the three-dimensional fluorescence deconvolution microscopy. Following the AZC treatment all the examined strains ( $\Delta tR(CCUG)$ ,  $\Delta tL(GAG)G$  and wild type) exhibited increased load on their quality control machinery resulting in multiple punctum per cell (**Figure 35**). Each population contained three distinct cellular appearances of VHL, diffused VHL, accumulation in a single punctum per cell and multiple puncta per cell. The significant elevation in appearances of multiple puncta per cell, indicates that the puncta-formation assay indeed measures unfolding stress. The cells were scored as before and divided into three categories, cells containing diffuse VHL, cells containing a single punctum and cells containing multiple puncta (two or more) (**Figure 35**). **Figure 36** shows a summary of the results obtained for the three strains, according to the three categories. The results showed a dramatic difference between the tRNA deletions and the wild type in the percent of single punctum and multiple puncta in the population.



**Figure 35 – Sorting of CHFP-VHL by the quality control machinery after AZC treatment in  $\Delta tR(CCU)J$  and Wild type cells.**

Representative fields of CHFP-VHL unfolded protein sorting after AZC treatment in  $\Delta tR(CCU)J$  cells (left) and wild-type cell (Right). Cells were treated with 2.5mM AZC for 30 minute prior to imaging.

The fundamental difference between the wild type and tRNA deletion strains was in the percent of cells containing a single and multiple puncta per cell. Following AZC application the wild-type population exhibited 13% fold increase in number of cells that display a single punctum and 37% fold increase in cells exhibiting multiple puncta. At the same time  $\Delta tR(CCU)J$  and  $\Delta tL(GAG)G$  showed 4% and 8% increase in single punctum and 26% increase in multiple puncta per cell in the population (**Figure 36**).



**Figure 36 – Percent of puncta appearances after AZC treatment in wild type,  $\Delta tR(CCU)J$  and  $\Delta tL(GAG)G$  cells.** The percentage of cells displaying the following phenotypes: no puncta, a single punctum and multiple puncta, in the population are displayed for each strain. The displayed percents are average of 2 biological repetitions standard deviation between repetitions is indicated for every cellular phenotype in each strain.

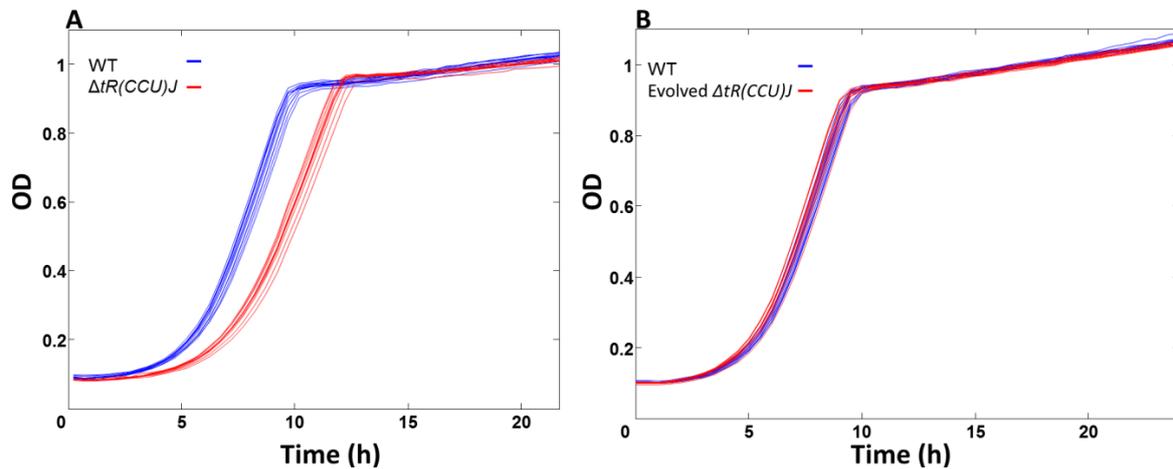
The two single copy tRNA deletions showed an improved capability to control and sort the formation of aggregates (puncta) in the cell. Taken together these results indicate that the deletion of certain tRNAs exposes the cells to a folding stress, followed by up-regulation of the cellular quality control machinery. This elevation in the quality control machinery leads to auto-protection against unfolding stress, in the cells once a similar stress is applied with a drug. In the wild type cells the quality control system is saturated and cannot handle the load of misfolded proteins upon application of the AZC drug, the outcome is multiple unsorted aggregates. The single copy tRNA deletions exhibit an improved sorting capacity upon AZC stress and thus more unfolded VHL is found in organized inclusions in the population (presumably IPOD), and less in multiple unstructured aggregates.

#### 5.14.2. Lab evolution of $\Delta tR(CCU)J$ results in changes to the tRNA pool

One of the most puzzling questions that arise from the tRNA deletion library concerns the survival of single copy tRNA deletions. Given that the deletion of most single copy tRNA genes is lethal, the question arises how the cell survive the deletion of these single gene tRNAs. One answer was already given in this work, examining the contribution of backup through wobble provided by other tRNAs, I found a backup mechanism that allows the cell to sustain deletion even of a single copy tRNA gene.

Using modifying enzymes which can alter the decoding properties of a given tRNA and

allow reading of multiple codons. And yet when co-deleting the modifying enzyme, along with the *tR(CCUG)* gene, the double deletion was still viable. Thus there might be other factors allowing the viability of this single copy tRNA deletion. The nature of such potential factors could be exposed in a lab evolution experiment in which  $\Delta tR(CCUG)$  would gradually evolve until the growth defect of the ancestor strain is completely alleviated. Analyzing the evolutionary solutions that would be found during such adaptive evolution can shed light on the backup mechanisms involved in decoding by tRNAs, on the effect such genetic perturbation has on cellular pathways, and possible cellular response mechanisms. I thus decided to perform a lab evolution experiment using  $\Delta tR(CCUG)$ . This project was done in collaboration with Avihue Yona a Ph.D. student in the lab. The *tR(CCUG)* deletion strain suffers from about 10% reduction in growth rate relative to the wild type, upon growth in rich medium, thus the evolution project was performed until wild type growth was reached. The *tR(CCUG)* deletion was evolved under optimal conditions, i.e. rich medium at 30°C, in 4 parallel experiment repetitions, using a daily serial dilution protocol. Every 10 days, i.e. about 70 generation, we evaluated the growth of the evolved strain in comparison to the its ancestor, i.e.  $\Delta tR(CCUG)$  and to the wild type strain, using the OD based robotic method. We observed a gradual improvement in growth rate of all 4 evolved strains compared to the ancestor strain  $\Delta tR(CCUG)$ , eventually reaching the growth rate values of the wild-type. The first dramatic improvement in the population was observed after 70 generations, and after 140-210 generations all 4 repetitions evolved to exhibit the same growth rate as the wild type (**Figure 37**). We hypothesized that at least part of the solution found by the evolved strains would involve the tRNAs that provide the backup through wobble to *tR(CCUG)* tRNA. We thus turned to sequence the 11 *tR(UCU)* gene copies which decode the relevant AGG codon through wobble interaction. We sequenced all 11 genes in each of the 4 evolved lines. Strikingly, in all 4 replicates we found a point mutation in a *tR(UCU)* gene, located at the anti-codon loop.



**Figure 37 - Growth curves of  $\Delta tR(CCU)J$  vs. Wild type and evolved  $\Delta tR(CCU)J$  vs. Wild type in rich medium.**  
**A-**Growth curves of the Wild type (blue) vs.  $\Delta tR(CCU)J$  (red). **B-**Growth curves of the Wild type (blue) vs. the evolved  $\Delta tR(CCU)J$  deletion (red). The X axis in both figures indicates time in hours. The Y axis indicates the OD values. The experiment was performed using the robotic OD based method in rich medium at 30°C. Each curve is composed of 12 technical repetitions of the experiment.

The point mutation was a change from a T to a C, which changed the anti-codon in that gene from UCU to CCU. As a result the cells now contained 10 copies of  $tR(UCU)$  along with a "new"  $tR(CCU)$  like gene. It should be noted that the mutation did not recover a canonical  $tR(CCU)$  gene, as there are 20 additional nucleotide bases which are different between  $tR(UCU)$  and the  $tR(CCU)J$  genes. Should this single base substitution suffice to rescue the growth rate defect exhibited by  $\Delta tR(CCU)J$ , introducing this point mutation to a  $tR(UCU)$  gene in the genetic background of  $\Delta tR(CCU)J$  would rescue the observed growth defect exhibited by this strain. Thus we are currently in the process of replacing one  $tR(UCU)$  gene in  $\Delta tR(CCU)J$  with a copy containing this point mutation to verify this point. In addition we intend to sequence the entire genome of one such evolved strain. This would enable ruling out the contribution of additional mutations in the evolved genome, to the observed phenotype.

It is interesting to mention that the single mutation acquired in each of the four evolution replicates effected in total 3 different genes out of the 11  $tR(UCU)$  genes, i.e. one  $tR(UCU)$  gene was mutated in two out of the four evolution repetitions. The mutations were found in the following genes,  $tR(UCU)K$ ,  $tR(UCU)G1$  and twice in  $tR(UCU)D$ . In light of the complete sequence identity between these genes and the observation of differential contribution in this family, one is tempted to speculate that not all  $tR(UCU)$  gene copies are free to evolve to the same extent. Two of these genes,  $\Delta tR(UCU)K$  and  $\Delta tR(UCU)G1$  are found in the tRNA deletion library and appear to have no effect on either growth rate or growth yield once grown in rich medium. We are currently performing additional evolution experiments which have the potential to shed more light on this subject.

Further, we see that the evolved cells can proliferate like the wild-type if they have 10 copies out of the original 11 *tR(UCU)*, also we notice that the conversion of one of the 11 *tR(UCU)* to contain the (CCU) anti-codon occurs within a relatively short time of evolution. That observation leads to a fundamental question, why does *S. cerevisiae* contain only a single copy of *tR(CCU)* when in principle it can convert very fast to contain more *tR(CCU)* copies without compromising the growth. We hypothesized that retaining a single copy of *tR(CCU)*, is crucial for proper control of translation. Farther support for this hypothesis comes from the strong connection observed in this study between translation (rate, accuracy) and protein folding. *S. cerevisiae* might retain *tR(CCU)* as a single copy gene to assist proper protein folding. Research in our lab, and additional works have shown that slowly translating codons that often correspond to low-copy-number tRNA genes are needed for accurate and efficient translation<sup>12, 37</sup>. If this hypothesis is correct then overexpressing *tR(CCU)* in a wild type background would be deleterious to the cells due to a substantial effect on translation and protein folding. In order to test this hypothesis we constructed high copy number plasmids containing either *tR(CCU)J* or *tR(UCU)E* (see materials and methods) and introduced them into the wild type strain. Since both encode for the same amino acid, Arginine, the major difference measured in this system would be the difference in copy number between isoacceptors. The growth of these multi-copy strains was compared to that of the wild type strain carrying an empty vector. The results of this experiment demonstrated that a strain containing the multi-(CCU) plasmid suffered from a severe growth rate defect relative to the strains containing multi-(UCU) plasmid, or empty plasmid. Showing that overexpression of this tRNA in the cell is indeed deleterious we aim to examine the effect such a plasmid would confer on protein folding in the cell. To examine this the growth of these strains will be examined in the presence of folding agents as well as by visualizing the localization of a fluorescently tagged VHL protein, as an indicator to a cellular folding stress.

## 6. Discussion

### 6.1. Creation of a tRNA deletion library

The copy number of tRNA genes correlates with codon usage as well as with amino acid usage in the proteome of *S. cerevisiae*<sup>110</sup>. In addition the tRNA gene copy number correlates with measured intracellular content of tRNA species in both *S. cerevisiae* and *E. coli*. Despite the huge effect tRNA quantities have on translation efficiency few studies made an attempt to quantify tRNA molecules in the cell. The studies by Ikemura T. 1981, 1982, quantified a large number of *E. coli* tRNA molecules and half of the tRNA isoacceptors found in *S. cerevisiae* using two-dimensional polyacrylamide gel electrophoresis<sup>39,40</sup>. More recent attempt to quantify tRNA molecules was done by Tuller *et al.*, 2010 in *S. cerevisiae* using specially designed microarrays<sup>71</sup>. In this method all tRNA molecules in a total RNA mixture extracted from the cell, are labeled selectively with a fluorophore, followed by hybridized to an array containing probes for all tRNA isoacceptors. This is not able to distinguish between tRNA gene copies and suffers from a high degree of cross hybridization between isoacceptors of the same amino acid and perhaps even between different amino acids. A different attempt to measure the binding of pol III to the tRNA genes in six different mammals provides a much more reliable proxy for tRNA transcription<sup>114</sup>. Nevertheless such measurements while providing a proxy for the transcription levels of each tRNA gene are completely unable to address the importance of each gene or isoacceptor to the cells fitness. The tRNA deletion library I created in *S. cerevisiae* provides a novel mean to study the fitness contribution of each tRNA gene, in rich medium and across conditions of environmental stress. It allows a system wide acquisition of the effects of small genetic perturbations in the translation machinery on the entire organism and provides an advance in our understanding of various cellular systems. In addition the library provides a functional measure to study genetic interactions establishing the effect of a single tRNA deletion on known or potential partners in the translation process. Analysis of such genetic interactions can serve as a powerful tool to study the function and contribution of each gene in the system allowing wiring of biological networks.

The creation of the tRNA deletion library established once again that in *S. cerevisiae* four tRNA genes are essential, while at the same time proved that the remaining 270 gene are dispensable. Following this initial observation I set to systemically determine the contribution of each tRNA gene to the tRNA pool, by means of growth analysis. The growth

of each strain, which can be evaluated by various parameters, represents the final outcome of cellular processes that occur during cell division and can thus serve as a proxy for gene contribution. The assumption was that in the case where all tRNA genes contribute the same, the growth parameters would be similar, whereas in cases where one tRNA gene contributes more to the cellular pool, the corresponding deletion strain would experience a growth aggravation. Screening the library in rich medium conditions using the colony size based method resulted in only five deletion strains which exhibited a reduction in colony size. The more sensitive OD based method resulted in additional strains which exhibit a growth defect. All in all about 20% of the strains in the library exhibited growth phenotypes in either growth rate or growth yield, when relatively more strains exhibit growth yield phenotypes. The relative change in growth (increase or decrease) was rather small, and most strains exhibited between 4-5% difference compared to the wild type strain. This result demonstrates that there is substantial backup between tRNA genes of a given tRNA isoacceptor family, in addition to backup through wobble, between different tRNA isoacceptors of a given amino acid. In rich growth condition, when the environment is plentiful, the tRNA deletion imposes only a relatively small burden on the cell, which is disguised by backup provided from other copies of the gene. Nevertheless the exposed phenotypes, mild as they may be, provided a first glimpse at the differences in contribution between the tRNA genes, and emphasized the need to screen the library in stress conditions.

## **6.2. Screening tRNA deletion library in stress conditions**

One of the main cardinal questions in my study was whether the ever changing environment which *S. cerevisiae* encounters during its growth results in changes in the tRNA pool. Should the tRNA pool be constant upon stress then the relative growth of each tRNA deletion compared to the wild type will be maintained. On the other hand if the contribution of each gene to the pool changes upon environmental stress then the relative growth should change as well. Screening across a number of conditions would allow us to better define possible dynamics within the tRNA pool and determine whether there are condition dependent isoacceptors, which change their contribution to the pool upon stress conditions or perhaps upon stress the entire library would contribute more. Given that severe stress conditions result in shut down of the translation machinery, in addition to degradation and sequestering of tRNA molecules into the nucleus, the stress had to be calibrated. The

stresses were calibrated using the wild type strain to be relatively mild. Using the colony size growth method we identified several conditions which exposed a relatively large number of strains exhibiting growth difficulties. These conditions were Cycloheximide, Heat shock of 40°C and Salinity. A similarity was observed between these stresses in the profile of exposed strains. Indications for a possible connection between Heat shock and Osmotic stress was put forth when the yeast high-osmolality glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway has been shown to be activated also by Heat stress. Yeast cells respond to a variety of stress responses by activating the mitogen-activated protein kinase (MAPK) pathways<sup>140</sup>. The response to severe salinity stress includes translation inhibition, change in polysomal profile, and certain changes in gene expression. Melamed et al., found that during salinity stress a group enriched with targets of the transcription factors Msn2/Msn4 exhibited a correlated change in the polysomal association and transcript levels<sup>25</sup>. There are several transcriptional programs which sense stress and enhance expression of molecular chaperons and degradation components; among them is the cytosolic ESR, which is regulated by the transcription factors HSF1 and MSN2/4<sup>123</sup>. MSN2 and MSN4 encode transcription factors that regulate the general stress response stress response of *S. cerevisiae*. Msn2p and Msn4p expression of ~200 genes in response to several stresses, including heat shock, osmotic shock, oxidative stress, glucose starvation etc., by binding to the STRE element, 5'-CCCCT-3', located in the promoters of these genes. MSN4 gene expression is itself Msn2/4p dependent and induced by stress; while MSN2 expression is constitutive. An elevation at the transcriptional level of MSN4 was observed for both single copy tRNA deletions *tR(CCU)J* and *tL(GAG)G*. Taken together these indications may point to a common cellular response which is activated during both Heat shock and salinity. This may lead to the resemblance in response to the stress and as a result the similarity between deletion strains aggravated by these stresses. No simple correlation was found between the identity of tRNA isoacceptor or amino acid exhibiting a growth defect and the applied stress. The Cycloheximide which is a protein synthesis inhibitor serves in a way as a competitive blocker of translation. Each of these three stress impose a further load on the translation machinery, and as such these stress library on various stress conditions in both colony size method and the OD based method. Under stress condition more tRNA deletions exhibit growth defect phenotype. These defects are not correlated with family size or amino acid.

### 6.3. Studying wobble interactions using the tRNA deletion library

The decoding properties of tRNA molecules are adjusted by various modification enzymes which enable the decoding of multiple codons by a single tRNA isoacceptor. Despite the models explaining the role of these modifications, and numerous revisions of the wobble hypothesis which have been put forth, the complete wobble rules along with the relevant modifications have not been fully defined yet. Thus far most of the studies in this field were based on structural data and *in vitro* translation systems. Only in recent years were *S. cerevisiae* mutations utilized for the purpose of understanding the role of modifications and wobble interactions. The tRNA deletion library provides a powerful systematic tool to fully establish the wobble rules in eukaryotes and provide the perfect platform for *in vivo* analysis of the precise contribution of the various components of the wobble including the various tRNA isoacceptor and modification enzymes. In this part of my study I aimed to better the understanding of wobble based interactions in supporting the viability by providing backup to those single copy tRNA genes deletions. Furthermore I aspired to evaluate the role of modifying enzymes in mediating these genetic interactions. The sensitivity and relative precision of the OD based growth method allowed me to characterize the two known dispensable, single copy tRNA genes, in a way which has never been done. Contrary to previously published results, I established a significant growth defect for the tR(CCU) deletion in numerous growth conditions. A previous study which failed to show the interaction between the Trm9 and the tR(CCU) concluded that the lack of wobble modification produced by Trm9 has a modest effect on the decoding properties of tRNA. My study provided on the one hand substantial supporting evidence for the existence of wobble interactions by demonstrating a strong genetic interaction between the tR(UCU) and tR(CCU) genes and between the *tL(UAG)* and *tL(GAG)* genes. While on the other hand revealed new evidence on the nature specificity and strength of these interactions through the double deletions of the Trm9 and the *tR(CCU)* genes. Taken together the results of the Trm9 deletion along with the *tR(CCU)* gene relative to the deletion of the Trm9 and the *tR(UCU)* genes indicate a strong tendency of this modification to promote the decoding of AGG codons rather than AGA codons. Modifications on the anticodon loop of tRNA molecules reduce conformational dynamics and shape the anticodon-domain architecture. The distinctive chemistry of modification, such as the one performed by the Trm9 enzyme, restricts tRNA to recognition of codons ending in A or G. Moreover they contribute order to the anticodon structure allows the recognition and binding of the tRNA anticodon to

cognate and wobble codons to occur accurately, rapidly and with a reduced entropic penalty to the ribosome . It is thus no surprise that the deletion of the Trm9 enzyme and double deletion of the enzyme along with tRNA genes result in defects in growth yield which shows a tight relation to glucose consumption and energy investment. Additional analysis of these strains showed that they better sustain growth in the presence of folding agents. Monitoring the folding stress in the  $\Delta trm9$  strain, revealed a substantial increase in the load on the quality control machinery. Such phenotypes are correlated with elevation of misfolded proteins which results in an up-regulation of members of the quality control system. My study demonstrates the significance of the modifying enzyme in mediating wobble based interactions to the translation machinery and cell's growth. It is an intriguing question arising from various parts of the study what determines defects in growth rate as opposed to defects in growth yield. This in discriminated decoding of the tL(UAG) which is unmodified is surprising and further strengthen the possibility that modifications were meant to prevent translation errors and restrict the decoding properties of certain tRNAs.

#### **6.4. Differential contribution of tRNA genes copies to the tRNA pool**

The tRNA genes are transcribed by RNA Pol III; their promoters are composed of two highly conserved sequence elements that reside within the transcribed region. The proximal A box and the more distal B box (Paule and White 2000). The presence of multiple copies of a particular gene, especially those that are transcribed by RNA polymerase III, seems to be common in eukaryotes <sup>42</sup>. The high demand for tRNA molecules, ~3 million per generation <sup>44</sup> can be met by multiple rounds of transcription and/or by multiple copies of the gene <sup>43</sup>. The naïve view is that all copies within a given tRNA isoacceptor family are transcribed to the same extent due to the internal promoter elements and the high degree of sequence similarity between them. This establishes a potential redundancy between the tRNA genes of the same isoacceptor family. If the multiplicity of *S. cerevisiae* tRNA genes is also functionally redundant then deletion of each gene should phenotypically equal that of the other family copies. To examine the contribution of identical tRNA gene copies to the tRNA pool in a genomic scale I used the data retrieved from growth analysis for the entire library. The data retrieved during the growth experiments on various media, indicated that that there was no functionally redundancy and that the tRNA genes contribute differential to the tRNA pool. That is deletion of tRNA genes from the same isoacceptor family resulted in differences in growth, manifested in both growth rate and growth yield. When grown on

rich medium the difference within a family could reach up to 5% difference in growth rate and about 10% in growth yield. This phenomenon was observed for multiple families and was found to be further accentuated during stressful growth conditions and reaches more the 15% difference between copies for growth rate and growth yield on low galactose or low glucose concentrations. Further analysis of a specific isoacceptor family tR(UCU) revealed once again that identical copies are aggravated to different extents upon double deletion with the modifying enzyme Trm9. Identifying the "major" tRNA gene contributors, based on this analysis, and double deletion of the these two "major" contributors resulted in a severe phenotype relative to the double deletions on "minor" copies. The ability to differentially express the tRNA genes may represent a novel, previously undiscovered level of translation regulation in the cell. In a recently published which mapped pol III occupancy genome-wide in six mammals it was found that pol III binding to individual tRNA genes varies substantially in strength and location. Moreover although the amount of pol III at each isoacceptor family was largely correlated with the number of copies of mouse tRNA genes in the family, there were many specific tRNA genes for the same isoacceptor that showed considerable variation<sup>114</sup>. Although the sequence similarity between the tRNA genes within a family, in human and mice isn't as high as the similarity within tRNA families in *S. Cerevisiae* tRNA genes, the phenomenon of differential transcription within an isoacceptor family exists for both identical genes and highly similar tRNA genes. The source of this phenomenon most likely lays in the sequences that flank the tRNA genes and are substantially different relative to the high degree of sequence similarity within the gene. All pol III transcription requires transcription factor IIIB (TFIIIB) which recruits pol III to its templates. TFIIIB binds sequences up-stream of the tRNA genes while TFIIIC binds the internal promoter, namely the two sequences elements, the A and B box within the tRNA gene<sup>49</sup>. TFIIIB assembly and binding on the DNA leads to pol III recruitment. As do all DNA-binding proteins, TFIIIB surely has sequence preferences, as it is very hard to believe that a DNA binding protein would be completely unbiased and promiscuous in the sequences it binds. If this is indeed the case then differential binding strength of TFIIIB to various loci in the genome may lead to differential pol III recruitment. The phenomenon of differential contribution observed for the tRNA deletion library might be a result of differential transcription. This transcriptional regulation of tRNA abundance may represent an alternative strategy for controlling intracellular tRNA levels. Past measurements of the distribution of essential components of pol III across the yeast genome identified pol III occupancy in 259 of the 274 tRNA genes in

yeast<sup>54</sup> thus it is most likely that all tRNA genes in *S. cerevisiae* are transcribed. Nevertheless there is still a possibility to maintain high quantities of a given isoacceptor by differentially regulating the expression of the entire gene family.

## 6.5. The molecular signature of tRNA deletion strains

Following the phenotypic growth characterization of the tRNA deletion library I decided to molecularly characterize several tRNA deletion strains. Analyzing the expression profile of tRNA deletions which exhibit a growth defect can expose the cellular pathways which change as a result of the deletion, and can provide insight to the molecular nature of the observed growth defects. The expression profiles of the tRNA deletion strains displayed a wide response both in the number of responsive genes and in the cellular function of these genes. Surprisingly we identified a molecular signature even for tRNA deletions which did not exhibit an appreciable reduction in growth. Positive relationships were observed between the growth defect and the extent of expression changes, when severe growth defects mostly result in a greater expression change. Interesting in that aspect were gene copies for the same amino acid for which differential contribution was observed, leading to differences in growth defects. Examining the expression profile for such a pair of genes (*tL(CAA)G1* and *tL(CAA)G2*) revealed that the differences depicted in growth were also manifested in the molecular signature, when the highly contributing gene (*tL(CAA)G2*) displayed a more substantial effect on expression upon deletion. The observed expression profiles for the collection of 13 tRNA deletions and the following analysis (GO enrichment, "compendium" correlations) revealed two distinct molecular signatures dividing the tRNA genes roughly according to the number of gene copies in the corresponding isoacceptor family. The tRNA deletions which belong to multi-copy families exhibited a specific effect on gene expression and cellular pathways, resulting in up-regulation of translation related process. For these tRNA deletions backup can be provided by the remaining copies in the family or by other isoacceptors decoding for the same amino acid through wobble. Once substantial backup can be provided, the cellular response is to "boost" the translation machinery (ribosome and tRNA molecules) possibly in attempt to compensate for the changes in the tRNA pool and their effect on translation. The tRNA deletions which are part of low-copy or single copy families resulted in an opposite response. The resulting cellular state in these deletions is of stress. Various extents of stress are displayed by the deletions when the most severe state is observed in the two single copy tRNA deletions, in which a

general change is observed once multiple cellular pathways are effected. As few backup possibilities exist for this group of tRNA deletions, the cellular response demonstrates a different strategy to compensate for hampered translation. In these strains stress related functions involving protein quality control and folding, in addition to transcription factors which are activated by stress are up-regulated. At the same time translation is somewhat down regulated as was observed upon severe obstructing of translation<sup>141</sup> and in severe stress conditions<sup>25</sup>.

An interesting question in systems biology is how different processes in the cell are coordinated, and how does damage to one process propagate and effect the entire cell. The cells must sense the defected process and respond in a way that would have a minimal effect on their fitness. The expression profiles obtained for various tRNA deletions may indicate that changes in the tRNA pool are monitored by the cell, thus creating defined molecular signatures. Several pathways which seem to be coupled to tRNA deletion may provide an answer as to how the cellular tRNA pool is monitored. One such example is the observed in the coupling between tRNA deletions and amino acid metabolism. For  $\Delta tR(CCUG)$  and the double deletion  $\Delta tR(CCUG)/trm9$ , I observed a strong reproducible reduction in the expression levels of 7 Arginine biosynthetic genes, and up-regulation in enzymes responsible for Arginine degradation. The Arginine biosynthetic genes are transcriptionally regulated by the general amino acid control and most of them are repressed by the presence of Arginine. This result might provide the first clue as to how a tRNA deletion is monitored by the cell. The deletion of the  $tR(CCUG)$  gene may lead to accumulation of Arginine in the cell which in turn represses the expression of the entire Arginine biosynthetic pathway. An additional interesting case is that of Methionine which shows an enrichment of several processes which are down-regulated all related to amino acid biosynthesis, such as cellular amino acid biosynthetic process (p-value  $2 \times 10^{-16}$ ) in addition to arginine metabolic process, glutamine, aspartate and lysine metabolic processes. The entire metabolic tree is undergoing shut down in this tRNA deletion. A tight connection between protein synthesis and amino acid levels has been observed in past studies when starvation for amino acids leads to a general inhibition of translation<sup>142</sup>. The proposed model is that accumulation of uncharged tRNAs activates the Gcn2 protein kinase, which phosphorylates the translation initiation factor eIF2 which in turn leads to translation inhibition<sup>142-144</sup>. The coupling observed between changes in amino acid biosynthesis and tRNA deletions points to possible involvement of amino acid levels in detecting the changes

in the tRNA pool. Additional processes which seem coupled to tRNA deletion and may provide a way to detect changes in the tRNA transcription are rRNA related processes. A possible connection between tRNA and rRNA has been proposed in the past where it was shown that mutants in Pol III also reduce the formation of the three rRNA species produced by Pol I. Thus adapting the production levels between rRNA and tRNA, keeping the steady state ratios between them to facilitate the translation machinery<sup>117</sup>. My results reinforce this possible co-regulation between the production of tRNAs and rRNAs and suggest that changes in the tRNA pool may lead to proportional changes in rRNA levels. Such a tight coupling would assist in the coordination of the translation process and its components. All in all the expression profiles of the tRNA deletions revealed that the tRNA pool is monitored and affects gene expression even when no phenotypic changes are observed. Several pathways were found to be coupled to such changes in the tRNA pool and provide possible candidates for detecting such changes.

## **6.6. Folding stress in tRNA deletion strains**

Proteins must fold correctly to maintain their biological function, to aid them in the process there are several mechanisms responsible to assist newly synthesized proteins and maintain a functional proteome. There is a tight connection of processes, production and quality control, between the translation machinery and the quality control machinery. The evidence for folding stress in tRNA deletion strains surfaced during analysis of their expression profile. To follow this lead in an indirect manner, I explored the growth of a sub-set of strains in the presence of various misfolding agents. The growth served as a proxy for the ability of the cells to cope and respond to the stress. These experiments revealed a remarkable phenomenon, in which tRNA deletion strains which exhibit a growth defect in default conditions (rich medium), display wild type growth parameters or, strikingly, perform better than the wild type in these stress conditions. Two explanations are possible for this observation, the first and more naïve one would be that these strains contribute less to the tRNA pool under these conditions and thus don't exhibit a growth defect, while the second explanation would be that these strains are auto-protected against the stress. To distinguish between the two possibilities and to examine the folding state in the cells in a more direct manner, I followed the sorting of an unfolded protein in the cell. In this experiment the overexpressed unfolded protein imposes a stress on the quality control system and allows delicate monitoring of its physiological state. These experiments revealed that certain tRNA

deletion strains experience a higher load on the quality control mechanism, and as a result contain more inclusions or puncta in the population. This in turn implies that the cellular quality control system is already engaged in assorting misfolded proteins, and the additional load brings them to saturation. Once the quality control system is saturated misfolded proteins are directed to inclusions in the cell. These results corroborated the hints from the expression profile and established a state of internal folding stress for some tRNA deletions; yet do not explain the results achieved in the growth experiments. To establish the possibility for auto-protection in the tRNA deletion strains, cells were treated with a folding agent to induce folding stress. While the quality control system in the wild type was unable to cope with the load, leading to accumulation of multiple unsorted aggregates in the cell, in the tRNA deletion strains the misfolded protein were sorted into inclusions. These results established an auto-protection of the tRNA deletion strains against folding agents by demonstrating a higher capacity of the quality control system to sort misfolded proteins. The nature of the folding stress in the tRNA deletions has yet to be established. There are a number of possible routes that can lead from a tRNA deletion to folding stress in the cell. A deficiency in a given tRNA might lead to hindered translation; while the ribosome awaits the next tRNA a number of scenarios are possible. The first would be translation errors, introduction of the wrong tRNA into the ribosome would lead to incorporation of the wrong amino acid in the protein. The second would be translation abortions; the ribosome awaits the correct tRNA and eventually disassembles and falls off. This in turn would lead to accumulation of truncated proteins which are misassembled and misfolded. The third possibility would be ribosome stalling, preventing the already produced protein from folding correctly due to the halt in translation. It is unclear which of these possibilities is the most relevant and perhaps the effect of various tRNA deletions on the translation machinery is varies as a factor of the absent tRNA. It was quite a surprise that these experiments exposed so many phenotypes, in both tRNA deletion strains with characterized growth defects in a selection of conditions and those which exhibit no growth defect. This once again emphasizes the fact that a tRNA deletion is sensed by the cell even when no growth defect is observed. An interesting comparison is the extent of protection which can be correlated the stress levels in the cell versus the effect on the translation machinery. The two single copy tRNA deletion strains, exhibit a severe and chronic folding stress, as was apparent in all the examined methods. These strains must rely on their wobble counterparts for proper translation, and it is obvious that they are struggling in the process of translation. As was

described before translation based signals play an important role in enhancing the levels of proteins that participate in the DNA damage, thus implicating tRNA modifications in the coordination of the DNA damage response <sup>103</sup>. In that aspect it is clear that modifying enzymes such as Trm9 carry out an important role in maintaining the proper translation in the cell. Analysis of the chaperon requirements for degradation of misfolded variants of a cytosolic protein, the VHL tumor suppressor, reveals that distinct chaperone pathways mediate its folding and quality control <sup>126</sup>. Both folding and degrading of VHL require Hsp70 and the chaperonin TRiC is essential for folding dispensable for degradation. The chaperone Hsp90 is essential for VHL degradation <sup>126</sup>. Successful partitioning to the ubiquitination and degradation machinery requires that the polypeptide remain soluble. Ubiquitination was demonstrated to be an important determinant for maintaining solubility of misfolded proteins, as non-ubiquitinated species are directed to the IPOD<sup>86</sup>. VHL accumulating in the IPOD correlates with a shift to the insoluble fraction, this compartment contains non-diffusing, insoluble species. The observation that amyloidogenic proteins appear to be targeted exclusively to the IPOD suggests that this compartment is the preferred cellular destination for protein aggregates<sup>86</sup>. The connection between tRNA and amyloid aggregates was suggested before and provides a fascinating connection between the translation machinery, the quality control machinery and amyloidosis and disease.

I would like to propose a hypothesis to the cellular outcome of a tRNA deletion, upon deletion of a tRNA the cellular amounts of that given tRNA isoacceptor change; as a result the process of translation is affected. The effect on the translation process leads to accumulation of misfolded proteins; these proteins in turn impose a load on the quality control machinery. The cell responds to this situation by up regulating at the transcriptional level genes which are members of the quality control mechanism to better handle and sort the misfolded proteins. The elevated capacity of the quality control mechanism leads to the phenomenon of auto-protection against mild external folding stress.

### **6.7. Adjusting the decoding properties in $\Delta tR(CC U)J$ through lab evolution**

The well-defined growth defect of the tR(CC U)J gene deletion, providing evidence for the wobble based backup interaction, yet manifesting that these interactions are not sufficient to provide full backup served as the basis for lab evolution. The aim of this project was to understand how on an evolutionary time scale the cells would cope with the severe effect on the translation machinery and on the entire cell as a result. The result the evolved population found in a rather short time window was a silent mutation, a transition of T to C,

converting the tRNA anticodon from UCU to CCU. The post-evolution population contained 10 gene copies of the tR(UCU) isoacceptor, and one copy which mimics the deleted tR(CCU)J. The growth of the evolved strain was identical to that of the wild type in both growth parameters, growth rate and growth yield. The evolution experiment was performed in quadruplicate, and all four evolved populations showed similar results, converting UCU to CCU by a point mutation each population at the expense of a different *tR(UCU)* gene. The resulting tR(UCU) genes which were lost were D, which was "lost" in two populations, K and M1. The deletions of tR(UCU)K and tR(UCU)M1 were constructed in the tRNA deletion library. The growth phenotypes of these deletions in rich medium (which was the medium during the evolution experiment), resembled that of the wild type strain. The extensive analysis performed during my study of  $\Delta tR(CCU)J$  revealed a complex physiological response indicating a certain state of stress. In light of the global impact of  $\Delta tR(CCU)J$  on the cell, and the solution found by the cells it is clear that the solution had to be found in the heart of the translation machinery and that the best and quickest solution would be to recreate the deleted tRNA. A number of questions arise from the outcome of this evolution, the first concerns the number of gene copies maintained for each tRNA in the genome. It is known that most tRNA genes are members of multi-copy families, there are only six single copy genes, nevertheless four of them are essential and the remaining two suffer from a growth defect. It is thus puzzling why this genomic state is preserved in the cell. A possible explanation would be that the single copy which translates to low tRNA amounts in the cell are beneficial in the process of translation to slow down translation speed on the mRNA where it is needed to allow proper folding of a protein domain or to allow unfolding of the following mRNA secondary structures. A state where by retaining a single copy of a gene benefits the entire translational system would preserve this situation through evolution. An additional question relates to the nature and speed of evolution. The resulting mutation dominated the growth of the population after only 20 days (approximately 140 generations) the mutation which provided the solution arose even sooner in the population. The robustness of the solution shows that generating such mutations within the tRNA genes is possible yet the 11 copies of the tR(UCU) isoacceptor are completely identical and preserved. The complete identity in this family which is very similar to the situation in other tRNA families is puzzling in light of the results from the lab evolution. It is unclear what is the evolutionary force which prevents mutations in the tRNA genes from fixating in the population. It could be strong selection on the sequence, secondary structure and coding

properties of the molecule, or it could be genetic drift.

## **6.8. Follow up experiments of this work**

As this thesis unraveled many new findings regarding tRNA function and the effect changes in the tRNA pool have on the cell, it is a fertile ground for future experiments. As the tRNA deletion library is of complete gene deletions, it would be extremely interesting to explore how a matching set of point mutations would effect the physiology and expression of the mutated genes. Such a comparison of the two libraries can provide the ability to differentiate between different functions of the tRNA gene. In addition it can serve as further validate that the effects observed for each gene deletion in the library can be attributed completely to the loss of the tRNA function.

Producing different combinations of tRNA deletions can assist in understanding the dynamics of the tRNA pool, and possible interactions between tRNA families and amino acids. In addition deciphering the wobble rules can be completely deduced as there would be no limitation on the number of consecutive deletions. To that end I only created double and triple tRNA deletion, yet it is now possible to create complete deletions even of multi-copy families using the Cre-Lox technology. Using this technology there would be no limitation to the number of deletions, as the antibiotic marker is introduced in the deletion and then extracted.

As the a connection between proteotoxic stress and tRNA deletions was established in this work it is possible to further characterize future connections between tRNA gene deletions and processes in the cell. In such a manner one can explore a possible connection between changes in the tRNA pool and drags that inhibit translation or drags that inhibit cell cycle progression.

## **6.9. Final conclusions**

During my Ph.D. studies I have created the tRNA deletion library in the yeast *S. cerevisiae*. This library is an innovative and powerful tool, consisting of minimalistic genetic perturbations to the translation machinery. This library provides a system based approach to the study of the tRNA pool, enabling exact measurement of the effects such perturbations impose on cellular fitness. Using this library I was able to deduce the contribution of each tRNA gene to the tRNA pool and to the translation process. I revealed a complex network of genetic interactions in which different gene copies within a family

contribute differentially to the tRNA pool. Screens of the library across stressful conditions enabled identification of condition dependent functional roles for multiple tRNA genes. Molecular characterizations of a subset of tRNA deletions revealed the remarkable capability of cells to monitor and respond to changes in the tRNA pool. The response to a tRNA deletion carries a specific molecular signature in which the cellular strategy corresponds to the isoacceptor family size and the ability of the remaining copies to provide backup. The molecular characterization revealed, for the first time, the cellular pathways which are effected by changes in the tRNA pool. Analysis of protein folding in the various tRNA deletion strains established a mechanistic connection between changes in the tRNA pool and their effect on translation, providing a new and exciting link between protein synthesis and quality control. The auto-protection of some tRNA deletion strains was revealed, allowing only a minimal effect of imposed translation hampering on growth, by elevating quality control components. Using lab evolution of a tRNA deletion strain to decipher possible solutions to the change in the tRNA pool, revealed a simple solution consisting of a single nucleotide change. This mutation changed the decoding properties of a synonymous isoacceptor, reconstructing the lost gene function. This solution further revealed the decoding preferences in the cell by preferring a perfect match between anti-codon and codon to the interaction through wobble.

Taken together, the results obtained in this study exposed many aspects in the biology of the tRNA pool and open multiple directions of research. In addition they establish the notion that the cellular tRNA pool is monitored and carefully regulated by mechanisms that still await further exploration.

## 6.10. Independent Efforts and Collaborations

I hereby declare that this thesis summarizes my independent efforts. Three projects were performed in collaboration as detailed below:

Bioinformatics analysis of tRNA genes and flanking genomic features was done in collaboration with Hila Gingold a fellow PhD student, from Yitzhak Pilpel's laboratory at Weizmann Institute of Science.

Analysis of microarray data for a collection of 13 tRNA deletion strains performed jointly with Sivan Navon a fellow PhD student, from Yitzhak Pilpel's laboratory at Weizmann Institute of Science.

Lab Evolution of  $\Delta tR(CCUG)$  and following analyses were performed jointly with Avihu Yona, a fellow PhD student, from Yitzhak Pilpel's laboratory at Weizmann Institute of Science.

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## 8. תקציר

שיטה מחקרית רבת עוצמה על מנת להבין את התפקיד והפעילות של מערכות ביולוגיות הינה יצירת מערכת של הפרעות גנטיות ובדיקת ההשלכות הפונקציונליות והמולקולריות. על מנת להטיב את הבנה של ההרכב התאי של מולקולות הרנא מוביל והשפעתן על תהליך התרגום, יצרתי ספריית חסר של מרבית גני הרנא מוביל מתוך 274 גנים הקיימים בגנום של השמר *S. cerevisiae*. בחינת הגידול והפנוטיפ המולקולרי של כל זן המכיל חסר של גן רנא מוביל איפשרה לי להסיק את תרומתו למאגר התאי, לתהליך התרגום וליכולת הגידול של התא. במהלך יצירת ספריית החסר של גני רנא מוביל גיליתי שמרבית הגנים המיוצגים על ידי עותק בודד בגנום הינם חיוניים אולם שניים מהם ניתן להחסיר. שני הגנים הללו מגובים באמצעות גני רנא מוביל ממשפחה אחרת המתימים לאותה חומצה אמינית בתהליך התנדודות. אנליזה מעמיקה של תהליך ההתנדודות חשפה כי מכניזם הגיבוי תלוי במתילציה המגבירה את יכולת ההכרה בין קודון לאנטי-קודון כאשר אין התאמה מושלמת בניהם. גנים השייכים למשפחות מרובות עותקים אינם חיוניים ומראים פנוטיפ מועט כאשר גידולם נבחן במצע עשיר, ככל הנראה בעקבות גיבוי הניתן להם מהעותקים הנוותרים של אותה משפחה. בכדי לחשוף פנוטיפים נוספים של זני הספריה סרקתי את הגידול של ספריית החסר במגוון תנאים של עקה. בסריקות אלו נחשפו גנים נוספים שתפקידם הינו תלוי תנאי גידול המתבטא בירידה בקצב הגידול או ביבול, כלומר מספר התאים בכניסה לפאזה הסטצינרית של הגידול. בנוסף תופעה מעניינת שנצפתה בהרבה משפחות, חשפה שחסר של גנים זהים מוביל לפגמי גידול שונים, וכונתה בעקבות זאת תרומה יחסית של עותקים. מחקר מעמיק של תופעת התרומה היחסית באמצעות זנים בעלי חסר כפול גילתה ירידה חדה בגידול בחסר של גנים בעלי תרומה גדולה למאגר לעומת כאלו התורמים פחות. איפיון מולקולרי של מבחר זנים מספריית החסר על ידי בדיקה של פרופיל ביטוי רנא שליח גילתה שתי חתימות מולקולריות שונות בגנום המחלקות את הזנים בהתאם לשתי תגובות תאיות לחסר. התגובה התאית לחסר של גני רנא מוביל ממשפחות מרובות עותקים המסוגלות לגבות חסר של גן בודד כללה עליה בתהליכים הקשורים לתרגום. לעומתם חסר בגני רנא מוביל השייכים למשפחות מעוטות עותקים הובילו את התאים למצב של עקה ואינדיקציות על בעיות קיפול חלבונים. בכדי לבדוק את האפשרות שחסר של רנא מוביל גורם לפגיעה בתהליך התרגום ומוביל לקיפול שגוי של חלבונים בדקתי את יכולת הגידול של זני החסר בתנאים המובילים לבעייה בקיפול חלבונים בנוסף לניתור של היווצרותם של חלבונים בעלי קיפול שגוי בתא. אנליזות אלו חשפו ממצאים מעניינים המצביאים על קשר אפשרי בין תהליך תרגום תקין של חלבון לקיפולו המרמז שכאשר אספקת מולקולות הרנא המוביל נפגעת הדבר מוביל לבעיות קיפול.

לפיכך ספריית החסר שיצרתי מייצגת את הספריה הראשונה שבאופן שיטתי פוגעת נקודתית בגנים השייכים למערכת התרגום וסוללת את הדרך לאיפיון התהליך המורכב של תרגום בתא.