Identification of the determinants of tRNA function and susceptibility to rapid tRNA decay by high-throughput in vivo analysis

Michael P. Guy,1,5 David L. Young,2,5 Matthew J. Payea,1 Xiaoju Zhang,1 Yoshiko Kon,1 Kimberly M. Dean,1 Elizabeth J. Grayhack,1 David H. Mathews,1 Stanley Fields,2,3,4 and Eric M. Phizicky1

1Department of Biochemistry and Biophysics, Center for RNA Biology, University of Rochester School of Medicine, Rochester, New York, USA 14642; 2Department of Genome Sciences, 3Department of Medicine, 4Howard Hughes Medical Institute, University of Washington, Seattle, Washington, USA 98195

Sequence variation in tRNA genes influences the structure, modification, and stability of tRNA; affects translation fidelity; impacts the activity of numerous isodecoders in metazoans; and leads to human diseases. To comprehensively define the effects of sequence variation on tRNA function, we developed a high-throughput in vivo screen to quantify the activity of a model tRNA, the nonsense suppressor SUP4oc of Saccharomyces cerevisiae. Using a highly sensitive fluorescent reporter gene with an ochre mutation, fluorescence-activated cell sorting of a library of SUP4oc mutant yeast strains, and deep sequencing, we scored 25,491 variants. Unexpectedly, SUP4oc tolerates numerous sequence variations, accommodates slippage in tertiary and secondary interactions, and exhibits genetic interactions that suggest an alternative functional tRNA conformation. Furthermore, we used this methodology to define tRNA variants subject to rapid tRNA decay (RTD). Even though RTD normally degrades tRNAs with exposed 5′ ends, mutations that sensitize SUP4oc to RTD were found to be located throughout the sequence, including the anti-codon stem. Thus, the integrity of the entire tRNA molecule is under surveillance by cellular quality control machinery. This approach to assess activity at high throughput is widely applicable to many problems in tRNA biology.

[Keywords: tRNA function; tRNA decay; high-throughput analysis; RTD; SUP4oc]

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loss, ataxia, myopathy, diabetes, epilepsy, neuropathy, and gastrointestinal dysfunction (Yarham et al. 2010); these mutations occur in all stems and loops.

Nonetheless, there are also data demonstrating that tRNAs can tolerate variant sequences in the stems and loops. For example, the yeast Saccharomyces cerevisiae tRNA^{Arg}_{GCG} (Geslain et al. 2003), and the yeast tRNA^{Glu}_{CCG} (Whipple et al. 2011). Similarly, several Escherichia coli variants of a partially impaired tRNA^{Ala}_{CUA} amber suppressor tRNA retain activity with individual mutations in the acceptor stem, the anti-codon stem, or the T stem (Hou and Schimmel 1992).

These seemingly conflicting data make it difficult to predict the effects on tRNA function of the numerous naturally occurring sequence variations in the metazoan tRNA isodecoders, which have the same anti-codon but altered tRNA bodies (Goodenbour and Pan 2006). In addition, most of the numerous disease-associated mitochondrial tRNA variants are poorly understood (Suzuki et al. 2011). Prediction of the function of variants is further complicated by the multiple modifications and quality control pathways that influence tRNA activity (Kadaba et al. 2004; Chernyakov et al. 2008; Hopper 2013; Kramer and Hopper 2013) and by the tRNA internal promoter, which is not quantitatively understood (Koski et al. 1980; Pearson et al. 1985; Kaiser and Brow 1995; Marck et al. 2006; Orioli et al. 2012).

Although there is a wealth of information on the effects of mutating individual tRNA residues on specific steps of tRNA processing and function (Normanly et al. 1986; Schultz and Yarus 1994; Yan and Francklyn 1994; Fechter et al. 2000; Schrader et al. 2009), there has been no quantitative analysis at a large scale of the effects of mutations on tRNA biology. Here we describe the use of a sensitive quantitative analysis at a large scale of the effects of mutations of yeast carrying a library of tRNA suppressor variants of a tRNA suppressor in the yeast S. cerevisiae and the use of this system to comprehensively define the biological substrates of a prominent tRNA decay pathway (Alexandrov et al. 2006). We identified a large number of mutated tRNAs that are functional, suggesting that tRNA structure is much more flexible than anticipated, and found that the tRNA decay pathway unexpectedly acts on many more classes of variants than previously known or predicted.

Results

Quantification of tRNA function by cell sorting of yeast carrying a library of tRNA variants

To analyze the effect of mutations on tRNA function in vivo, we sought a model system in which we could assay tRNA activity quantitatively, with high sensitivity and on a large scale. In yeast, suppression of a stop codon in the green fluorescent protein gene (GFP) allows fluorescence-activated cell sorting (FACS) of millions of cells based on their level of suppression by a nonsense suppressor tRNA. To test the feasibility of this approach, we integrated the nonsense suppressor SUP4_{oc} (tRNA^{Tyr}_{G34U}) into a yeast strain bearing GFP_{oc} in the RNA-ID reporter (Dean and Grayhack 2012); this strain allows a comparison of the expression of GFP_{oc} to the control red fluorescent protein gene (RFP) [Fig. 1A]. GFP\textsubscript{oc}/RFP was minimal without suppression (0.004 of GFP/RFP) but was nearly normal (0.94 of GFP/RFP) with SUP4\textsubscript{oc} [Fig. 1B], as anticipated for this stop codon because of its poor termination context (Bonetti et al. 1995; Dean and Grayhack 2012). Based on these data, this GFP\textsubscript{oc} expression assay discriminates with high resolution among tRNA variants, with a 235-fold dynamic range of expression and limited variation in GFP/RFP values for individual cells of a variant (Dean and Grayhack 2012). Moreover, the assay measures the net contribution of all steps of tRNA biogenesis and translation except fidelity.

We constructed a library of ~220,000 SUP4\textsubscript{oc} variants, each integrated into the yeast RNA-ID strain and bearing the yeast RNA-ID reporter used to quantify tRNA function (Dean and Grayhack 2012). [A] Schematic of the RNA-ID reporter used to quantify tRNA function. (B) SUP4\textsubscript{oc} efficiently suppresses GFP\textsubscript{oc}. Scatter plot of flow cytometry of cells with integrated RNA-ID reporter expressing GFP (green), GFP\textsubscript{oc} (red), and GFP\textsubscript{oc} and SUP4\textsubscript{oc} (blue). (C) FACS of SUP4\textsubscript{oc} variants. Cells were grown in YP galactose medium and sorted. (D) SUP4\textsubscript{oc} tolerates numerous mutations. Cloverleaf heat map showing GFP\textsubscript{SEQ} of single-mutant variants. Quadrant color around residues indicates variant activity. Active variants are white (GFP\textsubscript{SEQ} of 0.026) to blue (GFP\textsubscript{SEQ} of 1) gradient, and inactive variants are red. Modified bases are indicated in the figure.
~3% random mutations in nucleotides 1–33 and 38–73 (conventional numbering) [Supplemental Fig. S1A]. We grew this library at 28°C, sorted cells into four bins by FACS (Fig. 1C), PCR-amplified the SUP4oc allele from the pooled genomic DNA from each bin, and evaluated the bin distribution of individual variants by sequencing (Supplemental Table S1), similar to an approach used to measure gene expression from thousands of designed promoters [Sharon et al. 2012]. The fractional representation of reads for each variant in each bin was converted to the ratio of reads for each variant in each bin was converted to a GFP/RFP ratio, which was normalized to the SUP4oc ratio to define relative function (termed GFPSEQ). Filters were then applied to score only those variants with ≥100 reads and enough reads to measure the distribution of ≥30 cells (Supplemental Fig. S1B).

Overall, we scored 25,491 variants (Supplemental Table S2), including all 213 single variants. GFPSEQ was highly reproducible for single mutants of a biological replicate (Supplemental Fig. S1C), with R² = 0.99. We also confirmed tRNA activity of 60 variants by reconstruction and flow cytometry analysis of the variants; each activity, normalized to the SUP4oc ratio, yielded a GFP/RFP ratio termed GFPFLOW, which correlated highly with the corresponding GFPSEQ up to GFPFLOW of 0.4 (R² = 0.90) [Supplemental Fig. S1D]. To further enhance resolution of highly active variants, we used FACS to subdivide bin 1 into three fractions, extending the linear range of GFPSEQ values to GFPFLOW of 0.55. It is not clear why GFPSEQ is systematically approximately twofold higher than GFPFLOW, resulting in a correlation between GFPSEQ and GFPFLOW that only extends up to 0.55 [Supplemental Fig. S1D]. Some of the discrepancy is likely due to the limited resolution of high-fluorescence variants, even in the bin 1 subdivision data set. In addition, PCR chimerism in low-fluorescence bins can lead to spurious wild-type reads, thereby underestimating the function of the wild-type tRNA by the sequencing approach, which in turn leads to overestimation of variant function by GFPSEQ. Finally, minor systematic errors may be introduced by the use of different instruments for GFPSEQ and GFPFLOW measurements and the steps of bin collection and plating, PCR amplification, and sequencing.

SUP4oc is highly tolerant of mutations

To characterize the mutational consequences in SUP4oc, we initially analyzed the 213 single variants, given both their relative simplicity and the previous studies that examined single mutations in this tRNA. SUP4oc is remarkably tolerant of single mutations, with 44 highly functional variants [GFPSEQ ≥ 0.9] [Fig. 1D, dark blue] and 27 substantially functional variants [0.18–0.9] [Fig. 1D, blue], along with nine marginally functional variants [0.026–0.18] [Fig. 1D, light blue]. We note that there are minimal consequences due to the higher values of GFPSEQ relative to GFPFLOW. Thus, 26 of 32 nonfunctional or marginally active variants by GFPSEQ were correctly annotated based on reconstruction and GFPFLOW analysis, and six nonfunctional variants had trace amounts of GFPFLOW activity [Supplemental Fig. S1D]. Similarly, 13 of 16 of highly functional variants that were tested by reconstruction and flow cytometry had GFPFLOW values ≥0.7.

The highly or substantially active variants were heavily clustered in specific residues. These included each of the three possible mutations of all five D-loop uridine residues; U4 of the acceptor stem; A9, A13, and A22 of the D loop; residues 44, 45, and 47 of the variable loop; C59 of the T loop, and G62 of the T stem. These results are consistent with, and substantially extend, previous analyses of functional variants of SUP4oc [Kurjan and Hall 1982; Kohalmi and Kunz 1992], yeast tRNAArg(CCG) [Geslain et al. 2003], and an E. coli alanine amber suppressor tRNA [Hou and Schimmel 1992]. In contrast, residues that did not tolerate any single mutations included those in conserved tertiary pairs [U8–A14, R15–Y48, G18–U55, G19–C56, and U54–A58], emphasizing the requirement of the L-shaped tertiary fold of the tRNA for activity.

Although our data emphasize that the integrity of the four stems must be intact for tRNA to have full function, flexibility is observed at two locations. Single- and double-mutant variants that preserve canonical pairing were often functional [Fig. 2A], with the notable exceptions of the G53–C61 pair, which is highly conserved as part of the B-box of the internal promoter [Marck et al. 2006], C1–G72, which is a determinant for tyrosine charging [Fechter et al. 2000]; and the G10–C25 and C11–G24 pairs of the D stem, which is comprised of only 3 base pairs [bp]. In contrast, only eight of 140 stem variants with noncanonical pairing had a GFPSEQ > 0.5, and these eight included four variants of U4–G69 and two variants of C52–G62. Although functional variants with mismatches at U4–G69 might be anticipated because of the weak U–G pair and the known mismatches that occasionally occur among stem base pairs in tRNAs, it is unclear why SUP4oc tolerated mismatches at C52–G62, since this position is rarely occupied by a mismatched pair, G–U, or U–G [Marck and Grosjean 2002].

Our data also indicate that the tertiary fold must be intact, since little sequence variation is observed in the conserved tertiary pairs. Indeed, 45 of 47 variants with mutations in these pairs resulted in a completely nonfunctional tRNA, and the remaining two had only marginal activity [Fig. 2A].

Among the 9349 double-mutant variants, 1499 were active, including 685 substantially or highly functional variants. One important requirement for activity is a low ensemble defect (ED), which is a parameter that estimates the propensity of a tRNA to misfold [Zadeh et al. 2011a]. According to our data, almost all functional variants had an estimated per nucleotide ED < 0.21 [Fig. 2B, 95% cutoff, yellow], which is well within the range of native eukaryotic tRNAs [Supplemental Fig. S2A].

Unexpected positive interactions between residues

To identify previously unappreciated parameters important for tRNA function, we examined double-mutant variants that displayed positive (or negative) epistasis, indicating that they functioned substantially better [or
worse) than anticipated from the scores of the corresponding single-mutant variants. Epistasis within a protein or RNA can reveal interactions between residues when the phenotype caused by one mutation is dependent on mutation at another residue. Based on a multiplicative model, we calculated an epistasis score by subtracting the product of the GFPSEQ scores of two single variants from that of the corresponding double variant (Supplemental Tables S3, S4). Most double variants scored close to their predicted values (Fig. 2C), but 6.9% had substantial negative epistasis (defined as a score $< -0.18$) (Supplemental Fig. S2B), and 1.5% had positive epistasis ($> 0.18$) (Fig. 2D). As might be expected for a molecule with severe sequence constraints, there was a large excess of negative epistasis over positive epistasis. Indeed, of the double variants that had GFPSEQ scores that allowed the possibility of negative or positive epistasis, 62% were negatively epistatic, whereas only 1.4% were positively epistatic, and this excess was not dependent on the epistasis cutoff score used (Supplemental Fig. S2C). However, the 6.9% of total double variants with negative epistasis includes a remarkably large number (202 of 648, 31%) of completely nonfunctional doubles in which both singles were highly functional, suggesting that while the tRNA tolerates single mutations at multiple locations with little loss of function, it is extremely sensitive to a second mutation.

Many of the 1.5% of double variants displaying positive epistasis can be explained simply, such as by restoration of a base pair that was lost in both of the corresponding single variants. However, there were several striking examples of unexpected positive epistasis, four classes of which are highlighted below because they suggest structural rearrangements.

First, an alternative tRNA conformation appears to form in variants with mutations in the 26- to 44-nucleotide (nt) pair. The nucleotides at residues 26 and 44 are mismatched; 65% of the time in eukaryotes and, in known structures, often form a propeller-twisted noncanonical base pair in a Watson-Crick-like orientation (Fig. 3A; Kim et al. 1974); however, these nucleotides are also frequently canonically paired, with Watson-Crick [17%] or G–U [18%] pairings [Marck and Grosjean 2002]. We found that the A44U mutation [opposite G26] had nearly opposite effects on the function of double variants, dependent on the identity...
of the other mutation. Thus, the A44U mutation substantially rescued the function of variants with the destabilizing anti-codon stem mutations A29C, A29U, and A28U; in contrast, the A44U mutation had large negative epistatic effects with A9U, A9C, A22U, and G57A (Fig. 3B, C; Supplemental Table S4; Supplemental Fig. S3A), all of which often participate in the tertiary fold (Giege et al. 2012). One likely interpretation of these results is that A44U alters tRNA conformation by pairing with G26, strengthening the anti-codon stem and thereby countering other destabilizing anti-codon stem mutations while simultaneously causing structural shifts that impair the function of variants with otherwise benign mutations affecting the tRNA fold. It is notable that the 26–44 pair is in the “hinge” region of tRNA, which undergoes substantial conformational changes during ribosome passage in the A/T state with EF-Tu (Valle et al. 2003; Schmeing et al. 2009) and in the pe*/E state during translocation (Zhou et al. 2013). Flexibility in this region of the tRNA may also explain why the inactive G26U variant (opposite A44) was substantially rescued by mutation of G45 (Supplemental Table S4; Supplemental Fig. S3B–D). G45 sometimes interacts with the 10–25 pair (Westhof et al. 1985; Gautheret et al. 1995; Giege et al. 2012), suggesting that mutating G45 could alter or break this tertiary interaction, thus adding more flexibility to the hinge region and allowing for a Watson-Crick pair at 26–44.

Second, the virtually universally conserved U8–A14 pair (Randau et al. 2009) could be replaced by A8–G14, resulting in substantial function [Supplemental Table S4; Supplemental Fig. S4A–C], whereas none of eight other substitutions of this pair resulted in a tRNA that was functional [Supplemental Table S3]. Since U8–A14 forms a critical reverse Hoogsteen pair to help position the D stem, it seems plausible that A8–G14 is functional in part because it maintains this geometry (Sterner et al. 1995), perhaps with N1 of A8 protonated [Supplemental Fig. S4D; Leontis et al. 2002]. However, it is not clear why only the A8–G14 variant had function, since five of the other eight 8–14 pairs that we scored are predicted to accommodate this geometry, albeit with slightly differing spacing [Leontis et al. 2002].
et al. 2011; Dewe et al. 2012]. However, the full scope of sequence variants subject to RTD is not clear, as only the tRNA\textsuperscript{Ser} family has been examined in any detail; the roles of the anti-codon stem–loop, the D stem–loop, and the T loop have been only minimally examined; tRNA\textsuperscript{Ser} family members are in the minority class II of tRNAs that have a long variable stem; and acceptor stem/T-stem stability estimates do not always accurately predict RTD susceptibility for other tRNA species (Whipple et al. 2011).

We applied this library-based approach to comprehensively define SUP\textsubscript{4}oc\textsubscript{3} variants that are substrates for RTD. RTD is readily detected with the RNA-ID reporter, since the known substrate SUP\textsubscript{4}oc\textsubscript{3}-G62C (Whipple et al. 2011) had reduced GFP\textsubscript{FLOW} in MET\textsubscript{22}\textsuperscript{+} (wild-type) cells compared with that in met\textsubscript{22}\textsuperscript{Δ} cells [Fig. 5B, Supplemental Table S5], in which RTD is inactivated (Chernyakov et al. 2008). We made a SUP\textsubscript{4}oc\textsubscript{3} library in the met\textsubscript{22}\textsuperscript{Δ} strain, analyzed variants by FACS and sequencing (Supplemental Fig. S6A), and compared GFP\textsubscript{SEQ} of variants with that from wild-type cells. GFP\textsubscript{SEQ} from the met\textsubscript{22}\textsuperscript{Δ} strain was highly reproducible and correlated with GFP\textsubscript{FLOW} (Supplemental Fig. S6B,C).

This analysis revealed many single variants that were putative RTD substrates, with mutations surprisingly...
located throughout the tRNA body. In the met22Δ strain, 70 single variants were highly functional, including all 44 that we identified in the wild type (Supplemental Fig. S6D). Overall, 38 single variants were more than twofold more active in met22Δ cells than in wild-type cells (GFPSEQ RTD ratio >2), suggesting that they are RTD substrates [Fig. 5C, green; Supplemental Table S6], and for 16 of these, the increase in activity was >0.3 [Fig. 5C, dark wedge outlines]. Eleven of these 38 RTD candidates have mutations in the acceptor or T stem, as expected for RTD substrates [Whipple et al. 2011]. Remarkably, the other 27 RTD candidates have mutations in regions not previously associated with RTD, including 17 in the anti-codon stem and loop, six in the D stem, and one each in the D loop, V loop, and T loop and at N8 [Fig. 5C].

We determined that a number of these variants are RTD substrates by two approaches. First, we reconstructed individual RTD candidate variants with mutations in different regions of the tRNA and tested them by flow cytometry when integrated into met22Δ and wild-type reporter strains. Nineteen of 21 putative RTD substrates by two approaches. First, we reconstructed individual RTD candidate variants with mutations in different regions of the tRNA and tested them by flow cytometry when integrated into met22Δ and wild-type reporter strains. Nineteen of 21 putative RTD substrates with GFPSEQ RTD ratios ranging from 2.4 to 2.3 had GFPFLOW RTD ratios >2.0 (Supplemental Fig. S7A; Supplemental Table S5), whereas 26 of 30 putative non-RTD substrates with GFPSEQ RTD ratios ranging from 1.4 to 0.9 had GFPFLOW RTD ratios <2.0. We therefore conclude that RTD ratios determined by GFPSEQ scores have high predictive value for potential RTD substrates as measured by GFPFLOW. Second, a primer extension assay with dCTP instead of dCTP (which results in a G34 stop for tRNATyr wild-type cells; however, tRNA levels were substantially higher than in wild-type cells [Supplemental Fig. S8A,B]). Moreover, for each of three variants examined, the U4C double variants had similar tRNA levels in wild-type and met22Δ cells [Fig. 6C; Supplemental Fig. S8C]. This result suggests that U4C protects the 5’ end of variants subject to RTD from exonucleolytic attack, presumably by stabilizing the 4–69 base pair.

Discussion

Although tRNAs have evolved for their efficient transcription, processing, and modification, high structural stability, and accurate and efficient usage in the translation cycle, the analysis of >25,000 variants of the model yeast tRNA SUP4oc demonstrates that it is highly robust to mutation. This robustness was unexpected based on the numerous constraints on tRNA sequences but was consistent with models of RNA evolution in which sequences converge to those that are robust to mutation (van Nimwegen et al. 1999). Nonetheless, although many single-base changes are tolerated in SUP4oc, a second mutation is much more likely to abolish function than to rescue it (Supplemental Fig. S2C). Since this excess of negative epistasis was present also in met22Δ cells (Supplemental Table S3), RTD is not the primary cause of negative epistasis, although it is likely one contributing factor. Part of the reason that SUP4oc has a preponderance of negative epistasis may be that the multiple constraints on structure and function are too great to accommodate most double mutations.

The analysis of positive epistasis suggests a remarkable amount of flexibility allowed in the sequence of SUP4oc. The suggestion that an alternative tRNA conformation is provoked by mutation of the 26–44 pair in the hinge region [Fig. 3; Supplemental Fig. S3A] may be compatible with conformational changes during translation (Valle et al. 2003; Schmeing et al. 2009; Zhou et al. 2013), as previously proposed for a D-stem variant [Cochella and Green 2005]. In the met22Δ mutant, there is even more pronounced evidence for this alternative conformation based on additional examples of negative and positive epistasis for mutations affecting the 26–44 pair [Supplemental Table S3] and more extreme epistasis values. These data suggest that the large fraction [35%] of 1984 surveyed eukaryotic tRNAs with canonical 26–44 pairing
(Marck and Grosjean 2002) may have common compensatory features that distinguish them from tRNAs with unmatched residues at this position. The epistasis data derived from the met22Δ mutant underscore the flexibility of tRNA, since increased positive epistasis was observed for the C27U A28C variant and the U8A A14G variant. In addition, there are several examples of epistasis to preserve adjacent guanosine residues in the D loop (normally located at positions 18 and 19) (see Fig. 4A), including one variant that was not scored in wild-type cells (Supplemental Table S3).

Several other positive epistatic interactions cannot be explained easily by structural alterations (Supplemental Table S3). Since our approach measures the overall function of the tRNA, which includes all steps from its transcription by RNA polymerase III through its role in translation, such positive epistasis could arise because of altered function in the double mutant due to any combination of steps during the biogenesis of tRNA or its deployment in translation.

Our results suggest that RTD monitors the integrity of the entire tRNA molecule, greatly expanding the scope of variants subject to this pathway. Indeed, since 446 of 838 substantially functional double variants are likely subject to RTD, RTD is a major factor in determining the sequence limits to tRNA function (Supplemental Table S6). Since our analysis suggests that increased ΔΔG°28 correlates with susceptibility to RTD regardless of the stem that is affected (Fig. 5C, Supplemental Table S5, S6; Supplemental Fig. S7), this brings up the question of how overall tRNA stability influences RTD. Our suggestion that degradation occurs through the 5'9 end is supported by widespread U4C suppression of RTD (Fig. 6; Supplemental Fig. S8). Mutations to SUP4oc in the D stem, D loop, and T loop may trigger RTD by altering the tertiary fold, allowing increased 5' end attack. However, it is difficult to rationalize how mutations in the anti-codon stem expose the 5' end, since residues in this stem do not interact with other regions. These anti-codon stem mutations might trigger RTD due to cooperative unfolding of the acceptor stem, altered stacking with the D stem and consequent destabilization, or another sensing mechanism, perhaps an element of the translation machinery.

Our results provide a framework for understanding how sequence variation influences many aspects of tRNA biology, including the role and function of tRNA isodecoders in metazoans and the molecular basis of diseases caused by mitochondrial tRNA mutations (Yarham et al. 2010). An analysis of ED shows that tRNA structure prediction software may be useful for giving an upper bound on tolerated defects, but other parameters need to be incorporated for these programs to predict function successfully. Application of the high-throughput approach described here to define functional determinants of other tRNA species should lead to large improvements in our ability to predict function of variants.

The approach described here is generally applicable to many problems in tRNA biology. FACS followed by deep sequencing of tRNA genes can be used to score the effect of mutations that affect tRNA processing, modification, or translation by comparing the scores with those in a wild-type background. A prerequisite for this approach is that the tRNA can be made into a suppressor or that cells carrying the tRNA can be scored for growth or another activity. By the use of appropriate screens, this approach can also measure tRNA charging fidelity (Kramer et al. 2010). Furthermore, this overall approach...
can be adapted to study many problems in the biology of noncoding RNAs.

Materials and methods

Yeast strains

The BY4741 can1::P<sub>GAL1</sub>-GFP<sub>oc</sub>-P<sub>GAL1</sub>-RFP strain (YK380-1) was constructed by PCR amplification of the P<sub>GAL1</sub>-GFP<sub>oc</sub>-P<sub>GAL1</sub>-RFP reporter and its adjacent MET15 marker from plasmid EKDI302 (Dean and Grayhack 2012), using primers with sequence complementary to the 5′ and 3′ ends of CAN1, followed by linear transformation of the DNA into BY4741. A met22 derivative of the YK380-1 (YK391-1) was generated by PCR amplification of the met22-kanMX strain (Open Biosystems) followed by linear transformation. SUP4<sub>oc</sub> and SUP4<sub>oc</sub>-variant derivatives of strains YK380-1 and YK391-1 were generated by linear transformation to integrate the StuI fragment of the plasmid containing SUP4<sub>oc</sub> (derived from AB230-1) into the ADE2 locus, followed by selection on S-His dropout medium. Since the Stu fragment has different sequences of ADE2 DNA at each flanking the DNA containing SUP4<sub>oc</sub> and Schizosaccharomyces pombe his5<sup>+</sup>, linear transformation should not generate multiple integrants at this locus. For each variant strain analyzed, three individual transformants were constructed and used.

Plasmids

AB230-1 was constructed by replacement of the MET15 marker of JW132 [Whipple et al. 2011] with a fragment of DNA from pUG27 expressing S. pombe his5<sup>+</sup> [which complements S. cerevisiae his3</sup>] (Gueldener et al. 2002) followed by insertion of a 1508-base-pair (bp) fragment of FluC DNA into the BglIII and Xhol restriction sites to facilitate detection of inserts when SUP4<sub>oc</sub> variants were inserted into these sites.

Variant tRNAs were constructed by insertion of the appropriate SUP4<sub>oc</sub> tRNA sequence, flanked by the 22 bp 5′ of the +1 site and the 7 bp 3′ of residue 73 of mature tRNA<i>S</i>His<sup>AdoDH</sup> [Thi2 genuiss] G2], into the BglIII Xhol site of AB230-1, essentially as described previously [Whipple et al. 2011]. The final sequence inserted was as follows: S′-AACAAAGTCTATAAAGAATTACCTCGGATAGCCAATTTGATTAAAGCAGCAAGACCTTAAATTATCCTCACACGAAATCTTGAGAGTCTGACCCACCCCGGAATTTTTTCCTCCGAC-3′, with the SUP4<sub>oc</sub> exon sequence underlined, the anti-codon in bold, and the intron in italics.

Analytical flow cytometry

Strains were grown overnight at 28°C in S-His liquid dropout medium containing 2% raffinose and 2% galactose supplemented with 80 mg/L adenine, followed by growth for 24 h in YP medium containing 2% raffinose and 2% galactose supplemented with 80 mg/L adenine. Dilutions were made as necessary to maintain log phase growth. Cells were then diluted in the same medium to an OD<sub>600</sub> of 0.3 and grown to an OD<sub>600</sub> between 0.8 and 1.2. Samples were prepared and analyzed essentially as described previously. Briefly, 10,000 events were recorded after analysis on an LSR-II flow cytometer [BD Biosciences] using laser and fluorescence detection filter parameters as described previously, with filter voltages set so that both GFP and RFP fluorescence intensities were ~26,000 and with only those cells that passed an RFP cutoff of 5 × 10<sup>5</sup> analyzed [Dean and Grayhack 2012]. Data analysis was performed using FlowJo software [Tree Star]. GFPLOW scores for a culture of a given SUP4<sub>oc</sub> variant represent the ratio of the median GFP divided by the median RFP, normalized to the median GFP/median RFP for wild-type SUP4<sub>oc</sub>. Biological triplicates were used to obtain standard deviations.

SUP4<sub>oc</sub> library construction and analysis

The SUP4<sub>oc</sub> library was generated by annealing two partially complementary oligonucleotides (MPG P346 and MPG P347) with 3% random mutations in SUP4<sub>oc</sub> residues 1–33 and 39–73 [IDT] followed by filling in the unpaired overhangs [Supplemental Fig. S1] and cloning. The sequence of P346 was 5′-TTTTTGAGA TCTACCAAAAAGTTAAGAAATTTACTTTGCGTATGTTTTCAAAGCGAACACTTTTACAATTCACTCGAAG-3′, and that of P347 was 5′-AGTGGTCTCAGGGAAAGAAGTCTCTCCGGGGCGGATCGACAGCAAGATTTTCGATGATAAATTAA-3′, with the residues containing mutations underlined and the complementary sequence (comprising residues 34–38 and the intron) in bold. Annealing of the two oligonucleotides was done by heating for 5 min to 100°C followed by slow cooling to 30°C and then immediate placement on ice. The unpaired overhangs were then filled using the Klenow fragment of DNA polymerase at 37°C, and the reaction product was digested with BglII and Xhol, purified by gel extraction, and ligated into AB230-1, giving ~325,000 E. coli transformants. An aliquot of these transformants containing ~2.7 × 10<sup>7</sup> cells was amplified by ~4.3 generations of growth, and then plasmid DNA was extracted and digested with StuI for integration into yeast as described above. The yeast transformants were scraped, pooled, and frozen in aliquots for subsequent use.

To grow yeast SUP4<sub>oc</sub> libraries, ~4.9 million cells were thawed, inoculated into S-His medium containing 2% raffinose supplemented with 80 mg/L adenine, and grown for 24 h at 28°C followed by dilution to OD<sub>600</sub> of 0.04 and growth for 24 h in YP medium containing 2% raffinose and 2% galactose supplemented with 80 mg/L adenine. Dilutions were made as necessary to maintain log phase and ensure that at least 4.9 million cells were propagated at each step. Cells were then diluted in the same medium to an OD<sub>600</sub> of 0.4 and grown to an OD<sub>600</sub> of 1.1 prior to FACs into four bins on an Aria-II cell sorter [BD Biosciences] at the University of Rochester Medical Center Flow Cytometry Core facility. Laser and fluorescence detection filter parameters were set as previously described, and only those cells with a RFP > 5 × 10<sup>3</sup> were collected [Dean and Grayhack 2012]. Bin borders were set at a GFP<sub>LOW</sub> of 0.007 [the lowest activity readily distinguished for a strain containing SUP4<sub>oc</sub> variants as compared with strains with no SUP4<sub>oc</sub>, corresponding to GFP<sub>LOW</sub> of 0.026], with successive borders at 0.038 and 0.384. At least 2 million cells were collected [Supplemental Table S1] and then plated on YPD medium. After incubation for 3 d at 25°C, cells were scraped, pooled, and stored at ~80°C. Genomic DNA was then directly isolated from frozen aliquots of the stored cells in each bin. Libraries WT1 and WT2 are replicates of the SUP4<sub>oc</sub> library analyzed in wild-type cells, and libraries Δ1 and Δ2 are replicates of the SUP4<sub>oc</sub> library analyzed in <i>m</i>et22A cells. The GFP<sup>Δ1</sup> RTD analysis was based on comparing the WT2 and Δ2 libraries.

To enhance resolution of highly functional variants, aliquots of stored bin 1 cells [cells with high GFP expression] collected from the first sorting of the WT2 library were thawed, grown, and further sorted by FACs into four bins, three of which were subdivisions of the original bin1, and one of which was the original bin 2. Pooled cells were treated as described above prior to sequence analysis. Data from this analysis are referred to as WT2 6 bins and are the data set used for single, double, and epistasis analysis of SUP4<sub>oc</sub> in wild-type cells.
Sequencing

The SUP4<sub>oc</sub> construct, including 27 5' and 16 3' nucleotides, was amplified for 20 cycles (10 sec at 98°C, 30 sec at 52°C, and 30 sec at 72°C) from 1–3 μg of genomic DNA using Phusion polymerase and one of four sets of primers. Y19 (5'-AATGATACGGCGACCACCGAGATCTACCTCCTGCTTAAGCTGACGATAT TCGTCAGATAGTTGACAGCTCACACCATATGACACCTT CTTCTTCTCCTGCGCCACCAAGCTCGAGAAGAACCAAAAT C-3'), used in all four primer sets, contains the illumina adaptor sequence (1–29), a sequencing primer (30–56), and four Ns to disrupted if a nucleotide mutation prevented canonical pairing. The number of reads corresponding to each unique variant for all eukaryotic tRNA sequences for which the modification cation. For the assessment of the ED of natural tRNAs, we used all eukaryotic tRNA sequences for which the modification status is known from the following species: Bombyx mori, Bos taurus, Candida cylindracea, Drosophila melanogaster, Homo sapiens, Leishmania tarentolae, Lupinus albus, Lupinus luteus, Mus musculus, Nicotiana rustica, Nicotiana tabacum, Oryctolagus cuniculus, Pichia jadinii, Rattus norvegicus, S. cerevisiae, S. pombe, Tetrahymena thermophila, Trichium aestivum, and Xenopus laevis. Sequences were obtained from the Modomics database [http://modomics.genesilico.pl].

Calculation of ΔΔG<sub>28</sub>

The ΔΔG<sub>28</sub> is the folding free energy change difference between the mutant and wild-type tRNA structure at 28°C. The predicted ΔΔG<sub>28</sub> for each tRNA variant was computed with a custom program using the C<sup>2+</sup> classes from the RNAstructure package [Reuter and Mathews 2010]. ΔG's were calculated using nearest neighbor rules [Mathews et al. 2004], where base pairs were disrupted if a nucleotide mutation prevented canonical pairing.

Epistasis analysis

The formula to determine epistasis for double mutants that passed the read count filter was

\[ \text{epistasis} = \text{GFPSEQ}_{\text{double mutant}} - \left( \text{GFPSEQ}_{\text{single mutant 1}} \times \text{GFPSEQ}_{\text{single mutant 2}} \right). \]

The product of GFPSEQ of the two single mutants was correlated with the actual double-mutant GFPSEQ with an R<sup>2</sup> of 0.53 for Δ1, 0.56 for Δ2, 0.545 for WT1, and 0.534 for WT2. Epistasis can be >1 or less than −1, since some double-mutant variants have GFPSEQ values >1, because all sequencing reads for that variant were in bin 1, whereas some wild-type SUP4<sub>oc</sub> sequence reads occurred outside of bin 1.

Parameters that can be manipulated on the interactive SUP4<sub>oc</sub> Web site [http://depts.washington.edu/si/shields/tRNA_supplemental/ tRNA_interactive.html] are as follows: predicted fitness cutoff, only display epistatic interactions [links] in which the product of the GFPSEQ for the constituent single mutant variants is greater than this number; cell cutoff, only display interactions for double mutants with more total estimated cell counts than this number; read count cutoff, only display interactions for double mutants with more total sequencing reads than this number; epistasis color control, controls the point at which the negative and positive epistasis values switch from gray to color for links; opacity cutoff, the epistasis value below which link opacity is set to “minimum opacity”; and minimum opacity, a value between 0 (transparent) and 1 (opaque) that defines the transparency of epistatic links with values below the “opacity cutoff.” The default is 0.3.
ROCK (receiver operating characteristic) analysis
To assess the extent to which $\Delta G^\circ_{298}$ correlates with RTD, we performed ROC analysis. All of the single and double variants were filtered with the criterion requiring met22A $G^\circ_{298} \geq 0.052$ (twice the cutoff for a variant to be considered active). With that, all of the variants with a ratio of met22A $G^\circ_{298}$ to WT2 $G^\circ_{298} \geq 2.0$ were classified as RTD substrates. $\Delta G^\circ_{298}$ was taken as a predictor to compute the false positive rate and the true positive rate for ROC analysis. The plot was generated, and thresholds were computed by the R package pROC [http://www.R-project.org] (Robin et al. 2011).

Isolation of bulk RNA and tRNA purification
Wild-type and met22A cells containing integrated SUP4oc variants were grown as described for analytical flow cytometry. Bulk low-molecular-weight RNA was extracted from 300 OD-mL pellets by hot phenol extraction followed by two ethanol precipitations and resuspension in ddH$_2$O, as previously described (Jackman et al. 2003). tRNA$^{Gly}$ was purified using biotinylated oligomer MP129, which is complementary to residues 76–52 of endogenous tRNA$^{Gly}$, SUP4oc, and the variants analyzed (Jackman et al. 2003).

Primer extension of SUP4oc variants
Bulk low-molecular-weight RNA was subjected to a poison primer extension assay using a primer from nucleotides 57–37 or 62–43 of mature tRNA$^{Gly}$ that was 5’ end-labeled with T4 polynucleotide kinase and [$\gamma$-32P]ATP. Two-hundred nanograms of bulk low-molecular-weight RNA (or 7 ng of purified tRNA) was annealed to ~1 pmol of 5’ radiolabeled primer after incubation for 3 min at 95°C before slow cooling and incubation for 30 at 50°C. Annealed RNA was then incubated for 1 h at 50°C in the presence of 1 mM each ddCTP, dATP, dGTP, and dTTP and 2 U of AMV reverse transcriptase (Promega). After completion, the reaction was resolved on a 7 M urea and 15% polyacrylamide gel for ~4 h. The resulting gel was then dried and exposed to a phosphorimager plate for analysis, as previously described (Jackman et al. 2003).

An interactive Web site for analysis of GEP$^{18}$, epistasis, and RTD on tRNA cloverleaf maps is also available at http://depts.washington.edu/sfields/tRNA_supplemental/tRNA_interactive.html.

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