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Methodology for the high-throughput identification and characterization of tRNA variants that are substrates for a tRNA decay pathway

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Abstract

The rapid tRNA decay (RTD) pathway is a tRNA quality control pathway known to degrade several specific hypomodified or destabilized tRNAs in the yeast *Saccharomyces cerevisiae*. In this chapter we describe seven methods for identifying RTD substrates, with a focus on two new approaches: a high-throughput approach that utilizes a suppressor tRNA library, fluorescence activated cell sorting, and deep sequencing, and has greatly expanded the known range of RTD substrates; and a poison primer extension assay that allows for the measurement of levels of suppressor tRNA variants, even in the presence of highly similar endogenous tRNAs. We also discuss different applications of the use of the high throughput and poison primer extension methodologies for different problems in tRNA biology.

Keywords

rapid tRNA decay; poison primer extension; high throughput; yeast; RNA-ID; *SUP4_{oc}*

1. Introduction

tRNA folding and stability is crucial for efficient translation, and defects in either property can lead to reduced quantities of tRNA, resulting in growth defects in yeast and disease in humans (Hopper, 2013; Yarham, Elson, Blakely, McFarland, & Taylor, 2010). In the yeast *Saccharomyces cerevisiae* there are two major cellular quality control pathways known to degrade defective tRNA species. The first pathway is the nuclear surveillance pathway, which acts on pre-tRNA in the nucleus through use of the nuclear exosome and the TRAMP complex (Kadaba, Wang, & Anderson, 2006; Vanacova et al., 2005) by degrading pre-tRNA_i^{Met} lacking the m¹A₅₈ modification or with a misprocessed 3' trailer (Ozanick et al., 2009) and a fraction of wild type pre-tRNAs (Gudipati et al., 2012). The second pathway is the rapid tRNA decay (RTD) pathway, which degrades specific mature, hypomodified or destabilized tRNA species through the activity of the 5'–3' exonucleases Rat1 and Xrn1 (Alexandrov et al., 2006; Chernyakov, Whipple, Kotelawala, Grayhack, & Phizicky, 2008). RTD is elicited in mutants lacking any of several modifications in the body of the tRNA or

through destabilizing mutations, and for all identified RTD substrates, *MET22* deletion fully restores tRNA levels and growth (Alexandrov et al., 2006; Chernyakov, Whipple, et al., 2008; Dewe, Whipple, Chernyakov, Jaramillo, & Phizicky, 2012; Guy et al., 2014; Kotelawala, Grayhack, & Phizicky, 2008; Whipple, Lane, Chernyakov, D'Silva, & Phizicky, 2011). Suppression of RTD in *met22* strains is presumed to be due to inhibition of the exonucleases Rat1 and Xrn1 by the metabolite 3' phosphoadenosine-5' phosphate (pAp), which has increased levels when Met22 is inhibited (Dichtl, Stevens, & Tollervey, 1997; Murguia, Belles, & Serrano, 1996).

RTD is known to act on several specific tRNA species, which have been identified and studied using seven approaches (Fig. 1). The first approach was to use microarrays to compare the tRNA levels on a genome-wide scale in *trm8 trm4* temperature sensitive modification mutants (lacking m⁷G₄₆ and m⁵C) and in related strains under semi-permissive conditions. In this way, we identified the RTD substrate tRNA^{Val(AAC)}, since it had reduced tRNA levels in the *trm8 trm4* mutant relative to wild type (WT) or the corresponding single mutants (Alexandrov et al., 2006).

In the second approach, northern blots were used to examine both the rate and specificity of tRNA degradation for RTD substrates in temperature sensitive modification mutants. In this approach, RNA isolated from cells at different time points after temperature shift was analyzed for levels of specific tRNAs. From this analysis, we found that 50% of the tRNA^{Val(AAC)} was degraded in a *trm8 trm4* mutant within 30 minutes of a shift from 28°C to 37°C while the similarly hypomodified tRNA^{iMet}, tRNA^{Met}, and tRNA^{Phe} showed no decrease (Alexandrov et al., 2006; Chernyakov, Whipple, et al., 2008). Furthermore, the relative levels of charged and uncharged tRNA could be measured by performing the northern blot under acidic conditions, which showed that levels of charged tRNA^{Val(AAC)} were reduced by 50% within 25 minutes of temperature shift in a *trm8 trm4* mutant, and that the uncharged tRNA^{Val(AAC)} levels appeared unaffected (Alexandrov et al., 2006).

The third approach was through high copy tRNA suppression, wherein a high copy plasmid expressing a particular tRNA was introduced into a temperature sensitive tRNA modification mutant. If the tRNA was an RTD substrate and the temperature sensitivity was the result of a single tRNA species being degraded, then overexpression of the tRNA would suppress the defect. Thus, we found that the temperature sensitivity of a *trm8 trm4* mutant was suppressed by a high copy plasmid expressing tRNA^{Val(AAC)}, indicating that temperature sensitivity was primarily due to loss of tRNA^{Val(AAC)}, and that the missing modifications were important for tRNA stability (Alexandrov et al., 2006). Similarly, the RTD substrates of several other tRNA modification mutants have also been identified using this approach, including tRNA^{Ser(CGA)} and tRNA^{Ser(UGA)} in *tan1 trm44* mutants (lacking ac⁴C₁₂ and Um₄₄) and in *trm1 trm4* mutants (lacking m^{2,2}G₂₆ and m⁵C) (Chernyakov, Whipple, et al., 2008; Dewe et al., 2012; Kotelawala et al., 2008).

Fourth, we used a genetic replacement approach to identify RTD determinants in the tRNA^{Ser} family, by substituting the single essential tRNA^{Ser(CGA)} gene (*SUP61*) with different tRNA^{Ser(CGA)} variants in the WT and the *met22* strain, and then assaying for growth at different temperatures. Using this approach we determined that the combined

acceptor and T-stem stabilities were strong determinants for RTD susceptibility in the tRNA^{Ser(CGA)} gene family (Whipple et al., 2011). This conclusion was further supported by a fifth approach to measure RTD, in which we showed *in vitro* that tRNA^{Ser(CGA)} variants lacking ac⁴C₁₂ and Um₄₄, or with destabilizing mutations in the acceptor stem, were more prone to digestion by Xrn1 and more susceptible to 5' phosphate removal by calf intestinal phosphatase (Whipple et al., 2011).

In this review, we will discuss our recently developed sixth and seventh approaches for the study of RTD substrates, which have proven extremely valuable in broadening our understanding of the RTD pathway. The sixth approach uses a fluorescent reporter to comprehensively analyze libraries of thousands of tRNA variants in WT and *met22* strains. Through this approach, we have identified 643 likely RTD substrate candidates, many in regions not expected to elicit RTD based on previous work (Guy et al., 2014). We will show data demonstrating that this approach can be used to study tRNA function under different conditions, and will discuss other applications of the approach.

The seventh approach employs poison primer extension to measure the tRNA levels in a WT and *met22* strain, and is valuable in its ability to specifically measure a variant tRNA even in the presence of the WT tRNA whose sequence may differ by only a single residue. We provide a detailed methodology of this approach and discuss some of its other possible applications.

2. High-Throughput Identification of tRNA Substrates Degraded by the RTD Pathway

In this approach, a fluorescent reporter that is sensitive to the levels of functional tRNA is used to identify tRNA variants that are subject to RTD because there is less fluorescence in a WT strain (in which RTD is functional) than in a *met22* strain (in which RTD is inhibited). For this analysis, we used the previously developed RNA-ID fluorescent reporter, which contains the inducible P_{GAL1,10} bi-directional promoter expressing *RFP* in one direction and *GFP_{oc}* (GFP with a UAA nonsense codon) in the other direction (Dean & Grayhack, 2012). Expression of *GFP_{oc}* relative to RFP is 0.5% of that of the corresponding *GFP* reporter without a nonsense codon, and is increased to 94% if the strain has an integrated *SUP4_{oc}* gene (encoding tRNA^{Tyr} in which the GUA anticodon is mutated to UUA by a G34U mutation, Fig. 2A), which efficiently suppresses UAA nonsense codons (Guy et al., 2014). We tested the ability of our reporter to distinguish between substrates and non-substrates of the RTD pathway by examining a previously identified temperature sensitive RTD substrate, *SUP4_{oc}-G62C* (Whipple et al., 2011), for a significant increase in GFP fluorescence when expressed in a *met22* strain compared to a WT strain. We found that there was a ~2 fold increase in GFP fluorescence in the *met22* strain at 28°C, indicating that the levels of functional *SUP4_{oc}* tRNA were increased by a measurable level upon inhibition of the RTD pathway (Guy et al., 2014).

The use of the RNA-ID reporter for the identification of RTD substrates offered two distinct advantages over previously described genetic approaches to define substrates. First, a growth

or temperature sensitive phenotype would no longer be required to assay RTD, and second, a library of thousands of tRNA variants could be tested in parallel.

We used this approach to analyze a comprehensive library of approximately 220,000 independent *SUP4_{oc}* variants integrated into both WT and *met22* strains. We sorted the libraries into four bins based on GFP fluorescence using FACS, followed by deep sequencing of the extracted genomic DNA from each bin. Each variant was assigned a functional score (GFP^{SEQ}) based on its frequency of distribution in the four bins and the measured median GFP fluorescence of each bin, relative to that of *SUP4_{oc}*. This analysis resulted in GFP^{SEQ} scores for 9,563 single and double mutant variants in the WT library, of which 4,263 were also scored in the *met22* library (Guy et al., 2014). The GFP^{SEQ} scores for each variant were then used to calculate the fold increase in a *met22* strain compared to a WT strain (GFP^{SEQ} (*met22*)/GFP^{SEQ} (WT)), called the GFP^{SEQ} RTD ratio, which was used to identify variants as putative RTD substrates (Fig. 2B). Based on the comparison of GFP fluorescence for *SUP4_{oc}-G62C* and the overall trend of RTD ratios for the library, an RTD ratio cutoff of 2 was chosen to identify putative RTD substrates. For single mutants, this resulted in the identification of 38 putative RTD substrates among the 213 possible single mutant variants (Guy et al., 2014).

To validate the results from our deep-sequencing analysis, we further analyzed 51 *SUP4_{oc}* variants with a range of RTD ratios from 0.9 to 24.4 by reconstructing and re-testing the variants using flow cytometry (Fig. 3A). Our results showed that 19 of 21 putative RTD substrates identified by a GFP^{SEQ} RTD ratio of 2 or more also had an RTD ratio of 2 or more when measured by flow cytometry, and that 26 of 30 strains with a GFP^{SEQ} RTD ratio less than 2 also had an RTD ratio of less than 2 when measured by flow cytometry. These experiments validated the ability of our high-throughput and deep-sequencing analysis to accurately predict *SUP4_{oc}* variants as RTD substrates.

3. Measurement of tRNA levels of RTD Substrate *SUP4_{oc}* Variants in the Presence of WT tRNA^{Tyr}

To determine if the increased GFP^{SEQ} of a *SUP4_{oc}* variant in the *met22* strain (relative to the WT strain) was due to increased tRNA, we sought to compare the levels of *SUP4_{oc}* variants in each strain. However, this analysis was difficult because *SUP4_{oc}* differs from wild type tRNA^{Tyr} by only one nucleotide (the G34U mutation). Thus, it was not practical to either selectively purify *SUP4_{oc}* away from the endogenous tRNA^{Tyr}, or to selectively probe for *SUP4_{oc}* using a northern blot.

To solve this problem, we used a poison primer extension analysis (Driscoll, Wynne, Wallis, & Scott, 1989), which is performed similarly to conventional primer extension, except that one NTP in the reaction is replaced by a 2',3' dideoxy NTP that, once incorporated into the cDNA, prevents any further extension by the reverse transcriptase. This allows for one primer to extend two different tRNA species, in this case producing a signal unique to *SUP4_{oc}* variants even in the presence of contaminating wild type tRNA^{Tyr} (Guy et al., 2014).

Since *SUP4_{oc}* bears a G34U mutation in the anticodon that is four bases 3' of the next guanosine residue (G₃₀), we reverse transcribed RNA from a primer complementary to both WT tRNA^{Tyr} and *SUP4_{oc}* in the presence of 2',3' dideoxy CTP (ddCTP), which stops the extension when the reverse transcriptase inserts ddCTP opposite a guanosine residue. Thus, the primer extension product produced from *SUP4_{oc}* variants stops after G₃₀, whereas the product from wild type tRNA^{Tyr} stops after G₃₄ (Fig. 3B). As expected, in the absence of a *SUP4_{oc}* variant the ddCTP poison primer extension produces a stop primarily at G₃₄, corresponding to the WT tRNA^{Tyr}, whereas in the presence of *SUP4_{oc}* there is the expected increase at G₃₀ (Fig. 3C). Since both signals are easily distinguishable on a gel, this method also conveniently provides for an internal control for normalization of the *SUP4_{oc}* variant relative to wild type tRNA^{Tyr} (Fig. 3C).

We initially performed our poison primer extension analysis on tRNA that was purified using a biotinylated DNA probe complementary to residues 76–55 of tRNA^{Tyr}, which purified both wild type tRNA^{Tyr} and *SUP4_{oc}* variants. Although a poison primer extension assay of the purified tRNA discriminated between tRNA^{Tyr} and *SUP4_{oc}* with high resolution, the same analysis was equally discriminating with bulk low molecular weight RNA (Fig. 4A). We have used bulk RNA as our source for all further primer extensions, because it is simpler to make and because it is not subject to the possible anomalies that could occur during purification of different *SUP4_{oc}* variants together with endogenous tRNA^{Tyr}.

3.1 Preparation of Bulk Low Molecular Weight RNA

The bulk low molecular weight RNA was prepared by the hot phenol method as previously described (Chernyakov, Baker, Grayhack, & Phizicky, 2008) but modified slightly for the extraction from a smaller (2 OD-mL) pellet, harvested from 2 mL cells grown in rich (YP containing glucose or raffinose and galactose) media to OD₆₀₀ = ~1.0. The pellet is suspended in one volume of 200 µL of RNA Extraction Buffer (0.1M NaOAc pH 5.2, 20 mM EDTA, 1% SDS) and then mixed with an equivalent volume of phenol and lysed by the above method (Chernyakov, Baker, et al., 2008). The yield of RNA from 2 OD-mL cells is approximately 1.5 – 5.0 µg, based on A²⁶⁰.

3.2 Preparation of Labeled DNA Primer

The selection of a primer for the poison primer extensions with ddCTP of *SUP4_{oc}* variants was determined using two considerations. First, the primer had to anneal at a location in the tRNA where the first guanosine residue encountered during extension would be at residue 34, because any earlier encountered guanosine residue would result in only one signal for both *SUP4_{oc}* and WT tRNA^{Tyr}. Second, as with all primer extensions, the primer had to have a high enough T_m for efficient binding to the tRNA (70–75°C) but not too high so as to prevent nonspecific hybridization. Our first primer annealed to residues 57–37 (P5) of tRNA^{Tyr} and *SUP4_{oc}* variants. We also developed an alternative primer that annealed to residues 62–43 (P7) of tRNA^{Tyr}, still satisfying the above considerations, but allowing measurement of tRNA levels for variants with mutations at residues 42–38. For most of the examined variants, the two primers were interchangeable and although there was some variability in the measured read-through of variants in different experiments (possibly

attributed to differing RNA amounts or annealing efficiency), the ratios of read-through between WT and *met22* strains were largely unchanged for those variants (Fig. 4A, left panel, Fig. 4B). To label the primer for poison primer extension analysis, we did the following:

1. Incubate 2 μ L of 30 μ M primer (~60 pmol) suspended in TE 8 (10 mM Tris-HCL, pH8.0, 1 mM EDTA, pH 8.0) with 60 pmol [γ -³²P] ATP (ICN, 6000 Ci/mmol), and T4 polynucleotide kinase in the provided buffer (New England Biolabs, Cat# M0201L) for 1 hour at 37°C. The equimolar ratio of primer and ATP ensures high specific activity primers, which increases sensitivity of primer extension.
2. Remove proteins from labeled primer by eluting through a Micro BioSpin 6 column (BioRad, Cat# 732-6221) by spinning at 1000 $\times$ G for 4 minutes.
3. Discard the column and then mix the eluted sample with equal volume loading dye (100% formamide, 10 mM EDTA pH 8.0, 1 mg/mL Bromophenol Blue, 1 mg/mL Xylene Cyanol) and then heat at 95°C for 3 minutes.
4. Load sample onto a 15% 29:1 bis:acrylamide, 7 M urea, TBE pH 8.3 (0.88 M Tris base, 0.025 M Na₂EDTA, 0.9 M Boric Acid) sequencing gel and resolve the primer from contaminants by electrophoresis at 60 watts in TBE buffer. The gel should be warm to the touch under these conditions.

This step is important because we found that the primers used for our experiments (ordered from Integrated DNA Technologies) contained small amounts of contaminating species that were shorter than the designed primer. Although this would be a benign issue for northern blotting, it was a significant concern for the poison primer extension assay because the contaminating primers were also extended and therefore produced multiple bands of varying lengths for a single stop.

5. Expose the gel to radioisotope film (Carestream Biomax MS Film, Cat# 829 4985) for 30 seconds in a dark room to locate the purified primer band. Cut band out of gel and mechanically break the gel by forcing it through a 5 mL syringe using the plunger into a 14 mL round bottom tube (Falcon #2059).
6. Suspend the crushed gel in 500 μ L of extraction buffer (0.5 M NH₄OAC, 0.10% SDS, 5 mM EDTA, pH 8.0) and incubate while shaking at 37°C for 12 hours.
7. After incubation, spin the tubes in a clinical centrifuge at low speed for one minute. Remove the extraction buffer from the gel pieces by eluting through a Quik-Sep column (PerkinElmer Life Sciences, Cat# QS-PM) spun at 2000 RPM for 10 minutes in a clinical centrifuge at room temperature.

8. Add the eluted sample to a phase lock gel heavy (PLGH) tube and mix with 400 μ L of a 25:24:1 mixture of phenol:chloroform:isoamyl alcohol (equilibrated with 0.5 M Tris-HCl, pH 7.5) and spin at max speed for five minutes at room temperature in a microfuge.
9. Add the aqueous phase to a new tube containing 2 volumes of 100% ethanol with 5 μ L of 5 mg/mL glycogen and freeze on dry ice for 30 minutes, spin for 30 minutes at max speed in a microfuge at 4°C, and then discard the supernatant. A pellet should be visible at this point (from the glycogen carrier) and the discarded supernatant should contain only a relatively small amount of radioactivity compared to the pellet.
10. Wash with 100 μ L cold 70% ethanol without disturbing the pellet and then remove the supernatant.
11. Re-suspend the pellet in 120 μ L of water (~ 0.5 pmol primer/ μ L). The yield for 60 pmol of primer labeled with equimolar [γ - 32 P] ATP (ICN, 6000 Ci/mmol) is variable among probes, but is approximately 2.5×10^7 CPM as measured by a scintillation counter.

3.3 Poison Primer Extension

1. Add 200 ng of bulk low molecular weight RNA ($\sim 2.4 \times 10^{-4}$ mol tRNA, assuming the prep is 30% tRNA, corresponding to ~ 7 μ mol tRNA^{Tyr}) to ~ 1.0 pmol of [γ - 32 P] ATP labeled primer from a stock of ~ 0.5 pmol/ μ L (as described in Section 3.2) and dilute with water to a final reaction volume of 5 μ L.
2. Incubate the reaction at 95°C for 3 minutes using a heat block to melt the tRNA, and then slow cool to 50°C (about 1 hour) and incubate for another 30 minutes, to allow for specific annealing of the labeled primer to the tRNA target.
3. Remove the annealed reaction tube from the heat block and place directly on ice for 2 minutes, followed by quick spin of the tube to collect liquid that may have condensed on the lid.
4. Add 5 μ L of Extension Mix (1mM dATP, 1mM dGTP, 1mM dTTP, 1mM ddCTP, and 2 units AMV Reverse Transcriptase in the provided buffer (Promega, Cat# PR-M9004)) to the annealed reaction on ice and then incubate for 1 hour at 50°C, prior to storage of reaction mixtures at -20°C .

We compared Superscript III (Invitrogen) and AMV, and found that AMV had the most fidelity for incorporation of ddCTP and therefore the least background.

3.4 Analyzing Poison Primer Extension Using Polyacrylamide Gel Electrophoresis

1. Pre-run a large 7M Urea 15% 29:1 bis:polyacrylamide sequencing gel at 60 watts for 35–60 minutes so that it is warm to the touch. Clear precipitated urea from the lanes before and after pre-running the gel, by blowing out each lane using a syringe filled with running buffer.

2. Thaw poison primer extension reactions and add 5 μ L (half total reaction volume) to a 1.5 mL Eppendorf tube containing 5 μ L Loading Dye (100% formamide, 10 mM EDTA pH 8.0, 1 mg/mL Bromophenol Blue, 1 mg/mL Xylene Cyanol) and then heat at 95°C for 3 minutes, followed by a low speed spin.
3. Load samples onto the gel and resolve products by electrophoresis for 2.5 – 3 hours at 60 watts.
4. Remove gel from electrophoresis apparatus, remove glass plates, and then dry gel for 1.5 hours at 83°C using a gel dryer (BioRad Model 583 Gel Dryer) before exposing to a phosphorimager.

4. Conclusions and Additional Applications of these Approaches

The high-throughput method for identification of RTD substrates described here has greatly increased the number and type of known substrates for this cellular pathway, and has fueled experiments in our lab to understand the mechanisms of substrate recognition and RTD. Understanding these features of RTD in yeast may also shed light on specificity and mechanisms that occur during RTD in metazoans (Watanabe et al., 2013).

The high-throughput approach we described has a number of potential applications. We could use the same libraries to investigate temperature sensitivity of variants or to investigate RTD at different temperatures. As shown in Fig. 5A, the GFP/RFP value for *SUP4_{oc}* is reduced 2.18-fold after growth at 37°C (from GFP/RFP of 1.09 $\pm$ 0.02 at 28°C to 0.50 $\pm$ 0.03 at 37°C), whereas the GFP/RFP value for *SUP4_{oc}-A29U*GFP is reduced 3.75-fold (from 0.045 $\pm$ 0.003 to 0.012 $\pm$ 0.002), indicating that the *SUP4_{oc}-A29U* is slightly temperature sensitive. Media effects on suppression can also be investigated since they appear to differentially affect suppression of some variants. As shown in Fig. 5B, the GFP/RFP of *SUP4_{oc}* is reduced by 1.45-fold in minimal media containing galactose relative to YP media containing galactose (1.07 $\pm$ 0.02 in YP galactose and 0.74 $\pm$ 0.04 in S – His galactose), whereas the *SUP4_{oc}-A29U* variant is reduced 2.64-fold (from 0.066 $\pm$ 0.01 to 0.025 $\pm$ 0.001). Similarly, differential effects of UAA nonsense codon context can be examined. In setting up our high throughput analysis we used a *GFP_{oc}* construct in a “poor” context, since this results in poor termination and correspondingly good suppression (Bonetti, Fu, Moon, & Bedwell, 1995; Dean & Grayhack, 2012), giving us near complete suppression (94% of that with a GFP reporter with no stop codon) and therefore a large dynamic range (235-fold) for scoring suppression frequency (Guy et al., 2014). Since suppression by *SUP4_{oc}* is reduced to 35% in a good context *GFP_{oc}* reporter (Fig. 5C), one could use this reporter to examine a library of variants to identify those with differential suppression efficiency in this context, or with improved suppression efficiency relative to wild type. Additionally, we could use a *GFP-Y66_{oc}* construct to define determinants of TyrRS among our variants, since codon 66 of GFP is required to be tyrosine in GFP, and other variants do not fluoresce, or fluoresce with different excitation or emission maxima (Heim, Prasher, & Tsien, 1994). A *GFP-Y66_{oc}* construct has only ~3.1% of the GFP/RFP signal (Fig. 5C), which should be enough to define determinants, but if not, the signal might be improved by improving context or increasing copy number. We also have further applied

this approach to the identification of RTD substrates in another tRNA species: tRNA^{Ser(CGA)} (encoded by the *SUP61* gene) which, when mutated to a UUA anticodon is a relatively efficient suppressor of our *GFP_{oc}* construct (Fig. 5D).

In addition to the applications described above, variations of this high throughput approach could be used to identify tRNA variants with altered function in any mutant background that impacts tRNA processing or function. This could include, for example, screening for tRNA variants that function better in reporter strains lacking different modification genes, in strains with mutations that affect tRNA function during translation, in strains with mutations that affect intracellular trafficking, or in strains with mutations that affect other quality control pathways such as the nuclear surveillance pathway. With appropriate adaptation of this methodology one might also extend this approach to other RNA molecules for which a selection can be applied, based either on growth or on an appropriate reporter.

The poison primer extension methodology for measuring tRNA levels can be extended to analysis of relative levels of different isodecoders, tRNAs with the same anticodon but different tRNA bodies, which are commonly found in yeast and most other organisms (Goodenbour & Pan, 2006). A recent report shows that a specific isodecoder has a profound effect on the central nervous system in mice with a mutation in a factor that affects translation (Ishimura et al., 2014). In addition, poison primer extension methodology could be used to study the *in vitro* degradation of tRNA variants in complex RNA mixtures without the need for a previous purification step.

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References

- Alexandrov A, Chernyakov I, Gu W, Hiley SL, Hughes TR, Grayhack EJ, Phizicky EM. Rapid tRNA decay can result from lack of nonessential modifications. *Mol Cell*. 2006; 21(1):87–96. [PubMed: 16387656]
- Bonetti B, Fu L, Moon J, Bedwell DM. The efficiency of translation termination is determined by a synergistic interplay between upstream and downstream sequences in *Saccharomyces cerevisiae*. *J Mol Biol*. 1995; 251(3):334–345. [PubMed: 7650736]
- Chernyakov I, Baker MA, Grayhack EJ, Phizicky EM. Chapter 11. Identification and analysis of tRNAs that are degraded in *Saccharomyces cerevisiae* due to lack of modifications. *Methods Enzymol*. 2008; 449:221–237. [PubMed: 19215761]
- Chernyakov I, Whipple JM, Kotelawala L, Grayhack EJ, Phizicky EM. Degradation of several hypomodified mature tRNA species in *Saccharomyces cerevisiae* is mediated by Met22 and the 5′–3′ exonucleases Rat1 and Xrn1. *Genes Dev*. 2008; 22(10):1369–1380. [PubMed: 18443146]
- Dean KM, Grayhack EJ. RNA-ID, a highly sensitive and robust method to identify cis-regulatory sequences using superfolder GFP and a fluorescence-based assay. *RNA*. 2012; 18(12):2335–2344. [PubMed: 23097427]
- Dewe JM, Whipple JM, Chernyakov I, Jaramillo LN, Phizicky EM. The yeast rapid tRNA decay pathway competes with elongation factor 1A for substrate tRNAs and acts on tRNAs lacking one or more of several modifications. *RNA*. 2012; 18(10):1886–1896. [PubMed: 22895820]

- Dichtl B, Stevens A, Tollervey D. Lithium toxicity in yeast is due to the inhibition of RNA processing enzymes. *EMBO J.* 1997; 16(23):7184–7195. [PubMed: 9384595]
- Driscoll DM, Wynne JK, Wallis SC, Scott J. An in vitro system for the editing of apolipoprotein B mRNA. *Cell.* 1989; 58(3):519–525. [PubMed: 2758465]
- Goodenbour JM, Pan T. Diversity of tRNA genes in eukaryotes. *Nucleic Acids Res.* 2006; 34(21):6137–6146. [PubMed: 17088292]
- Gudipati RK, Xu Z, Lebreton A, Seraphin B, Steinmetz LM, Jacquier A, Libri D. Extensive degradation of RNA precursors by the exosome in wild-type cells. *Mol Cell.* 2012; 48(3):409–421. [PubMed: 23000176]
- Guy MP, Young DL, Payea MJ, Zhang X, Kon Y, Dean KM, Phizicky EM. Identification of the determinants of tRNA function and susceptibility to rapid tRNA decay by high-throughput in vivo analysis. *Genes Dev.* 2014; 28(15):1721–1732. [PubMed: 25085423]
- Heim R, Prasher DC, Tsien RY. Wavelength mutations and posttranslational autoxidation of green fluorescent protein. *Proc Natl Acad Sci U S A.* 1994; 91(26):12501–12504. [PubMed: 7809066]
- Hopper AK. Transfer RNA post-transcriptional processing, turnover, and subcellular dynamics in the yeast *Saccharomyces cerevisiae*. *Genetics.* 2013; 194(1):43–67. [PubMed: 23633143]
- Ishimura R, Nagy G, Dotu I, Zhou H, Yang XL, Schimmel P, Ackerman SL. Ribosome stalling induced by mutation of a CNS-specific tRNA causes neurodegeneration. *Science.* 2014; 345(6195):455–459. [PubMed: 25061210]
- Kadaba S, Wang X, Anderson JT. Nuclear RNA surveillance in *Saccharomyces cerevisiae*: Trf4p-dependent polyadenylation of nascent hypomethylated tRNA and an aberrant form of 5S rRNA. *RNA.* 2006; 12(3):508–521. [PubMed: 16431988]
- Kotelawala L, Grayhack EJ, Phizicky EM. Identification of yeast tRNA Um44 2'-O-methyltransferase (Trm44) and demonstration of a Trm44 role in sustaining levels of specific tRNA^{Ser} species. *RNA.* 2008; 14:158–169. [PubMed: 18025252]
- Murguia JR, Belles JM, Serrano R. The yeast HAL2 nucleotidase is an in vivo target of salt toxicity. *J Biol Chem.* 1996; 271(46):29029–29033. [PubMed: 8910555]
- Ozanick SG, Wang X, Costanzo M, Brost RL, Boone C, Anderson JT. Rex1p deficiency leads to accumulation of precursor initiator tRNA^{Met} and polyadenylation of substrate RNAs in *Saccharomyces cerevisiae*. *Nucleic Acids Res.* 2009; 37(1):298–308. [PubMed: 19042972]
- Vanacova S, Wolf J, Martin G, Blank D, Dettwiler S, Friedlein A, Keller W. A new yeast poly(A) polymerase complex involved in RNA quality control. *PLoS Biol.* 2005; 3(6):e189. [PubMed: 15828860]
- Watanabe K, Miyagawa R, Tomikawa C, Mizuno R, Takahashi A, Hori H, Ijiri K. Degradation of initiator tRNA^{Met} by Xrn1/2 via its accumulation in the nucleus of heat-treated HeLa cells. *Nucleic Acids Res.* 2013; 41(8):4671–4685. [PubMed: 23471000]
- Whipple JM, Lane EA, Chernyakov I, D'Silva S, Phizicky EM. The yeast rapid tRNA decay pathway primarily monitors the structural integrity of the acceptor and T-stems of mature tRNA. *Genes Dev.* 2011; 25(11):1173–1184. [PubMed: 21632824]
- Yarham JW, Elson JL, Blakely EL, McFarland R, Taylor RW. Mitochondrial tRNA mutations and disease. *Wiley Interdiscip Rev RNA.* 2010; 1(2):304–324. [PubMed: 21935892]

Figure 1.
Different approaches used to identify and analyze RTD substrates.

Figure 2. High throughput approach for identification of *SUP4_{oc}* RTD substrates
(A) Schematic representation of the sequence of *SUP4_{oc}*, a G34U mutant of a tRNA^{Tyr} gene. The anticodon residues are underlined. **(B)** General approach for identification of *SUP4_{oc}* RTD substrates using yeast libraries of *SUP4_{oc}* variants in WT and *met22Δ* RNA-ID reporter strains.

Figure 3. Methods for validating *SUP4_{oc}* RTD candidates identified through a high throughput approach

(A) Scatterplot of flow cytometry of the reconstructed *SUP4_{oc}-A29U* variant in WT (RTD on, median GFP/RFP = 0.045 $\pm$ 0.003) and *met22* cells (RTD off, median GFP/RFP = 0.47 $\pm$ 0.06) with the integrated RNA-ID reporter. (B) Schematic representation of the poison primer extension assay with ddCTP used for determining levels of *SUP4_{oc}* variants. (C) The poison primer extension assay measures the levels of *SUP4_{oc}* in bulk RNA. Bulk

RNA from the indicated strains was analyzed by primer extension in the presence of ddCTP. A sequencing ladder is on the left.

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Figure 4. Analysis of levels of the *SUP4_{oc}*-A29U variant by poison primer extension

(A) Comparison of poison primer extension assay using either bulk RNA (left panel) or purified tRNA^{Tyr} (right panel). P; primer (B) Comparison of poison primer extension assay using different primers. P; primer. Primer P5 is complementary to residues 57–37 of tRNA^{Tyr}, and primer P7 is complementary to residues 62–43 of tRNA^{Tyr}.

Figure 5. Effect of different variables on activity of suppressor tRNAs as measured by flow cytometry

(A) Flow cytometry analysis comparing suppression by the *SUP4_{oc}-A29U* variant at different temperatures. *SUP4_{oc}* has a median GFP/RFP of 1.09 ± 0.02 at 28°C and 0.50 ± 0.03 at 37°C, and the *SUP4_{oc}-A29U* variant has a median GFP/RFP of 0.045 ± 0.003 at 28°C and 0.012 ± 0.002 at 37°C. (B) Flow cytometry analysis comparing suppression by the *SUP4_{oc}-A29U* variant in different media. Wild type *SUP4_{oc}* has a median GFP/RFP of 0.74 ± 0.04 in S - His galactose medium and 1.07 ± 0.02 in YP galactose medium, and the *SUP4_{oc}-A29U* variant has a median GFP/RFP of 0.025 ± 0.001 in S - His galactose medium and 0.066 ± 0.01 in YP galactose medium. (C) Flow cytometry analysis comparing the effect of stop codon context on suppression by *SUP4_{oc}*. *SUP4_{oc}* has a median GFP/RFP of 1.04 ± 0.01 for a stop codon in a poor context, 0.36 ± 0.01 for a stop codon in a good context, and 0.032 ± 0.002 for the stop codon when inserted at amino acid 66 of GFP. (D) Flow cytometry analysis demonstrating that *SUP61_{oc}* suppresses *GFP_{oc}*. *SUP61_{oc}* has a median GFP/RFP of 0.22 ± 0.002 compared to 0.007 in the absence of a suppressor tRNA.