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In vivo cross-linking and co-immunoprecipitation procedure to analyze nuclear tRNA export complexes in yeast cells

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Abstract

tRNAs are small non-coding RNAs, that are predominantly known for their roles in protein synthesis, also participate in numerous other functions ranging from retroviral replication to apoptosis. In eukaryotic cells, all tRNAs move bidirectionally, shuttling between the nucleus and the cytoplasm. Bi-directional nuclear-cytoplasmic tRNA trafficking requires a complex set of conserved proteins. Here, we describe an *in vivo* biochemical methodology in *S. cerevisiae* to assess the ability of proteins implicated in tRNA nuclear export to form nuclear export complexes with tRNAs. This method employs tagged putative tRNA nuclear exporter proteins and co-immunoprecipitation of tRNA-exporter complexes using antibody-conjugated magnetic beads. Because the interaction between nuclear exporters and tRNAs may be transient, this methodology employs strategies to effectively trap tRNA-protein complexes *in vivo*. This pulldown method can be used to verify and characterize candidate proteins and their potential interactors implicated in tRNA nuclear-cytoplasmic trafficking.

Keywords

Co-immunoprecipitation; formaldehyde-cross-linking; Western blot; RT-PCR; RT-qPCR; primary tRNA nuclear export; tRNA retrograde nuclear import; tRNA nuclear re-export

1. Introduction

In eukaryotic cells, all tRNAs move bidirectionally between the nucleus and cytoplasm; these movements function in some of the steps to process primary tRNA transcripts into mature, functional tRNAs and also serve for tRNA quality control [1]. These bidirectional tRNA movements through the nuclear pores consist of three main steps (Fig. 1) (reviewed in [2]). (1) Newly transcribed and partially processed nuclear tRNAs are exported to the cytoplasm in a step called primary tRNA nuclear export. (2) Retrograde nuclear import constitutes the second tRNA trafficking step which relocates cytoplasmic tRNAs to the nucleus both constitutively and in response to environmental stresses. (3) tRNAs that were

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imported into the nucleus return to the cytoplasm by the tRNA nuclear re-export step. Each of the three steps of tRNA nuclear-cytoplasmic movements are evolutionarily conserved [3-5]. With recent discoveries of the roles of tRNAs in multiple human diseases [6], it is important to identify the gene products involved in tRNA biogenesis and subcellular traffic as well as to understand the regulation of these steps.

The best described exporter participating in primary tRNA nuclear export is the conserved β-importin family member Los1(Exp-t in vertebrates, Xpot in *S. pombe* and PAUSED in plants) [7-13]. Los1, like other β-importins, binds and exports cargo via a mechanism that is dependent on the GTPase Ran (Reviews [14-16]), [7, 13, 17-21]. However, Los1 and its orthologs are non-essential in all organisms tested, including haploid human cancer cell lines [10, 12, 22-25], indicating that there are parallel tRNA nuclear export pathways. Also, no ortholog of Los1 has been identified in *Drosophila sp.* and a recent study discovered that Los1-independent tRNA nuclear export pathways exist in *Trypanosomatids* [26, 27].

To identify tRNA nuclear exporters working in parallel to Los1, a genome-wide screen was conducted, and it identified three candidate proteins (Mex67, Mtr2, and Crm1) to be involved in the tRNA primary nuclear export step in yeast, *Saccharomyces cerevisiae* [28, 29]. The screen utilized northern hybridization to assess the cellular levels of individual end-processed, intron-containing pre-tRNAs upon individual deletion/mutation of nearly all yeast ORFs [28]. In budding and fission yeast, although pre-tRNA 5' and 3' end maturation occur in the nucleus (Review [30]), tRNA splicing takes place on the surface of mitochondria [31-33]; so, the accumulation of end-processed, intron-containing pre-tRNAs may indicate an inability of those pre-tRNAs to access mitochondrial splicing machinery due to faulty nuclear export. Employing RNA FISH, Mex67, Mtr2, and Crm1 temperature sensitive (ts) mutants were further shown to accumulate tRNAs in the nucleus when mutant cells were incubated at the non-permissive growth temperature [28, 29]. However, neither northern analyses nor RNA FISH can distinguish whether these proteins function directly or indirectly in tRNA nuclear export. To function as a *bona fide* tRNA nuclear exporter, the candidate tRNA nuclear exporter must interact with tRNA *in vivo* to form nuclear export complexes.

Therefore, we developed a methodology that allows for the isolation of tRNA-nuclear export complexes formed *in vivo* [34]. The method employs formaldehyde cross-linking and immunopurification of candidate tagged tRNA nuclear exporter proteins via magnetic beads conjugated with antibodies specific to the tags (Fig. 2). It is imperative to assure that tagging does not affect the function of tRNA nuclear exporters. This can be accomplished by assessing the consequences of the tags on cellular viability, growth rate, and pre-tRNA splicing [29, 33]. The formaldehyde cross-linking and immunopurification assay can be performed either by over-expressing the tagged putative nuclear exporter protein from a multi-copy plasmid [20, 29] or by expressing the protein from its endogenous chromosomal locus (Chatterjee *et al.*, 2022, in revision).

We prefer to perform the formaldehyde cross-linking and immunopurification assay utilizing endogenously expressed proteins for three reasons: i) over-expression of certain putative tRNA nuclear exporter proteins, like Crm1, is detrimental to cell growth (Chatterjee *et al.*,

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2022, in revision); ii) over-expression of tRNA nuclear exporters may incorrectly assess the efficiencies of nuclear exporter interactions with tRNAs. For example, we reported that the heterodimeric Mex67-Mtr2 complex substitutes for Los1 in yeast cells when over-expressed ~5-fold, but not when expressed at endogenous levels [29]; iii) over-expression of proteins may lead to cellular mis-localization [33].

To obtain tRNA-exporter complexes, which do not dissociate during isolation, two strategies have been adopted. The first strategy is to employ formaldehyde cross-linking. Formaldehyde treatment results in formation of covalent bonds between RNA and protein as well as between interacting proteins, thus stabilizing transient interactions between the tRNA and nuclear exporter proteins and their putative adaptors. This strategy is especially effective for maintaining tRNA binding to nuclear exporters like the Mex67-Mtr2 heterodimer, which is not a β-importin complex and does not employ the RanGTP-RanGDP nuclear_cytoplasmic gradient for tRNA nuclear export.

The second strategy combines formaldehyde crosslinking and inhibition of RanGTP hydrolysis to RanGDP (Fig. 2). Since Los1 and Crm1 are β-importins, they bind their RNA cargoes in the presence of GTP bound form of Ran. Dissociation of exportins from their cargos results from the hydrolysis of RanGTP to RanGDP. Therefore, inhibition of hydrolysis of RanGTP to RanGDP results in the nuclear exportins remaining associated with cargo. Thus, a non-hydrolysable RanGTP (Gsp1-G21V)-locked mutant construct is employed to maintain export complexes. In contrast, a predominantly GDP bound Ran will prevent exportin-cargo complex formation [35] and thus, a RanGDP (Gsp1-T24N)-locked mutant construct expressed in yeast cells serve as an internal negative control. Since expression of Ran-locked mutant proteins results in dominant lethality [36], Ran constructs encoding RanGTP-locked or RanGDP-locked mutants should be expressed from inducible promoters for a limited time. It is also important to verify whether the subcellular distributions of the exportins are maintained for the duration of Ran mutant protein induction, as prolonged expression of RanGTP-locked mutant has been shown to alter the subcellular distribution of β-importins tRNA nuclear exporters [34].

Cross-linked yeast cells expressing tagged exporter proteins and, when appropriate, the Ran-locked mutants are harvested and cryogenically ground using a planetary ball mill cell following the well-established protocol from the Rout lab (http://lab.rockefeller.edu/rout/pdf/protocols/Cryogenic_Disruption_of_Yeast_Cells_PM100.pdf) [37]. Manipulation of the yeast cell "grindates" at very low temperatures (ranging from −80 °C during storage to −196 °C when cooled in liquid nitrogen) prevents ribonucleoprotein particles (RNP) of interest from being degraded by proteases and/or nucleases. The resultant lysates containing the tagged exporter proteins crosslinked to RNA are then bound and subsequently immunopurified by anti-tag antibody coated magnetic beads (See Notes 1 and 2).

The co-immunoprecipitation fractions are divided into two pools - one for protein and the other for RNA analyses. The pull-down protein fractions are heated to reverse cross-linking and then the proteins are resolved on polyacrylamide gels for staining and western blot analyses. Protein staining assess enrichment of proteins with the anticipated molecular weight of tagged exporters, as shown in Fig. 3A . The identity of the enriched

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proteins observed by protein stain are verified by western blots using antibody against the tags (Fig. 3B). Moreover, for pull-down experiments using β-importins, successful co-immunoprecipitations should show substantial co-enrichment of Ran as determined by the employment of anti-Ran (Gsp1) antibodies (Fig. 3B). In contrast, the level of Ran co-purifying with tRNA exporters in GDP-locked form should be low.

The identity of the RNAs that co-purified with the tagged tRNA nuclear exporters are revealed by analyzing the RNA fractions using RT-PCR (Figs. 3 C-F). For those yeast tRNAs that are encoded by intron-containing tRNA genes, the primary tRNA nuclear exporter proteins must bind intron-containing tRNAs. To identify whether unspliced pre-tRNAs co-enrich with immunoprecipitated proteins, reverse transcription reactions (RT) are performed on RNAs obtained after reversing the formaldehyde covalent crosslinks; these reactions employ reverse primers complementary to the intron sequences of the tRNAs, followed by PCR reactions using the same reverse primer as used in RT reaction and a forward primer that corresponds to sequences in the 5’ exon (Fig. 3C). For studies of protein members of the β-importin family, the RT-PCR assay should demonstrate robust enrichment of intron-containing pre-tRNA with the tRNA nuclear exporter in the presence of RanGTP-locked mutant but not in cells expressing GDP-locked mutants (Fig. 3C). For conducting assays with tRNA nuclear exporters that are not members of the β-importin family such as the heterodimeric Mex67-Mtr2, a tagged, mutated form of the protein, *mex67-5* [38], previously shown to display tRNA nuclear export defects [29], can be employed as a negative control

Since in yeast, pre-tRNA splicing occurs on the cytoplasmic surface of mitochondria after the primary tRNA nuclear export process [31], a nuclear exporter will be implicated in tRNA re-export step if it is able to bind the spliced form of a tRNA encoded from an intron-containing tRNA gene (Fig.1, **Arrow #5**). Using a reverse primer complementary to sequences spanning the 5’ and 3’ exon junction and a forward primer corresponding to the 5’ exon (Fig. 3D), the RT-PCR can assess whether spliced tRNA co-immunoprecipitate with a tRNA nuclear exporter in the tRNA re-export step. Using this strategy, all the three nuclear exporters (Los1, Mex67-Mtr2 and Crm1) in yeast have been shown to form nuclear export complexes for both the tRNA primary and the tRNA re-export steps ([20, 29] and Chatterjee *et al.,* 2022, in revision) (Fig. 3C,D).

Various other controls are included in RT-PCR experiments to validate the co-enrichment of unspliced and spliced tRNAs with the various nuclear exporters. Since nuclear–cytoplasm shuttling proteins such as Los1, the Mex67-Mtr2 heterodimer, and Crm1 are not known to associate with the mitochondrial matrix *in vivo*, mitochondrial-encoded $tRNA^{Ile}{}_{GAU}$ has been employed as negative control to assess contaminating RNAs that may co-purify with the protein that has been enriched. To detect low levels of mitochondrial RNAs, RT-PCR reactions are amplified by increasing the number of PCR cycles or by increasing the reverse primer concentrations used in reverse transcription reactions. For β-importin such as Crm1, equivalent low levels of mitochondrial $tRNA^{Ile}{}_{GAU}$ in the pull-down fractions from cells with exportins and Ran in the GTP-locked form as well the GDP-locked form, document that there are equivalent by very low levels of contamination from the mitochondrial compartment in the co-IP enrichments (Fig. 3E). For non β-importins like Mex67-Mtr2,

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equivalent low levels of mitochondrial $tRNA^{Ile}_{GAU}$ should be observed in the pull-down fractions from cells with functional exportins as well as the non-functional mutant form of the protein, demonstrating equivalent background contamination in both the pull-down fractions.

Previously characterized RNA cargoes exported by individual tRNA exporters can be amplified from the pull-down total RNA fractions and thereby serve as positive controls. For example, *TLC1, a known non-coding* RNA, cargo for the Crm1 nuclear exporter [39], was found to bind to GFP-tagged Crm1 expressing Ran in the GTP-locked but not Ran in the GFP-locked form [39] (Fig. 3F).

Overall, the potential of the *in vivo* co-immunoprecipitation methodology to contribute to field of tRNA biology is extensive. For example, we demonstrated that individual tRNA nuclear exporters exhibit tRNA family preferences for nuclear export and that there is cooperation among the three nuclear exporters to maintain the cytoplasmic pool of tRNAs (Chatterjee *et al.*, 2022, in revision).

The formaldehyde cross-linking and immunopurification assay can also be utilized to assess whether potential candidate participate in the tRNA retrograde import. For example, co-IP studies of Mtr10 revealed that this putative nuclear importer may not be directly involved in the tRNA retrograde import step, despite a host of genetic and cytological data suggesting otherwise [20, 40, 41]. Overall, use of the *in vivo* cross-linking and coimmunoprecipitation method will not only lead to the discovery of novel tRNA-protein interactions, but it should lead to the identification and characterization of additional proteins involved in various aspects of tRNA biology.

2. Materials

2.1 Formaldehyde cross-linking and cryolysis of yeast cells

1. Yeast cells expressing tagged tRNA nuclear export proteins. Cells, expressing tagged tRNA nuclear exporters utilizing the Ran gradient for tRNA nuclear export such as Los1 and Crm1, are transformed with a single copy plasmid (pRS415, Leu^{+}) encoding either the RanGTP (Gsp1-G21V)-locked mutant or the RanGDP (Gsp1-T24N)-locked mutant protein, expressed from an inducible Gal promoter. Transformation of yeast cells with Ran-locked mutants is not necessary while performing co-immunoprecipitation assays with non-karyopherin tRNA nuclear exporters such as Mex67-Mtr2.

2. 1.35 L of synthetic defined media lacking appropriate selectable nutrients and containing 2% Raffinose (final concentration) as the carbon source. Dissolve 9.0 g of Yeast Nitrogen base without amino acids (DIFCO), 1.74 of amino acid premix without uracil (or 1.66 g of amino acid premix without uracil and leucine, where applicable) and 27 g of Raffinose (Thermo Fisher Scientific) in 900 ml double distilled water. Adjust volume to 1.35 L.

3. 20% galactose stock solution (only for cultures that require galactose-mediated induction of Ran mutants). Dissolve 100 g of galactose to 350 ml of autoclaved double distilled water with very gentle heating; adjust volume to 500 ml.

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4. 37% Formaldehyde stock solutions (Fisher BioReagents).

5. 2 M Glycine stock solution. Dissolve 75 g of glycine in 400 ml of autoclaved double distilled water; adjust the volume to 500 ml.

6. Resuspension buffer. 20mM HEPES, pH 7.5, and 1.2% polyvinylpyrrolidone (PVP). Dissolve 2.383 g of HEPES and 6.0 g PVP in 500 ml of autoclaved double distilled water. Adjust the pH to 7.5 using 1 N NaOH. Add the following solutions to the volume of buffer to be used.

- 1:100 dilution of PIC (Protease inhibitor cocktail IV, Millipore Sigma)
- 1:100 dilution of Solution P (Stock solution: 90 mg of PMSF + 500 µl of Pepstatin A dissolved in 4.5 ml of 100% Ethanol).
- 1:1000 dilution of 1 M DTT (Dithiothreitol), stored at −20°C.
- PIC, Solution P, and DTT should be added just before use.

2.2 Co-immunoprecipitation.

All buffers and solutions should be ice-cold before use, unless indicated otherwise.

1. 1 M HEPES, pH 7.4.

2. 1 M KOAc

3. 1 M $MgCl_2$

4. 5 M NaCl.

5. TritonX 100.

6. 10% Tween 20 solution. Dissolve 100 ml of Tween 20 solution in 800 ml of autoclaved double distilled water with gentle stirring. Adjust volume to 1 L.

7. 1 M DTT solution. Dissolve 1.54 g of DTT in 10 ml of autoclaved double distilled water. Filter sterilize. Store at −20°C as 1 ml aliquots.

8. 100 µg/µl Heparin Solution.

9. 40 mM of GTP stock solution: Add 40 µl of 100 mM GTP (stored at −20°C) to 60 µl of autoclaved double distilled water.

10. Extraction buffer: 20 mM HEPES, pH 7.4, 110 mM KOAc, 40µM $MgCl_2$,100 mM NaCl, 0.5% Triton, 0.1% Tween-20, 0.05 mM DTT, 0.24 µg/µl Heparin Sodium,1:1000 dilution of protease inhibitor cocktail set IV (EMD Millipore), 1:1000 dilution of Solution P (see 2.1), 1:1000 dilution of Antifoam A (prevents foam formation while dissolving aqueous systems containing protein) and 1:5000 dilution of RNase inhibitor (RNaseOUT, Invitrogen). This is made by combining 1 ml of 1 M HEPES, pH 7.4, 5.5 ml of 1 M KOAc, 1 ml of 5 M NaCl, and 2 µl of 1 M $MgCl_2$ in 25 ml of autoclaved double distilled water. Mix. Add 2.5 ml of 10% Triton X-100 solution, 500 µl of 10% Tween20 solution, 2.5 µl of 1 M DTT solution,

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120 μl of 100 μg/μl Heparin solution. Mix. Just before use, add 50 μl of Solution P, 50 μl of PIC, 50 μl of antifoam A and 10 μl of RNaseOUT ™ Recombinant RNase Inhibitor. Adjust volume to 50 ml (See Note 3).

11. Wash buffer A: 20 mM HEPES, pH 7.4, 110 mM KOAc, 40 μM $MgCl_2$, 0.5% Triton, 0.1% Tween-20, 0.05 mM DTT, 0.24 μg/μl of Heparin Sodium,1:1000 dilution of protease inhibitor cocktail set IV (EMD Millipore), 1: 1000 dilution of Solution P, 1: 1000 dilution of Antifoam A and 1:5000 dilution of RNase inhibitor (RNaseOUT). Mix 1 ml of 1M HEPES, pH 7.4, 5.5 ml of 1M KOAc, and 2 μl of 1M $MgCl_2$ in 25 ml of autoclaved double distilled water. Add 2.5 ml of 10% Triton X-100 solution, 500 μl of 10% Tween20 solution, 2.5 μl of 1 M DTT solution, 120 μl of 100 μg/μl Heparin solution. Mix. Just before use, add 50 μl of Solution P, 50 μl of PIC, 50 μl of antifoam and 10 μl of RNaseOUT ™ Recombinant RNase Inhibitor. Bring volume to 50 ml.

12. Wash Buffer B: 0.1 M NH_4OAc, 0.1 mM $MgCl_2$, 0.02% Tween-20. Add 100 μl of 1 M NH_4OAc and 1 μl of 1M $MgCl_2$ to 5 ml autoclaved double distilled water. Mix. Add 20 μl of 10% Tween 20, volume to 10 ml with autoclaved double distilled water.

13. Protein elution buffer: 0.5 M NH_4OH, 0.5 mM EDTA. Add 690 μl of 7.4 M NH_4OH and 10 μl of 0.5 M EDTA in 9.3 ml of autoclaved double distilled water to make a total volume of 10 ml working solution. Protein elution buffer should be made just before use.

14. RNA elution buffer: 50 mM Tris-HCl, pH 7.4,10mM EDTA, 1% SDS, 10 mM DTT. Add 1 ml of 10% SDS solution to 8 ml autoclaved double distilled water. Mix. Add 500 μl of 1M Tris-HCl, pH 7.4, 200 μl of 0.5 M EDTA, pH 8.0 solution, 100 μl of 1M DTT solution. Bring volume to 10 ml.

15. Proteinase K (20 mg/ml) (NEB)

16. Phenol saturated with Tris buffer (pH 4.4) (Fisher Scientific).

17. 100% ethanol (Store at 4°C).

18. Glycoblue precipitant 15 mg/ml (Ambion).

19. 70% ethanol (Store at 4°C)

20. 1.6 μm Whatman GD/X sterile glass microfiber syringe filter (Sigma Aldrich).

2.3. Gel Electrophoresis of proteins.

1. 4-12% NuPAGE Novex Bis-Tris precast gels (Life Technologies) or equivalent gel systems.

2. NuPAGE™ MOPS SDS Running Buffer stock solution (Life Technologies). Add 25 ml of stock solution to 475 ml double distilled water, mix. Store at 4°C. Add 1.25 ml of NuPAGE™ Antioxidant (Life Technologies) before electrophoresis.

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3. 4X LDS protein-loading buffer: Lithium dodecyl sulfate (LDS), pH 8.5, SERVA Blue G250 and phenol red (Invitrogen).

4. 1 M DTT solution (Dissolve 1.54 g of DTT in 10 ml of autoclaved double distilled water. Filter sterilize. Store at −20°C as 1 ml aliquots).

5. Novex™ Sharp Unstained Protein Standard (Invitrogen) or equivalent for protein stain.

6. Chameleon® Duo Pre-stained Protein Ladder (LI-COR Biosciences) or equivalent for Western blot.

2.4. Protein Staining.

1. SYPRO® RUBY Protein Gel Stain (Molecular Probes).

2. Fix solution: 50% methanol, 7% acetic acid. Add 500 ml of reagent grade methanol and 70 ml of reagent grade acetic acid to 430 ml double distilled water, for a final volume of 1 L. Mix.

3. Wash solution: 10% methanol, 7% acetic acid. Mix 100 ml of reagent grade methanol and 70 ml of reagent grade acetic acid to 830 ml with double distilled water for a. final volume of 1 L. Mix.

2.5 Western blot

1. PVDF membrane (BIORAD).

2. Western blot transfer buffer: 0.025 M Tris–HCl, 0.192 M glycine, 20 % methanol. Mix 6.04 g of Tris, 37.6 g of glycine and 400 ml of reagent grade methanol with 1.5 L double distilled water, after mixing, bring total volume to 2L with double distilled water.

3. Tris-buffered saline (TBS; 1X): 120 mM NaCl, 20 mM Tris–HCl, pH 7.4. Add 20 ml of 1M Tris-HCl, pH 7.4 stock solution and 24 ml of 5M NaCl stock solution and bring up volume to 1 L with double distilled water.

4. TBS containing 0.1% Tween-20 (TBST). Use 995 ml of 1X TBS from recipe above. Add 5 ml of 20% Tween20 solution for a total volume of 1 L.

5. Blocking solution: 5 % milk in TBST. Dissolve 5 g of non-fat instant dry milk in 100 ml of TBST buffer. Store at 4 °C.

6. Anti-GFP/Protein A antibody (Mouse) (AbCam)

7. Anti-Ran primary antibody (AbCam).

8. IR dye tagged anti-IgG secondary antibodies (LI-COR Biosciences) (or Horse radish peroxidase conjugated secondary antibodies, if employing Chemiluminescence (AbCam)).

9. Filter paper (Cytiva).

10. Horse-radish Peroxidase (HRP) substrate (Pierce).

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2.6 RT-PCR and RT-qPCR

1. TURBO DNA-*free*™ Kit (Invitrogen) containing DNase enzyme, buffer and DNase inactivation agent to terminate DNase activity.

2. Phenol, saturated with TE Buffer (pH 4.4) (Fisher Scientific).

3. 100% ethanol

4. GlycoBlue precipitant (15mg/ml) (Thermo Fisher Scientific).

5. 4X Superscript III Reverse Transcriptase First strand buffer (Invitrogen).

6. 100 mM DTT solution (Invitrogen).

7. Superscript III Reverse Transcriptase Enzyme (Invitrogen).

8. RNaseOUT ™ Recombinant RNase Inhibitor (Invitrogen).

9. 5X GoTaq Flexi Polymerase Buffer (Promega)

10. 25 mM $MgCl_2$ (Promega)

11. 10 mM dNTP Mix.

12. 5X GoTaq Flexi Polymerase enzyme (Promega).

13. PowerUp SYBR Green Master Mix.

14. Oligonucleotide primers for tRNA analyses

In choosing primers for tRNA analyses the following are considered:

a. To detect unspliced tRNAs, reverse primers are designed to hybridize to tRNA introns and forward primers bind to the 5' exons.

b. To detect spliced tRNAs, the reverse primers are designed to hybridize to sequences spanning tRNA splice junction and forward primers bind to the 5' exons.

c. The primers shall be at least 18 bases in length, with a preferable GC content between 40 and 60% and less than 10 degrees C difference in melting temperature between the primer pairs.

d. Regions in tRNA sequences that have modified nucleotides such as $m^2{}_2G$ can inhibit or slow the progress of Reverse Transcriptase enzyme [1, 42]. To avoid such problems, the reverse probe sequence can include this region so that the modified nucleotide is "covered" or the position of the probe should be complementary to positions such that RT extension does not encounter such modified nucleotides.

For example, we provide the primer sequences employed for RT-PCR and RT-qPCR reactions for unspliced and spliced $tRNA^{Ile}_{UAU}$, mitochondrial $tRNA^{Ile}_{GAU}$ and *TLC1* RNA (Table 1).

2.7 Agarose Gel Electrophoresis

1. 10X TBE buffer. Dissolve 108 g Tris and 55 g Boric acid in 900 ml distilled water. Add 40 ml 0.5 M Na_2EDTA, pH 8.0. Bring volume to 1 L.

2. 2 % Agarose Gel. 2.0 g Agarose is dissolved in 10 ml of 10X TBE buffer and 90 ml of double distilled water.

3. 25 bp ladder (Promega).

3. Methods

3.1 *In vivo* cross-linking

1. Inoculate from glycerol stocks (stored at −70°C) of yeast cells expressing tagged tRNA nuclear exporters (and RanGTP/GDP-locked mutants, where applicable) in 10 ml of appropriate selection media containing 2% raffinose as the carbon source. Incubate for a day in a shaker incubator at 30°C until 0.4-0.6 OD_{600} (See Note 4).

2. Inoculate ~7.5 x 10^7 cells to 1.35 L of appropriate selective media containing 2% raffinose and incubated at 30°C until 0.4-0.6 OD_{600}. This takes approximately 16 to 18 hr.

3. Add 150 ml of 20% galactose solution to the cultures (final volume 1.5 L, 2% final concentration) to induce expressions of the RanGTP-locked or RanGDP-locked proteins for 1hr at 30°C. This step is only applicable for cells expressing tRNA exporters that are β-importins like Los1 and Crm1 and RanGTP/GDP-locked mutants.

4. Add 12.1 ml of 37% Formaldehyde stock solution (0.3 % final concentration) into the cultures to induce chemical cross-linking of RNA and protein.

5. After 30 minutes (min), quench extra formaldehyde by adding of 49.5 ml of 2 M glycine solution to a final concentration of 66 mM. Incubate for 10 min.

6. Harvest cells and generate cell pellet noodles using the protocol from the Rout lab (See Note. 5). The cells can be stored at −80°C at this point for a few months.

7. Cryogenically lyse the frozen cells using a planetary ball mill following the protocol from Rout lab (See note 6). The powdered cells can be stored at −80°C.

3.2 Immunoprecipitation of tRNA-tagged export complexes

1. Suspend 0.5 mg of frozen, ground cells in 4.5 ml of extraction buffer.

2. Add 1.25 μl of 40 mM of GTP to the extracts from tagged export proteins and from untagged WT cells that contain RanGTP-locked constructs.

3. Preclear lysates by centrifugation at 3000Xg at 4°C for 10 min.

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4. While the lysates are being centrifuged, equilibrate 30 µl of antibody-conjugated magnetic beads (per sample) twice with equivalent amount of binding buffer.

4. The soluble extract (supernatant) was further clarified by passing through a 1.6 µm GD/X sterile glass microfiber syringe filter.

5. Transfer 50 µl of the total lysate into fresh 1.5 ml micro-centrifuge tubes and flash freeze in liquid nitrogen. Store tubes at −80°C.

6. Incubate the remainder of the lysate (~ 4.4 ml) with the 30 µl equilibrated antibody-conjugated magnetic beads (from Step 4) at 4°C for 30 min on a nutator.

7. Collect beads with a magnet, wash six times with 1 ml of ice-cold Wash buffer A and once with 1 ml ice-cold Wash Buffer B. The beads are suspended into 1 ml Wash Buffer B and divided into two equal fractions of 500 µl.

8. 500 µl of one bead fraction is employed for protein extraction. Collect beads with a magnet.

9. To elute the proteins, incubate the beads with 1ml of freshly prepared protein elution buffer for 20 min at room temperature in 1.5 ml microcentrifuge tubes.

10. Transfer the eluant into a fresh 1.5 ml tightly capped microcentrifuge tube and flash freeze in liquid nitrogen. Before freezing, puncture a small hole on the cap of the tubes.

11. Lyophilize eluates overnight employing a Speed Vac.

12. Suspend the lyophilized pellets in 50 µl of autoclaved double distilled water, intermittently keeping the tubes on ice while suspending.

3.3 SYPRO® RUBY staining

1. Mix 5 µl of total lysate (frozen in Section 3.2, Step 5) with 5 µl of 4X LDS protein-loading buffer containing 50 mM DTT and 10 µl of autoclaved double distilled water. Heat at 95°C for 30 min to disassemble the putative complexes cross-linked by formaldehyde in a total volume of 20 µl.

2. For protein staining using SYPRO® RUBY (Molecular probes), mix 15 µl of immunoprecipitated protein samples (3.2 step 12) with 5 µl of 4X LDS protein-loading buffer containing freshly added 50 mM DTT and heat at 95°C for 30 min. Put samples on ice. Add 5 µl of Novex pre-stained protein ladder (or equivalent) in a separate well from wells with 20 µl co-immunoprecipitated protein samples and lysates.

3. Resolve the protein samples employing 4-12% NuPAGE Novex Bis-Tris precast gels or equivalent and 1X NuPAGE™ MOPS SDS Running Buffer containing antioxidant at 200 V for 45 m in a cold room (4°C).

4. For gels containing protein samples for SYPRO® RUBY staining, after electrophoresis, pry the plastic gel plates open. Cut off the wells of the gel and just below the dye front.

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5. Place the gel into a clean container with 100 ml of the Fix solution (See section 2.4) and agitate on an orbital shaker for 30 min. Repeat once with fresh Fix solution. Pour off the used Fix solution.

6. Add 60 mL of SYPRO® RUBY gel stain. Agitate on an orbital shaker overnight.

7. Transfer the gel to a clean container and rinse with 100 mL of the Wash solution (See Section 2.4) for 30 min. This washing step minimizes background staining irregularities and stain speckles on the gel.

8. Rinse gel in double distilled water a minimum of two times for 5 min to prevent possible corrosive damage to the imager before imaging.

9. Visualize gels using a 300 nm UV transilluminator (See note 7).

3.4 Western blot of immunoprecipitated proteins

1. Mix 5 μl of immunoprecipitated protein extracts (from 3.2, step 12) in 5 μl of 4X LDS protein-loading buffer containing freshly added 50 mM DTT and 10 μl of autoclaved double distilled water and heat at 95°C for 30 min. Put samples on ice.

2. Resolve the protein samples employing 4-12% NuPAGE Novex Bis-Tris precast gels or equivalent and 1X NuPAGE™ MOPS SDS Running Buffer containing antioxidant at 200 V for 45 min in a cold room (4°C).

3. After electrophoresis, pry the plastic gel plates open. Cut off the wells of the gel and just below the dye front. Remove any gel fragments that could interfere with the subsequent transfer step.

4. Cut a piece of PVDF membrane to the size of the gel. Cut a small piece off the top left corner of the membrane. Assemble the transfer cassette in a glass container containing 1x Transfer buffer. The order of assembly on the transfer cassette is as follows: one foam pad, 3 sheets of filter paper (cut approximately to the same size as the membrane), the membrane (with the cut corner oriented at the top left), the gel, 3 more sheets of filter paper and another foam pad to form a supported "gel sandwich". Seal the cassette. The cassette is placed vertically in a tank between platinum wire electrodes and the tank is filled with transfer buffer. Transfer electrophoretically at 0.3 A for 1hr at 4°C.

5. Following transfer of proteins to PVDF membrane, disassemble the cassette. Orient the membrane with the protein side facing up (the cut corner should be at the top left). It is very important that the blot doesn't dry at this point.

6. Place the membrane in a clean container and submerge membrane with 10 ml of blocking solution for 1h at room temperature on an orbital shaker with gentle mixing.

7. After 1h, pour off blocking solution and rinse membrane with 10 ml of 1X TBST for 5 min.

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8. Add 10 ml of 1X TBST solution containing 0.5% milk and manufacturer recommended dilutions of primary antibodies to the membrane. Incubate for 1h at room temperature on an orbital shaker with gentle mixing.

9. Pour off antibody solution and wash membrane three times with 1X TBST solution for 5 min each on an orbital shaker with gentle mixing at room temperature.

10. Incubate the membrane with 1X TBST solution containing 0.5% milk and manufacturer's recommended dilutions of InfraRed Dye tagged secondary antibodies (LICOR) or horse radish conjugated secondary antibodies (Abcam) for 1hr at room temperature on an orbital with gentle shaking.

11. Pour off antibody solution and wash membrane three times with 1X TBST solution for 5 min each on an orbital with gentle shaking at room temperature.

12. If using LI-COR ODYSSEY platform machine for imaging, rinse membrane twice with 1X TBS for 5 min each on an orbital with gentle shaking at room temperature as residual Triton X100 can damage the machine.

13. After step 12, the gel can be imaged immediately if using LI-COR ODYSSEY platform machine. If gel is imaged by chemiluminescence, the blot can be imaged after addition of HRP substrate, according to manufacturer's instruction.

3.4.1 RT-PCR analysis of co-immunoprecipitated RNA—1. The second bead fraction from Section 3.2, Step 7 is used to obtain co-immunoprecipitated RNA. Collect the magnetic beads suspended in wash buffer B at 4°C using a magnet and re-suspend the beads in 200 μl of RNA elution buffer, followed by 70°C incubation for 45 min.

2. Incubate samples with 1 μl of Proteinase K (20 mg/ml) for 30 min at 30°C.

3. Extract enriched RNAs using 200 μl of acid phenol pH 4.4. Briefly vortex, centrifuge at 7,840 X g, for 10 min at 4°C.

4. Carefully collect supernatant in a fresh 2 ml autoclaved microcentrifuge tube and precipitate RNA with 600 μl of ice-cold 100% EtOH and 0.5 μg of GlyoBlue co-precipitant, overnight at −80°C.

5. Collect RNA pellet by centrifuging at 17,860 x g, for 10 min at 4°C. A tiny blue pellet should be visible after centrifugation.

6. Carefully remove and discard supernatant without disturbing the pellet and wash the pellet with 600 μl of ice-cold 70% ethanol solution.

7. Centrifuge at 17,860 X g, for 10 min at 4°C.

8. Carefully remove and discard supernatant without disturbing the pellet.

9. Air-dry the tubes at room temperature for 10 min to remove residual ethanol.

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10. Add 43 µl of autoclaved double distilled water to the tubes and gently tap to dissolve the pellet.

11. Incubate the tubes at 55°C in a water bath for 15 min. Briefly spin to collect condensate. Put tubes on ice.

12. Add 5 µl of Turbo DNase buffer and 2 µl of Turbo DNase in each tube. Incubate tubes at 37°C for 30 min.

13. Add 5 µl of DNase inactivation reagent and incubate at room temperature for 5 min with intermittent tapping.

14. Centrifuge samples at 7840 X g for 90 seconds (sec).

15. Carefully transfer supernatant to a fresh tube.

16. Measure total RNA concentration using a NanoDrop microvolume spectrophotometer (Thermo Fisher Scientific) or equivalent machine (See Notes 8 and 9).

17. Prepare samples for reverse transcription reactions: In a 1.5 ml tight capped micro-centrifuge tube, add 10 ng of co-immunoprecipitated total RNA, 1 µl of 2 µM of reverse primer (specific to the tRNA specific sequences as described in Section 2.7), 1 µl of 10 mM dNTP solution and adjust volume to 13 µl with autoclaved double distilled water (See Note 9).

18. Heat tubes at 65°C for 5 min. Chill on ice for 5 min. Briefly centrifuge to collect condensate.

19. To each tube, add 4 µl of 5X First strand buffer, 1 µl of 0.1 M DTT, 1 µl RNaseOUT ™ Recombinant RNase Inhibitor and 1 µl of Superscript III reverse transcriptase to make a total volume of 20 µl. Mix gently. Incubate tubes at 42 °C for 1 hr.

20. After 1hr, briefly centrifuge the tubes to collect condensate.

21. Inactivate the reaction by incubating the tubes at 70°C for 15 min. Briefly centrifuge the tubes to collect condensate.

22. Prepare for PCR reaction: In a 0.2 ml PCR tube, mix 5 µl of GoTaq® DNA Polymerase buffer, 1 µl each of forward and reverse primers (specific to the tRNA species of interest, see Step 23, Fig. 3E), 3 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP, 1 µl of GoTaq® DNA Polymerase (5u/µl), 1 µl of cDNA and 12 µl of autoclaved double distilled water for a total volume of 25 µl.

23. Example primer sets and PCR conditions are as follows:

i) For unspliced pre-$tRNA^{Ile}_{UAU}$: 2 min at 95°C; 27 cycles of 30 sec at 95°C, 20 sec at 52°C, and 20 sec at 72°C; 30 sec at 72°C; 2 picomoles of primer sets IVY1 and IVY3.

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ii) For spliced $tRNA^{Ile}{}_{UAU}$, 2 min at 95°C; 25 cycles of 30 sec at 95°C, 20 sec at 52°C, and 20 sec at 55°C; 30 sec at 72°C; 2 picomoles of primer set IVY1 and IVY149.

iii) For mitochondria encoded $tRNA^{Ile}{}_{GUA}$, 2 min at 95°C; 35 cycles of 30 sec at 95°C, 20 sec at 43°C, and 20 sec at 72°C; 30 sec at 95°C; 10 picomoles of forward and reverse primers. For pull-down reactions involving GFP-tagged exporters, 2 picomoles of forward and reverse primers are used.

iv) For *TLC1* RNA, 2 min at 95°C; 30 cycles of 30 sec at 95°C, 20 sec at 57°C, and 20 sec at 72°C; 2 picomoles of forward and reverse primers.

24. After PCR, resolve 15 μl of PCR products on a 2% Agarose gel at 100V for 1.5hr. Apply 2.5 μl of 25 bp DNA step ladder in a separate well to serve as molecular size markers.

25. Image the resolved products by employing a UV-transilluminator.

3.4.2 RT-qPCR analysis of co-immunoprecipitated RNA—1. For RT-qPCR, in a 96 well-plate, 1 μl of 100-fold diluted cDNA (performed in triplicates and obtained in Step 21 of 3.4.1 after completion of reverse transcription reaction) was added to 5 μl of the PowerUp SYBR Green Master Mix and 0.4 μl each of the same forward and reverse primers as were employed for the RT-PCR reactions. Bring volume to 10 μl/well with 3.2 μl autoclaved double distilled water.

2. No-template controls are included for all primer sets by adding all components described in Step 22 except cDNA. No-RT controls are analyzed for each sample with each primer set.

3. Standard curves are prepared with 10-fold serial dilutions of gel-extracted, RT-PCR products as templates to determine the concentrations of co-immunoprecipitated RNAs (See Note 11).

4. The samples are analyzed by employing in Quant Studio 3 (Applied Biosystems) or equivalent machine. The RT-qPCR conditions were as follows: a) Hold Stage: 2 min 30 sec at 50°C, 20 sec at 95 °C; b) PCR Stage: 40 cycles of 1 sec at 95°C, 20 sec at 60°C c) Melt curve stage: 1 sec at 95 °C, 20 sec at 60°C, 1 sec at 95°C. A melt curve analysis verifies specific amplification of the target.

Notes:

1. Beads should be used within 3 months of conjugation.
2. Co-immunoprecipitation assays performed with exporters with different tags may have differential background contaminating RNAs and proteins.
3. Mix all the salt components with autoclaved double distilled water before adding detergent components. Adding Triton X100 and Tween 20 in concentrated solutions of KOAc and NaCl will lead to precipitation of detergent.
4. Yeast cells expressing tagged β-importins such as Los1 or Crm1 are co-transformed with a plasmid encoding galactose-regulated RanGTP or RanGDP-

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locked mutant constructs. It is important that the cultures are maintained < 0.8 OD_{600} to avoid stationary phase of growth in order to prevent expression of Gal-regulated Ran mutants upon depletion of favored carbon sources, like glucose obtained from the hydrolysis of trisaccharide raffinose.

5. Link to Rout lab protocols for harvesting and cryogenic disruption of yeast cells (https://lab.rockefeller.edu/rout/protocols). Briefly, yeast cells are collected by centrifugation and the pellets are passed through a syringe into a 50 ml tube with filled liquid Nitrogen to form noodles. These frozen yeast cells are lysed using a planetary ball-mill in stainless steel grinding jars, with stainless steel balls in presence of liquid nitrogen. Grinding is done in 8 cycles, each cycle is set in the following manner: 400 rpm for 3 min, followed by 1 min reverse rotation with no breaks between rotations. Between each cycle the jars are removed and cooled in liquid nitrogen. When 8 cycles are complete the lysed yeast powder is removed with a sterilized spatula, and stored in 50 ml tubes, pre-chilled in liquid nitrogen, at −80°C.

6. Sometimes during cryolysis, the powdered cells cake in the bottom the cryolysis jars. Using a sterilized spatula, break the caked materials to ensure optimal grinding of the yeast cells.

7. Gels stained with the SYPRO® RUBY protein gel stain can also be visualized using imaging systems equipped with lasers that emit at 450, 473, 488, or 532 nm.

8. To measure RNA by Nanodrop, 1 μL of autoclaved double distilled water is added on the sample reader and dried with a KimWipe. Then, following software instructions, 1 μL of water (blank) is loaded onto the sample reader to initialize the system. The computer's settings are changed to RNA and the blank button is clicked. The blank is wiped off using KimWipe. 1 μL of sample is subsequently loaded onto the sample reader and the measure button is clicked. After the read is complete, A260/A280 and A260/A230 ratios and the amount of RNA present in sample (in ng/μL) are recorded. The reader is wiped with clean KimWipe between subsequent sample reads.

9. Reverse primer specific to only one species of tRNA are added in each reverse transcriptase reaction. Although it is theoretically possible to add multiple primers to generate cDNA of multiple tRNA species simultaneously, we have not carried out multiplex reverse transcriptions.

10. For each sample, ~ 200 nanograms of co-immunoprecipitated RNA are obtained from the pull-down assays, which should be sufficient to carry out ~ 20 RT-PCR reactions.

11. To prepare standard curves for RT-qPCR assays, each tRNA species was reverse transcribed from co-immunoprecipitated RNA, followed by PCR using the primer sets as described above. PCR products were resolved on an agarose gel and extracted using QIAquick Gel Extraction Kit (Qiagen). Gel extracted PCR products were quantitated using Nanodrop Microvolume UV-Vis

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spectrophotometer (See Note 8). 10-fold serial dilutions of gel-extracted, RT-PCR products were used as templates in RT-qPCR reactions and standard curves were prepared. These standard curves were used to determine the concentrations of co-immunoprecipitated RNAs.

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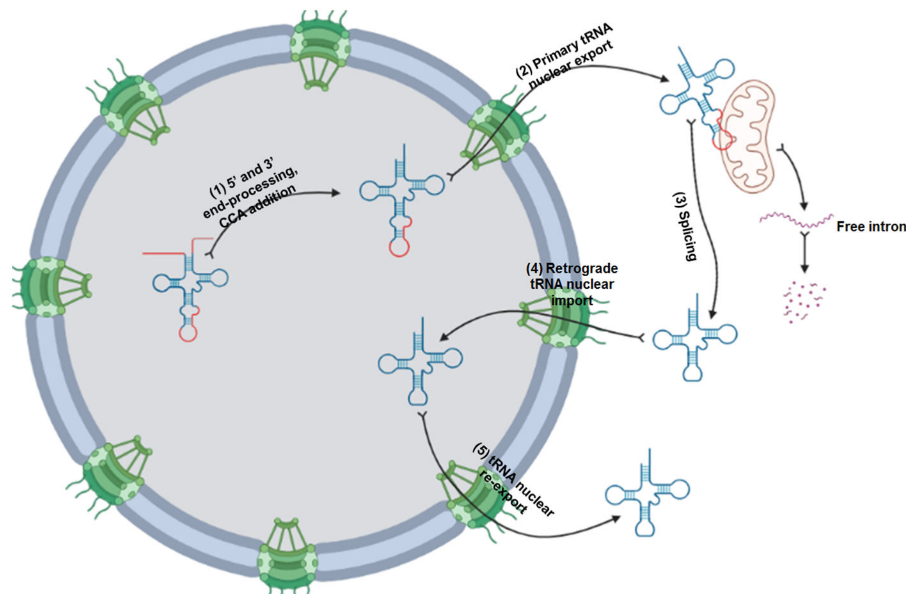

Fig.1. Bidirectional nuclear-cytoplasmic shuttling of tRNAs in yeast cells.

The diagram depicts tRNA nuclear-cytoplasmic movement for tRNAs encoded by intron-containing tRNA genes. 1. Precursor tRNAs are transcribed with 5' leader and 3' trailer sequences that are removed by RNase P and 3' end processing enzymes, respectively. 2. End-processed intron-containing pre-tRNAs are exported to the cytoplasm by at least three nuclear exporters: Los1, Mex67-Mtr2, and Crm1. 3.The introns are removed by the tRNA splicing endonuclease complex that is located on the mitochondrial surface; free introns are efficiently destroyed. 4. Cytoplasmic tRNAs are imported back to the nucleus via the tRNA retrograde nuclear import step. 5. tRNAs are returned to the cytoplasm by the tRNA nuclear re-export step. Blue lines indicate exons; red lines indicate the intron and the 5'-leader and 3'-trailer sequences.

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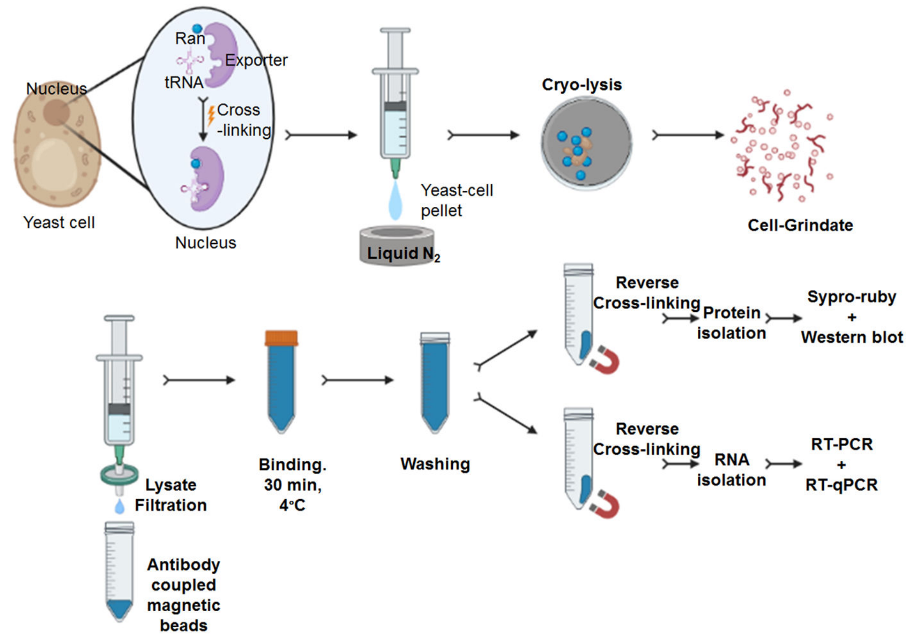

Fig.2. Strategy for the *in vivo* cross-linking co-immunoprecipitation process.

Cross-linked yeast cells co-expressing tagged putative tRNA nuclear exporters are harvested, lysed by cryo-milling and stored at −80 °C. The powdered cells are mixed with extraction buffer, centrifuged, and filtered. After incubation with anti-GFP antibody-conjugated magnetic beads, RNP complexes are isolated via a magnet, washed, uncrosslinked, eluted under denaturing conditions and protein and RNA components are analyzed.

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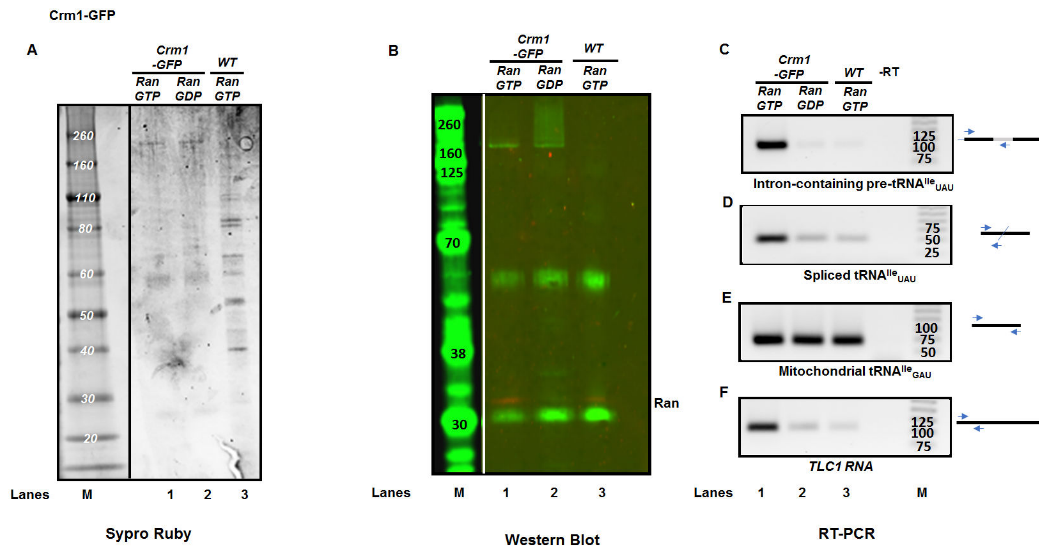

Fig. 3. *In vivo* co-immunoprecipitation assay to identify tRNA pulled down by tagged tRNA nuclear exporters.

(A) SYPRO® RUBY staining of proteins enriched by immunoprecipitation employing anti-tag antibody-conjugated magnetic beads. Lane M: Molecular size markers. Lane 1: yeast cells co-expressing GFP-tagged Crm1 and RanGTP (Gsp1-G21V) locked-mutant. Lane 2: yeast cells co-expressing GFP-tagged Crm1 and RanGDP (Gsp1-T26N) locked-mutant Lane 3: BY4741 yeast cells expressing RanGTP -locked mutant. (B) Western blot analyses to confirm the identities of the proteins enriched and stained in (A). Note: (A) and (B) are two separate gels. Lane M: Molecular size markers. Lane 1: yeast cells co-expressing GFP-tagged Crm1 and RanGTP (Gsp1-G21V) locked-mutant. Lane 2: yeast cells co-expressing GFP-tagged Crm1 and RanGDP (Gsp1-T26N) locked-mutant Lane 3: BY4741 yeast cells expressing RanGTP -locked mutant. (C-F) RT-PCR analyses of RNAs co-immunoprecipitated with RNA nuclear exporters: (C) intron-containing tRNA; (D) spliced tRNA; (E) mRNA; (F) mitochondrial tRNA; Lane 1: yeast cells co-expressing GFP-tagged Crm1 and RanGTP (Gsp1-G21V) locked-mutant. Lane 2: yeast cells co-expressing GFP-tagged Crm1 and RanGDP (Gsp1-T26N) locked-mutant. Lane 3: BY4741 yeast cells expressing RanGTP -locked mutant. Lane M: Molecular size markers. Diagrams of primers utilized for RT-PCR reactions are indicated next to panels (C-F). Black boxes: exons; gray box: intron sequences; slash: splice junction.

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Table 1

Primer sequences for RT-PCR and RT-qPCR reactions

Primer Name	Sequence
Ivy 1 Forward Primer for 5' exon of $tRNA^{Ile}_{UAU}$	5'-GCTCGTGTAGCTCAGTGGTTAG-3'
IVY3 Reverse primer for intron of $tRNA^{Ile}_{UAU}$	5'-CTTTAAAGGCCTGTTTGAAAG-3'
IVY149 Reverse primer for splice junction of $tRNA^{Ile}_{UAU}$	5'-ACGGTCGCGTTATAAGCACGA-3'
KC107 Forward primer for mitochondrial $tRNA^{Ile}_{GAU}$	5'-GAAACTATAATTCAATTGGTT-3'.
KC108 Reverse primer for mitochondrial $tRNA^{Ile}_{GAU}$	5'-TGGTGAAACTAACAGG-3'.
KC057 Forward primer for *TLC1* RNA	5'- AAGCCTACCATCACCACACC -3'
KC058 Reverse primer for *TLC1* RNA	5'- AAACAGCGAACTCGTGCAAA -3'