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Samika Joshi April 1, 2019

Understanding the Functional Consequences of RNA Exosome Disease Mutations, in a Budding Yeast Model System.

Functional Consequences of RNA Exosome Consequences of

by

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Abstract

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All cells in the human body possess the same genome, yet different cells in the body have different structure, composition, and function due to the cell-specific gene expression, which is achieved through transcriptional and post-transcriptional regulation. Ribonucleases, which are key regulators of post-transcriptional events, are conserved through evolution. One key ribonuclease machine is the RNA exosome, which is a multi-subunit 3'-5' riboexonuclease complex. This complex, which is responsible for the processing and/or degradation of many classes of RNA, is comprised of 10-subunits, termed *EXOSC* proteins in humans. This core complex interacts with a variety of cofactors that are thought to confer specificity for the many different RNA targets. Recently, mutations in the genes encoding multiple RNA exosome subunits have been linked to human diseases with surprisingly different phenotypes. For example, mutations in the *EXOSC2* gene cause a novel syndrome characterized by hearing loss, mild intellectual disability, and retinitis pigmentosa. In contrast, mutations in *EXOSC3* cause pontocerebellar hypoplasia type 1b affecting the pons and cerebellum. How mutations in genes that encode components of the same complex cause such distinct phenotypes is not known. A potential hypothesis is that the amino acid substitution within the RNA exosome result in specific functional consequences by impacting co-factor interactions, thereby disrupting proper targeting and processing/degradation of target RNAs. My studies focus on a patient mutation, G198D, on the EXOSC2 subunit. This residue corresponds to G226 in the budding yeast orthologue of EXOSC2, Rrp4.This mutation has shown to be be temperature sensitive at 37°C, and the phenotype has been used to interaction of the RNA exosome and co-factors. The research took an exploratory approach to identify new suppressors via a High Copy Suppressor Screen, as well as a more targeted approach of overexpression of known co-factors to restore the slow growth phenotype. A genetic interaction between Rrp4 and the TRAMP complex, and the targeted screen identified Trf4 as a potential suppressor. Deletion of the *TRF4* gene, showed growth defect while the *rrp4-G226D* cells do not show viability. The research provides insight about how amino acid changes in RNA exosome subunits alter function and cause disease.

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Understanding the Functional Consequences of RNA Exosome Disease Mutations, in a Budding Yeast Model System.

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April 1st, 2019

Introduction:

Though all cells in a given organism possess the same genetic information, different cells in the body have distinct structure, composition, and function. This diversity and versatility of the cells in the body is due to cell-specific gene expression (1). Key to this regulation of gene expression are post-transcriptional events that regulate RNA levels through processing and decay pathways. One critical post-transcriptional regulatory machine is the RNA exosome, which is an evolutionarily conserved 3'-5' ribonuclease complex. First identified in a budding yeast screen for ribosomal processing defects, the subunits in *S. cerevisiae* are referred to as Rrps (Ribosomal RNA Processing) (2). The RNA exosome is vital for proper ribosomal RNA processing and subsequent studies showed that this complex can affects nearly every class of RNA both in the nucleus and in the cytoplasm (3). As shown in **Figure 1**, the complex is comprised of 10 subunits; the RNA exosome complex forms a barrel like shape with a three-member cap and six-member core ring. The only catalytic subunit of the RNA exosome, DIS3, associates with the barrel and both degrades and processes RNA substrates that are threaded through the complex in a 3'-5' direction. Thus, the RNA exosome performs a duel role in mediating both precise processing of some RNAs and complete destruction of other RNAs.

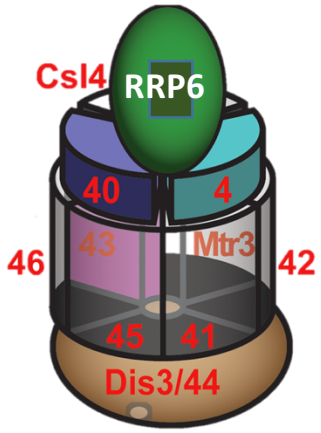

Figure 1: Schematic of the *S. cerevisiae* RNA exosome complex. The complex is composed of a nine-subunit core, a three-subunit cap at the top and a six-subunit ring in the middle. Dis3/44 and Rrp6 represent the catalytic ribonuclease subunits (Morton et. al. 2017).

The RNA exosome interacts with a variety of cofactors that are thought to confer specificity for the many different RNA targets of the RNA exosome in both the nucleus and cytoplasm (4) (2). Recent research has defined RNA exosome co-factors as proteins, that aid in target recognition, unwinding, and degradation of RNA substrates (5). A co-factor that aids the catalytic

functions of the exosome is the TRAMP complex. This complex is composed of one of two non-conventional poly(A) polymerases Trf4/Trf5, one of two homologous RNA-binding proteins Air1/Air2, and the RNA helicase called as Mtr4 (6). Co-factors include RNA helicases Mtr4 and Ski2 that are suspected to play a role in unwinding the RNA substrates in the nucleus and cytoplasm, respectively (7) (8). Both helicases have orthologues in mammalian systems and interact within larger complexes (7). The helicase Mtr4 is a member of the TRAMP complex. The TRAMP complex is responsible for nuclear quality control of several types of RNA and is thought to aid in the targeting and degrading of nuclear RNA exosome targets (9) (10) (6) (11). Additional nuclear RNA exosome co-factors include the exoribonucleases Rrp6 and a required stabilizing partner Rrp47. Rrp6 and Rrp47 interact directly with the exosome and are important in the processing of complex RNA (12) (13). The interaction between the RNA exosome and Rrp6/Rr47 is mediated by a small protein, Mpp6, which provides an interface platform for direct interactions with the exosome cap as revealed in recent solved structures of the human RNA exosome (7). Many of these interactions are well studied and reveal the important role RNA exosome co-factor interactions play in determining the substrate specificity of both nuclear and cytoplasmic RNA exosome targets, and in providing additional catalytic functions to aid the RNA exosome *in vivo*.

All RNA exosome subunits are essential for the viability of all organisms where their function has been studied (2). This is not surprising give the critical role of this complex in producing many important cellular RNAs. Thus, it was surprising when recent studies identified mutations in the genes encoding RNA exosome subunits that are linked to human disease (14-16). In all identified cases, these RNA exosome mutations cause amino acid substitutions within evolutionarily conserved domains of the subunits (2). Mutations in *EXOSC2,* which encodes a cap subunit of the RNA exosome, are linked to a novel syndrome characterized by hearing loss, mild intellectual disability, and retinitis pigmentosa (16). In contrast, mutations in *EXOSC3,* which encodes a different cap subunit, cause pontocerebellar hypoplasia type 1b (PCH1b), an early onset neurodegenerative disease characterized by atrophy of the pons and cerebellum within the

brain (14). Meanwhile, mutations in *EXOSC8*, which encodes one of the structural core subunits, are linked to a different type of PCH (type 1c), distinct with further psychomotor deficits, cerebellar and corpus callosum hypoplasia, hypomyelination, and spinal muscular atrophy (SMA) (15). Finally, mutations in the EXOSC9 gene cause cerebellar atrophy with spinal motor neuronopathy (17). While a number of these mutations impact the function of the cerebellum, they have distinct pathology raising the question of how changes within different subunits of the same complex can have such different consequences.

Due to the vast differences in the phenotypes as well as the different tissues affected, a question fundamental to RNA exosome biology arises—how do mutations in genes that encode structural components of the ubiquitously expressed and conserved RNA exosome result in distinct human diseases? One potential model is that each amino acid substitution results in specific functional consequences. These amino acid substitutions could alter RNA processing or degradation of key targets. Additionally, each amino acid substitution may impact specific RNA exosome co-factor interactions, further impacting targeting of specific RNA substrates. In fact, a previous study demonstrated that an amino acid substitution in EXOSC3 that is linked to disease decreases binding to the Mpp6 cofactor (18). Therefore, I hypothesize that amino acid substitution within the RNA exosome result in specific functional consequences by impacting co-factor interactions, thereby disrupting proper targeting and processing/degradation of target RNAs.

Thus far, we can only speculate about the *in vivo* consequences of these disease-linked amino acid substitutions in RNA exosome subunits. However, the lab recently analyzed *in vivo* consequences of the disease-linked amino acid changes in the cap subunit EXOSC3 by modeling the amino acid changes found in patients in the *Saccharomyces cerevisiae* orthologue Rrp40 (19). The studies presented here employ similar techniques, modeling human disease-linked *EXOSC2* mutations in the *S. cerevisiae* orthologue Rrp4. Using the *S. cerevisiae* model system has many distinct advantages. As the RNA exosome is highly conserved both in structure and function across all eukaryotes (2), we can readily employ the genetic tools available through the

S. cerevisiae system to model human disease-associated mutations and analyze the functional consequences of these amino acid substitutions *in vivo.*

This study focuses on the novel syndrome associated with single amino acid changes in the EXOSC2 protein, which is termed Rrp4 in budding yeast. This syndrome affects a variety of different tissues and organs including the ear, the brain, and the eye. The syndrome seems to affect a large range of tissues, ultimately with different effects in each organ. Each tissue, whether it be brain, ear, or eye, is affected in a unique manner and is related to the tissue type. This indicates a level of specificity. For the RNA exosome, RNA target specificity is thought to be conferred by cofactors. This investigation focuses on a patient mutation that causes a glycine to aspartic acid (G198D) change in the EXOSC2 subunit (**Figure 2**). This residue corresponds to G226 in the budding yeast orthologue of EXOSC2, Rrp4. Previous research shows that when the *RRP4* gene has been edited to encode the G226D amino acid substitution, the cells show temperature sensitive growth at 37°C (Fasken et al, unpublished results). This temperature sensitive growth phenotype can be exploited to determine and understand the effect of the interactions of the RNA exosome and various co-factors.

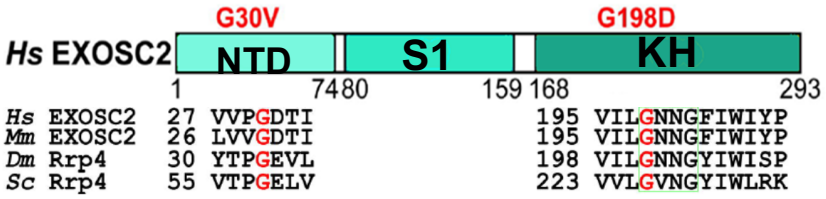

Figure 2: Amino acids altered in disease are conserved from human to yeast. The domain structure of EXOSC2 is shown with the N-terminal (N), S1 RNAse, and KH domains indicated. Regions surrounding residues altered in disease (G30 and G198) are shown for human (*Hs*), mouse (*Mm*), fruit fly (*Dm*) and budding yeast (*Sc*) EXOSC2/Rrp4 with the amino acid change in disease G198D, which maps to G226D in budding yeast (Glycine to Aspartic acid) is the primary focus of the work presented here.

For this study, I exploited the temperature sensitive growth of *rrp4G226D* cells. If the G226D amino acid substitution alters interactions with RNA exosome cofactors, increasing the dose of specific cofactors may alleviate the growth defect. For these studies, I examined a series of known and candidate RNA exosome cofactors from yeast. I complemented this approach with an unbiased high copy suppressor screen. The results of this study provide support that amino acid substitutions in the RNA exosome can modulate interactions with RNA exosome cofactors.

Materials and Methods

Saccharomyces cerevisiae strains, plasmids, and chemicals

The chemicals for all experiments were obtained from Sigma-Aldrich or Fisher Scientific. The techniques and manipulations of the yeast and the DNA were performed according to the procedures described in Fasken *et. al.* 2017 (19). The Δ*rrp4* strain (yAV1104) was previously described (19). The *LEU2 CEN6 RRP4* plasmid was constructed via PCR of the *RRP4* from *S. cerevisiae* genomic DNA, and cloning into pRS415 plasmid (20). The *LEU2 CEN6 rrp4-G8A* (pAV1181) and *rrp4- G226D* (pAV1183) mutant plasmids were previously described (19) and were generated with QuikChange Site-Directed Mutagenesis Kit (Stratagene).

S. cerevisiae growth assays for Δrrp4 mutations

The *in-vivo* function of Δ*rrp4* mutants was tested by the use of Δ*rrp4* (rrp4 ACY2420) cells which had a covering plasmid containing *RRP4-URA3.* The cells were transformed with a plasmid with mutants – *rrp4-RRP4*, *rrp4-G58A*, and *rrp4-G226D* encoded in a *LEU2* plasmid. The cells were grown overnight at 30°C to saturation in Ura$^-$ Leu$^-$ Glucose minimal medium. The overnight cell cultures were normalized to OD_{600}= 1, and serially diluted in 10-fold dilutions, and spotted on Ura Leu$^-$ Glucose media plates, and 5-Fluoroorotic Acid Leu$^-$ (5-FOA Leu$^-$) Glucose media plates. Plates were grown at 25°C, 30°C, and 37°C. The plates were imaged after 2 days of growth.

S. cerevisiae growth assays for Δrrp4-G226D with over-expressed co-factors

The Δ*rrp4* cells were transformed with the plasmid containing Δ*rrp4-G226D.* The cells were then struck onto 5-FOA Leu$^-$ minimal media plates, to remove the *RRP4-URA3* maintenance plasmid. The cells were then grown in Leu$^-$ minimal liquid media in an overnight culture at 30°C and then transformed with plasmids encoding known RNA exosome cofactors and some candidate cofactors – Mpp6, Rrp47, Rrp6, Trf4, Trf5, Air1, Air2, Mtr4, Ski7, Nab3, Nrd1, and Sen1, all of which were on *URA3* plasmids. The transformed mutants were allowed to grow for 2 days at

30°C. These transformants were then grown in overnight cultures 30°C to saturation in Ura$^-$ Leu$^-$ Glucose minimal medium. The overnight cell cultures were normalized to OD_{600}= 1, and serially diluted in 10-fold dilutions, and spotted on Ura Leu$^-$ Glucose media plates.

High Copy Suppressor Screen

The yeast cells with *RRP4*-patient mutation were transformed with a *LEU2* 2 micron genomic library. Following transformation, the cells were grown and assessed for any suppression of the *rrp4G226D* temperature sensitive growth phenotype. As a control, a LEU-selectable marker plasmid with the wild-type *RRP4* gene was inserted to show rescued growth. The transformed cells were grown at 37°C, and any growth was re-struck on Ura$^-$ Leu$^-$ Glucose media plates. The cells were spotted to assess if there was any rescued growth of the Δ*rrp4-G226D* similar to *RRP4* growth. The yeast cells with rescued growth were prepped based on the plasmid rescue protocol (21). As a control, cells with a wild-type recue plasmid and cells with an empty vector plasmid (plasmid with no genes) were also subjected to the extraction procedure.

Generation of Δrrp4- ΔTrf4-deletion mutants

The yeast cells with the Δ*rrp4*-deletion were used as a base to add the Δ*trf4* deletion via homologous recombination. The *TRF4* gene was replaced by the *KAN* gene and was amplified by PCR and co-transformed with a marker that contained an overlapping homology and homology with target locus. The cells were grown on kanamycin media plates to select for the cells with the deletion. The cells were then transformed with Δ*rrp4*-mutants plasmids.

Results:

Functional consequences of rrp4 mutations

To test the functional consequences of amino acid substitutions that occur in patients in EXOSC2, I analyzed the corresponding amino acid changes in the budding yeast orthologue Rrp4 (See **Figure 2**). To perform this analysis, I used S. cerevisiae cells lacking the endogenous *RRP4* gene (*rrp4*Δ cells), which are maintained by a copy of RRP4 on a *URA3* plasmid. Cells were then transformed with a control plasmid encoding wildtype Rrp4, or rrp4-G226D or a variant at position 58 (rrp4-G58A), which models the G30V change found in patients. We employed the G58V variant rather than G58V as previous studies demonstrated that the G58V-rrp4 variant is not functional as the sole copy of Rrp4. The cells expressing the Rrp4 test plasmids as the sole copy of Rrp4 were then analyzed for any growth defects by a serial dilution and spotting assay. **Figure 3** shows that the yeast that express wildtype Rrp4 or any of the variants show similar growth at 25°C and 30°C. However, at 37°C the *rrp4*-G226D mutation shows a slow growth phenotype. In contrast, the *rrp4-G58A* cells show growth similar to the wild type cells. The negative control, Vector alone, cells (not shown) are not viable as Rrp4 is essential for yeast viability (22).

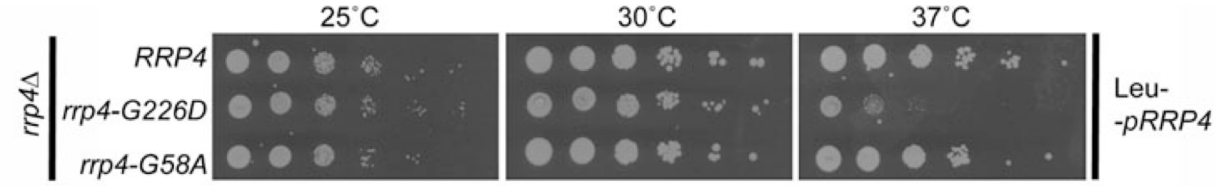

Figure 3: Functional consequences of amino acid substitution in Rrp4. Cells were deleted for the *RRP4* gene (*rrp4*Δ*)* and express wildtype *RRP4* or the mutant version of RRP4 indicated. The *rrp4-G226D* cells show a slow growth phenotype at 37°C, while the *rrp4-G58A* cells show growth similar the wildtype cells.

Overexpression of candidate RNA exosome cofactors to suppress the temperature sensitive growth of Δ*rrp4-G226D cells*

I next tested whether overexpression of previously identified RNA exosome cofactors (4) can suppress the growth defect observed for *rrp4-G226D* cells. For this analysis, I tested a series of RNA exosome cofactors on 2 micron overexpression plasmids that were transformed into the *rrp4-G226D* cells. Growth was then assessed by serial dilution and spotting as described in Material and Methods. As shown in **Figure 4**, overexpression of the co-factor *TRF4* suppressed the *rrp4-G226D* slow growth observed as assessed at one day of growth at 25°C. The overexpression of *RRP6* and *NAB3* also caused some increase in growth compared to the Vector control. These observations show that overexpression of some RNA exosome cofactors can suppress the growth defect caused by amino acid changes that model those found in human disease.

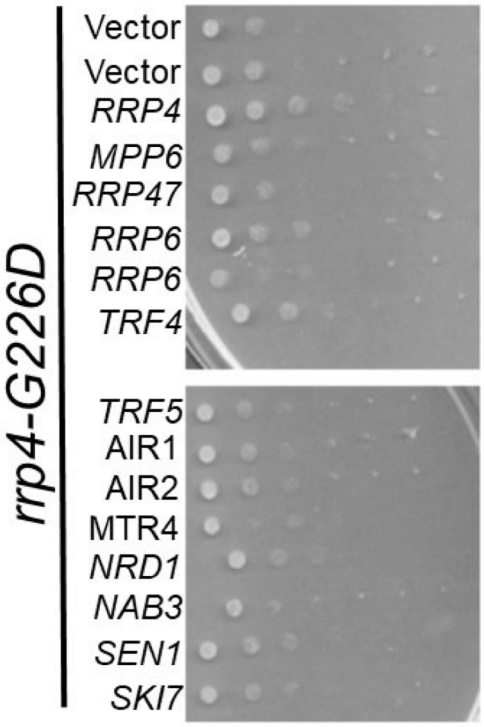

Figure 4: Overexpression of candidate RNA exosome cofactors. Overexpression of RNA exosome co-factors at 25°C, one day after spotting reveals some mild suppression of the *rrp4-G226D* growth defect with *NAB3, RRP6*, and *TR45*.

High Copy Suppressor Screen

As a complement to the candidate-based overexpression test, I also implemented an unbiased high copy suppressor screen to identify any novel interactors with the *rrp4-G226D* allele. For this set of experiments, I performed a pilot screen where I transformed the *rrp4-G226D* cells with a 2 micron genomic S. cerevisiae library and screened for colonies that could grow at 37°C. From an initial plating on ten plates, I identified several colonies that could grow at 37°C (**Figure 5**). To identify the gene encoded in the suppressor plasmid, I rescued the plasmid from the yeast cells and sequenced the insert. Thus, far, I have identified multiple copies of the wild-type RRP4. While this means that no new suppressors have been identified yet, the results do show that the screen

is working. Future experiments will screen more colonies and focus on those that show growth that does not appear identical to the *rrp4-G226D* cells transformed with the control *RRP4* plasmid.

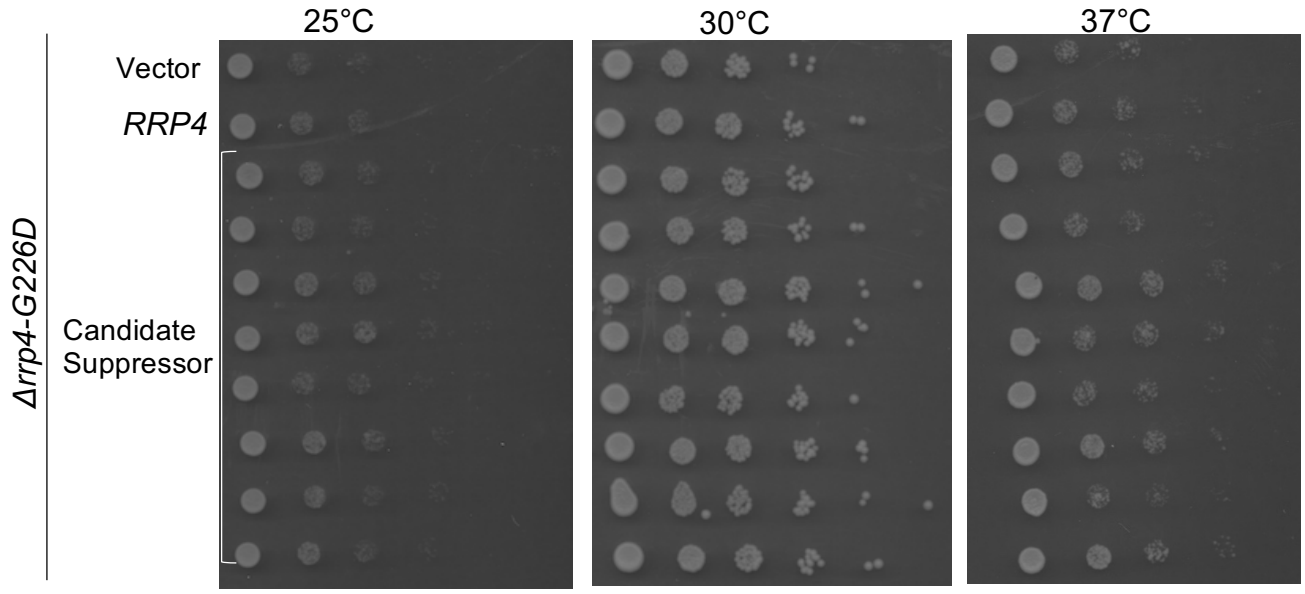

Figure 5: Screen for high copy suppressors of *rrp4-G226D* cells. The *rrp4-G226D* cells were transformed with an *S. cerevisiae* 2 micron genomic library. Some candidate suppressors improve cell growth at 37°C.

Genetic analysis of RNA exosome cofactors

To extend the analysis of RNA exosome cofactor function, I created cells where the *TRF4* gene is deleted. I selected the *TRF* gene family for this analysis because I observed growth suppression with overexpression of *TRF4* in *rrp4-G226D* cells (See **Figure 4**) and another lab member also

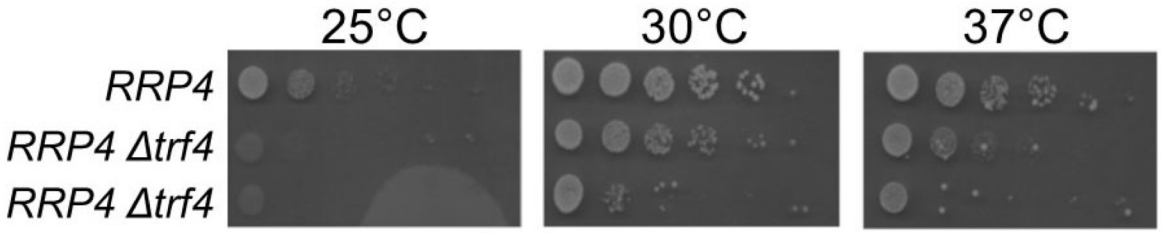

Figure 6: Analysis of RNA exosome cofactor function. Deletion of TRF4 causes a slow growth phenotype at 25°C compared to the wildtype control (*RRP4*) cells. Furthermore, the *trf4*Δ cells show a slightly reduced growth at all temperatures compared to control cells.

detected suppression with overexpression of *TRF4 (*Sterrett and Corbett, unpublished results). These cells were prepared to examine a genetic interaction between cells with the rrp4-G226D

amino acid change and loss of a cofactor. Deletion of the *TRF4* gene in cells which were already deleted for the *rrp4* gene (*Δrrp4 Δtrf4*) (but contain a covering plasmid *URA3-RRP4*), the cells, which are effectively deleted for *TRF4*, show severe cold-sensitivity (25°C) with modestly slowed growth at other temperatures tested (30°C and 37°C) (**Figure 6**) when compared to the wildtype *RRP4* control cells. To examine genetic interactions between RNA exosome amino acid changes and cofactors, the double mutant *Δrrp4Δtrf4* cells (maintained by a covering *URA3-RRP4* plasmid*)* were transformed with plasmids encoding Rrp4 variants (Rrp4-G58A, Rrp4-G226D, Rrp4-L136P). As controls, these cells were also transformed with an empty Vector and with a control wildtype *RRP4* plasmid. The transformed cells were streaked on plates containing 5-FOA to remove the covering *URA3-RRP4* plasmid (**Figure 7**). As expected, *Δrrp4Δtrf4* cells with Vector alone are not viable and cells with wildtype *RRP4* can grow. The cells that express Rrp4-G58V are able to grow, but several other alleles of RRP4 cannot support growth when the cells also lack Trf4. This is true for the *rrp4-G226D* mutant, which usually shows growth at the permissive temperature of 25°C. Future experiments will use the more quantitative serial dilution and spotting assay to further assess genetic interactions between RNA exosome mutations and various cofactor deletions and mutants.

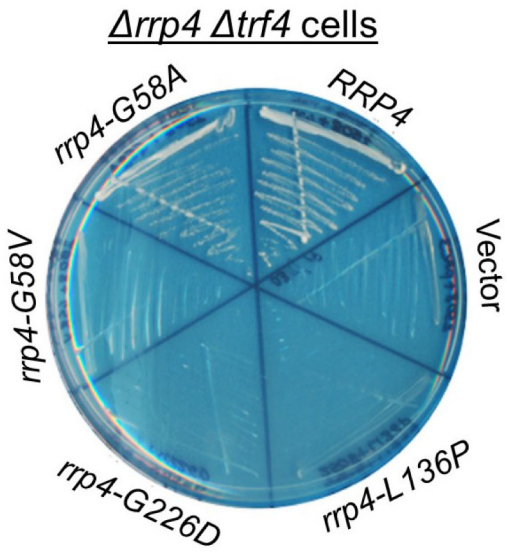

Figure 7. Genetic interactions between *rrp4* mutants and RNA exosome cofactors. Double mutant *Δrrp4Δtrf4* cells were transformed the indicated plasmids and streaked on a plate containing 5-FOA at 25°C, which is the permissive temperature for the *rrp4-G226D* allele. Only the *RRP4* control and *rrp4-G58A* are functional as the sole copy of Rrp4 in cells that lack Trf4.

Discussion:

The results of these studies demonstrate that the overexpression of certain RNA exosome co-factors can suppress the growth defect observed in *rrp4-G226D* mutant cells. Furthermore, I detected genetic interactions between specific RNA exosome mutants and RNA exosome cofactors. Taken together, these results are consistent with a model where the amino acid changes that occur in the RNA exosome in disease could impair interactions with RNA exosome cofactors. This altered interaction with cofactors could mean that specific RNA targets of the exosome are not properly processed or degraded. A major remaining question is why particular tissues are affected in patients. An even larger question is why mutations in different RNA exosome subunits cause such different disease phenotypes.

One possible answer could be due to the cell specific-gene expression in multicellular organisms. It is possible that due to differential gene-expression, different co-factors maybe expressed in different tissues. These could be responsible for the unique phenotypes caused by mutations, as and depending on how and where the co-factors interact with the exosome complex. As co-factors interact with the RNA exosome distinctly, based on the type of cell and type of co-factor expressed in the cells, different mutations would affect different tissues in unique manners.

While it is not possible to study the tissue-specific nature of the disease in budding yeast, the simplified model offered by the yeast allows us to conduct broad-based exploratory studies which may give us insight into the basic scaffold that might be present in more complex multicellular organisms. Any possible evidence gathered via exploratory studies such as these in *S.cerevisiae* can be adapted in higher order organism such as *Drosophilla*, which is an approach that is already underway in the Corbett Lab.

Though the screen performed to this date has not yielded potential suppressors, another potential approach would be the inclusion of the patient exosome mutation endogenously. This could be achieved by using CRISPR/Cas9 system to edit the yeast genome. This would allow for

closer examination of the effects of the mutation under the endogenous integrated G226D mutation in the RRP4 locus to better perform a genetic suppressor screen as it would also reduce any complications that could occur with transforming two plasmids into a yeast cell. At the Corbett lab we are currently in the process of creating these mutants with another known *RRP4* mutation with a stronger phenotype. If successful, the methodology can be readily used to create the rrp4-G226D which would indicate a more accurate representation of yeast phenotypes and thus a better analysis of the in vivo consequences of these disease-linked amino acid substitutions.

In conclusion, the approaches performed here in this study demonstrate a way to assess the consequences in co-factor interactions resulting from these disease-linked mutations in RNA exosome subunit genes. Through the use of a yeast system, we can easily use genetics and unbiased screens to assess which co-factor interactions are impacted by these RNA exosome amino acid substitutions. These conclusions can further be assayed in more complex systems—such as *Drosophila melanogaster* or human cell lines—which may shed light on the tissue specific disease pathologies observed in patients.

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