Exploring fungal-specific aspects of ribosome production: finding new ways to protect global food security and human health.

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- This project aimed to investigate how ribosomes are produced in fungal cells, particularly to try and identify fungal-specific aspects of ribosome production.
- Functional ribosomes are essential, as the ribosomes produce all the proteins needed for the cell to survive.
- Drugs that interfere with the ribosome production machinery can be used in order to kill cells. Therefore, investigating this process in fungal cells could provide insights into new targets for antifungal drugs.

Addressing Sustainability Goals

- **Fungal contamination is a significant contributor to food spoilage globally.**
  - It has been estimated that fungi are responsible for up to 20% loss of global crop yield, enough food to feed up to 600 million people annually. (1)
  - Antifungal drugs that specifically target fungal cells could reduce spoilage, without impacting the food itself.
  - This addresses UN sustainability goal 2, as it aims to prevent food wastage.

- **Pathogenic fungi present a globally significant healthcare issue.**
  - Per year over 150 million people contract severe fungal infections worldwide, resulting in approximately 1.7 million deaths. (2)
  - Finding new drug targets to address this issue is critical and addresses UN sustainability goal 3.

Background

The mature eukaryotic ribosome contains four ribosomal (r)RNAs and ≈80 proteins. During ribosome production three of the rRNAs required come from a single precursor transcript (pre-rRNA). The pre-rRNA precusor must be cut up correctly, and assembled with the ribosomal proteins. This produces the mature 18S rRNA of the 40S ribosomal subunit, and the 5.8S and 25S rRNAs of the 60S ribosomal subunit, see Figure 1 below:

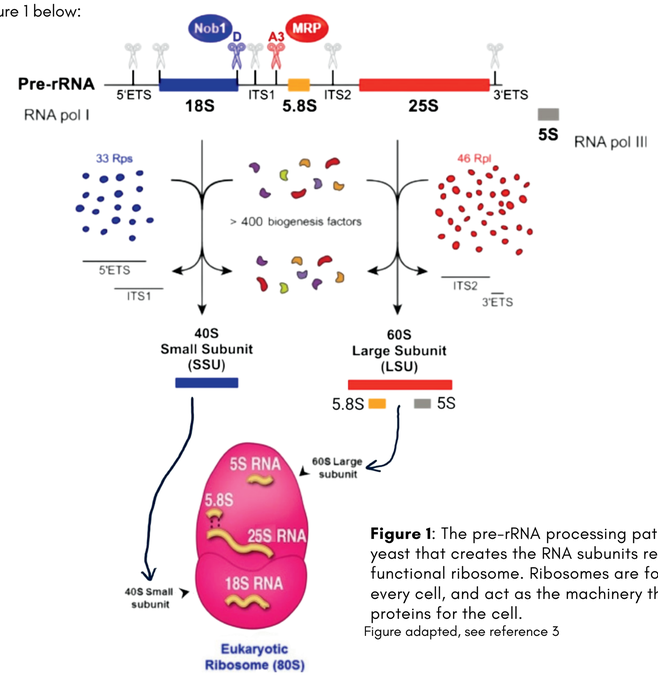


Figure 1: The pre-rRNA processing pathway in yeast that creates the RNA subunits required for functional ribosome. Ribosomes are found in every cell, and act as the machinery that produce proteins for the cell.
Figure adapted, see reference 3

Yeast cells differ to human cells in the way they remove the internal transcribed spacer (ITS1). ITS1 removal in yeast can be initiated by cleavage at two sites, one of which is called A3. The enzyme RNase MRP cleaves at the A3 site. The catalytic mechanism of MRP in both humans and fungi is not completely clear. Importantly, fungal MRP contains two essential proteins (Snm1 and Rmp1) which are not found in humans, which makes MRP a promising target for new fungicides.

Aims:

To explore fungal-specific aspects in ribosome production as new drug targets, using a "modification-interference" method in budding yeast to shed light onto the catalytic mechanism of RNase MRP.

Methodologies

In order to dissect the enzymatic mechanism of RNase MRP in pre-rRNA cleavage we made small alterations to the pre-rRNA transcript in the IST1 region - in and around where MRP cleaves:

- By applying the *in vivo* "modification-interference" method we can generate plasmids expressing small nucleolar RNAs (snoRNAs) designed to 2'-*O*-methylate nucleotides at or around the RNase MRP pre-rRNA cleavage site *in vivo*.
  - We decided to create 6 different plasmids to introduce to yeast strains. Each of the 6 plasmids conferred the addition of a methyl group either on or around the A3 cleavage site.
  - The 'A3' strain refers to where a methyl group was added at the A3 site. A3-1 refers to where a methyl group was added 1 nucleotide downstream, and A3+1 refers to 1 nucleotide upstream, and so on for all 6 strains, see Figure 2 below.

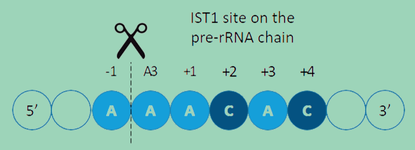


Figure 2: The nucleotide chain around the A3 cleavage site in the IST1 spacer, showing where methyl groups were added for each yeast strain, and where RNase MRP usually cleaves - see scissor line.

- RNA modification can be carried out by small nucleolar RNPs (snoRNPs), which refers to a small nucleolar RNA-protein complex.
- Using artificial box C/D small nucleolar RNPs (snoRNPs) we can block individual pre-rRNA cleavage events from occurring without depleting or mutating the endonuclease.
  - i.e.; without making any additional changes to the cell that may effect the results.
- snoRNPs can be targeted to a specific location by altering the guide sequence in its snoRNA component - which can be designed to guide modification at specific locations within the pre-rRNA.
  - We cloned in guide sequences that target the snoRNA to methylate at the aforementioned 'A3' sites in the pre-rRNA (Figure 2)
  - The pre-rRNA at the cleavage site therefore is directly methylated by the snoRNP.
- The production of the snoRNA is induced by switching the yeast strain from media containing glucose (it's preferred sugar, which represses snoRNA expression) to galactose which induces expression - see Figure 3 below.

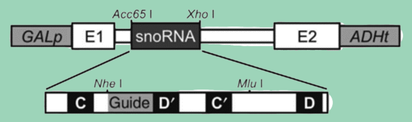


Figure 3: Diagram of the plasmid used in the artificial snoRNA system. GALp represents the Galactose inducible promoter, which the expression of the snoRNA is controlled by.

Transform snoRNA into Yeast

- We transformed the snoRNAs on a plasmid we generated into yeast to produce 6 strains, each capable of expressing a snoRNA that methylated at a specific site on or around the A3 cleavage site.
  - We compared each new strain to a strain transformed with a control plasmid; not expressing any snoRNA.
- We then performed drop testing and RNA analysis to test effect on survival, snoRNA expression and pre-rRNA processing in the yeast.
  - We also grew the yeast at different temperatures to assess if this had any effect on yeast survival.
- We then quantified the effect of different structural by using a robot to generate growth curves, and analysing the data produced by this.

Results

When we carried out the drop tests for all of our yeast strains it was clear that cell growth and survival was effected in the A3 and A3-1 strain.
  - This result made us want to ensure that all strains were expressing the snoRNA that we had transformed into them.

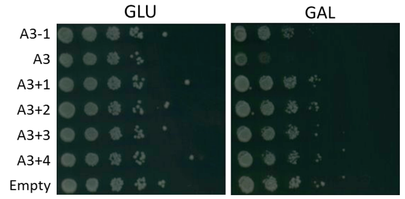
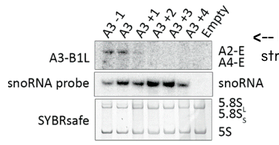


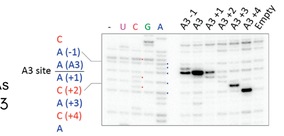
Figure 4: Drop tests comparing the survival of each yeast strain in glucose (GLU) - no snoRNA expression, with galactose (GAL) - snoRNA expressed and causing interference.

The reduced survival in the A3 and A3-1 strain can be seen. In this test yeast were grown at their optimum temperature (30°C).



<-- Figure 5: Northern blot testing if snoRNAs are expressed in all strains. All snoRNAs are being expressed - so we know they are present in all strains not just A3 and A3-1.

Figure 6: --> Primer extension analyses to ensure that the snoRNAs are targeting methylation at the correct sites on or surrounding A3 site.



From tests shown in Figures 5 and 6 we can conclude that in our yeast strains the snoRNAs are both present, and methylating in the right place.

Quantification and Effect of Temperature

We then used a robot to generate growth curves to quantify the effect of different snoRNAs variants.

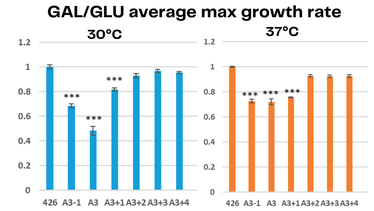


Figure 7: Bar charts show the max growth rate at both 30°C and 37°C. Most notably, this data showed that cellular survival and growth is significantly reduced in three strains: A3-1, A3 and A3+1.

The data produced by the same experiment at 37°C shows that an increase in temperature may 'rescue' the A3 strain; as growth rate is significantly increased.

p-value for graphs adjacent: p < 0.001=***
Error bars show standard error (SEM), statistical analysis was performed using an unpaired t-test

Final Conclusions

- When 2'-*O*-methyl groups are generated at nucleotides A3-1, A3 and A3+1 cellular survival and growth is reduced.
  - This suggests that methylation at these sites directly interferes with the catalytic activity of RNase MRP.
- Interestingly, defects are partially rescued by growth at 37°C compared to 30°C.
  - This suggests that methylation at these sites also impairs substrate recognition, which is facilitated at higher temperatures.
- In addition, methylation at the surrounding sites, which would also be predicted to interfere with RNase MRP pre-rRNA substrate binding, but not cleavage, had no significant effect.

Outcomes:

This project provided new insights into the catalytic mechanism of the essential pre-rRNA cleavage enzyme MRP. Understanding the process of ribosome production in fungi on a molecular level will be beneficial for future anti-fungal drug discovery.

References

1. Davies CR, Wohlgemuth F, Young T, Violet J, Dickinson M, Sanders J-W, et al. Evolving challenges and strategies for fungal control in the food supply chain. Fungal Biology Reviews. 2021;36:15-26.
2. Kainz K, Bauer MA, Madeo F, Carmona-Gutierrez D. Fungal infections in humans: the silent crisis. Microb Cell. 2020;7(6):143-5.
3. Kressler D, Hurt E, Baßler J. A Puzzle of Life: Crafting Ribosomal Subunits. Trends Biochem Sci. 2017;42(8):640-54.

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