UNIVERSITY OF CALIFORNIA SAN DIEGO

Investigating how Translation is Impacted, After Fermentative Growth, in *S. cerevisiae*

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Chemistry

by

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The Thesis of Gabriel Bracamontes is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California San Diego

2019

DEDICATION

I dedicate this to my nephews and future nieces, do not be afraid to trek through unfamiliar territory. Challenge yourselves. Develop tenacity. Become self-sufficient.

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LIST OF ABBREVIATIONS

Ado1	adenosine kinase 1
AMP	adenosine monophosphate
ATP	adenosine triphosphate
ATP1	adenosine triphosphate synthase protein 1
ATP2	adenosine triphosphate synthase protein 2
bp	base pair
C	celsius
Dcp2	mRNA decapping protein 2
Ded1	dead-box protein 1
DNA	deoxyribonucleic acid
eGFP	enhanced green fluorescent protein
eIF4F	eukaryotic initiation factor 4F
eIF4E	eukaryotic initiation factor 4E
eIF4GI	eukaryotic initiation factor 4GI
FITC	fluorescein isothiocyanate
His3	histidine 3
Hph	hygromycin B phosphotransferase
hr	hour
Hsp12	heat shock protein 12
kB	kilobase pair
Leu2	leucine 2

LLPS	liquid-liquid phase separation
mCherry	monomeric red fluorescent protein
min	minute
mRNA	messenger ribonucleic acid
mRNP	messenger ribonucleic protein
ms	millisecond
mL	milliliter
nanoLuc	nanoluciferase
nanoLucP	nanoluciferase pest
nm	nanometer
OD_{600}	optical density at 600 nm
RPM	rotations per minute
Pab1	poly(A) binding protein 1
PCR	polymerase chain reaction
PEG	polyethylene glycol
pmol	picomolar
prUTR	promoter-UTR
Pub1	polyuridylate binding protein 1
qPCR	qualitative PCR
RT	reverse transcriptase
RNA	ribonucleic acid
s	second

SAM	s-adenosyl-methionine
SC	synthetic complete media
TE	translation efficiency
TRITC	tetramethylrhodamine isothiocyanate
Ura3	uracil 3
UTR	untranslated regions
µL	microliter
WT	wild-type
w/v	weight per volume
YNB	yeast nitrogen base
YPD	yeast extract, peptone, dextrose media

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ACKNOWLEDGEMENTS

Thank you Dr. Brian Zid for supporting my research and mentoring me. Special thanks go to the Zid lab members for helping me with my research and making my tenure at UCSD enjoyable.

ABSTRACT OF THE THESIS

Investigating how Translation is Impacted, After Fermentative Growth, in *S. cerevisiae*

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Gabriel Bracamontes

Master of Science in Chemistry

University of California San Diego, 2019

Professor Brian Zid, Chair

When yeast cells endure environmental changes, they respond transcriptionally and translationally causing a fluctuation of gene expression dependent on the environmental changes. Understanding how yeast cells molecularly respond to environmental changes can give us insight into certain molecular mechanisms that contribute to cellular responses. We investigated how yeast cells molecularly respond during a 5-day growth period in which they will endure glucose

deprivation causing metabolization of ethanol that produces a diauxic shift resulting in respiratory growth, before the cells reach stationary phase. During the growth period, yeast develop stress granules during respiratory growth. The functions of stress granules are not known but are indicated to have a role in gene regulation during environment changes. Ded1 is an RNA helicase important for translation initiation that is a contributing factor to the assembly and disassembly of stress granules by co-localizing to them. A recently discovery found deleting *Ado1* completely disrupts co-localization of Ded1 to stress granules. Giving us the opportunity to use the disruption to understand how yeast cells endure the 5-day growth period but also attempt to discover a possible function for stress granules.

I. Introduction

A. *Saccharomyces cerevisiae* growth curve

The yeast *Saccharomyces cerevisiae* is a eukaryotic microorganism that utilizes fermentable carbohydrates, such as glucose, as an energy source. During fermentation, carbohydrates are metabolized for the production of ATP, the energy currency of cells. The fermentative growth produces ethanol and carbon dioxide as by-products (Fig. A) [1]. A traditional growth curve for microorganisms grown in optimal growth conditions would contain a lag phase, log phase and stationary phase (Fig. 1B). During lag phase, cells are adjusting to the environment, synthesizing biochemicals and growing in size. In log phase, cells are metabolizing metabolites and doubling exponentially at a constant rate. And stationary phase occurs when the growth rate plateaus due to the rate of cellular division equaling the rate of cellular death. Yeast have a second growth spurt that occurs after log phase during fermentation, known as respiratory phase. As glucose levels become depleted, a diauxic shift occurs because the yeast cells utilize the built-up ethanol as an energy source (Fig. A) [2]. A 120-hr growth curve, of yeast grown in YPD at 30˚C, captures fermentative growth, the diauxic shift, respiratory growth and stationary phase (Fig. 1C). Once ethanol is completely consumed, yeast cells enter stationary phase [3]. Not only does the switch from fermentative growth to respiratory growth cause a change in cellular metabolism but many genes undergo changes in expression [4].

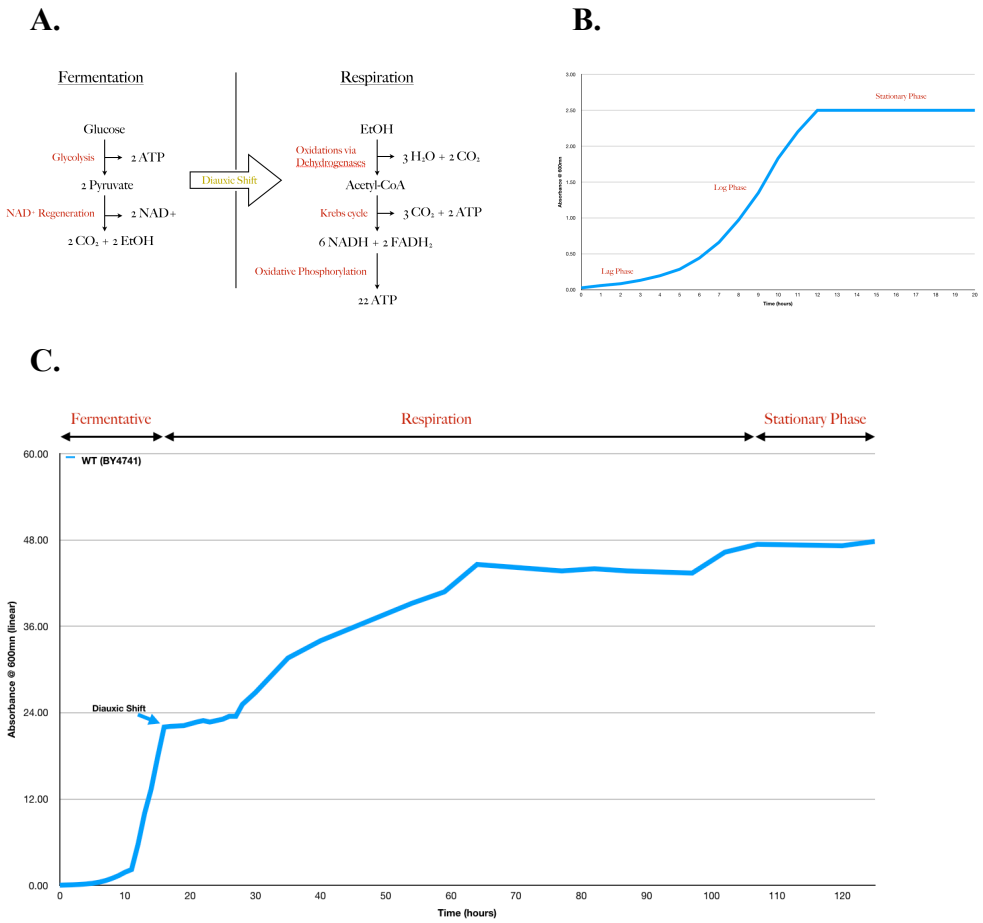

Figure 1. OD_{600} measurements of yeast, *BY4741*. A) Schematic of the metabolic pathways during fermentation and respiration B) A typical microorganism growth curve. Measurements were taken hourly using a spectrophotometer C) 120-hr yeast growth curve. Measurements were taken hourly for the first 24 hours and five hour intervals for the remaining 96 hours. Cultures were grown aerobically at 30˚C, measured and diluted with YPD.

B. Importance of Ded1 for translation initiation

Ded1 is a highly conserved DEAD-box protein, a family of proteins that are implicated in all processes involving RNA. DEAD-box proteins contain at least nine conserved motifs required for adenine recognition and ATP and RNA binding [5]. Most of the DEAD-box proteins are ATP-dependent and their roles contribute to disrupting RNA secondary structures and remodeling mRNP complexes [5, 6]. Functionally being a RNA helicase, Ded1 has also been shown to promote translation initiation by interacting with eIF4F complex [7]. The proposed mechanism is a two-step process, first forming a complex minimally containing eIF4F, Ded1 and RNA, followed by Ded1, in ATP dependent fashion, unraveling the 5'UTR and disassociating itself, allowing entry of mRNA into active translation (Fig. 2a) [7]. Another form of evidence demonstrating Ded1's importance for translation initiation suggests translation efficiencies decrease for a large set of genes when Ded1 is inactivated [8]. Further looking into those Ded1 sensitive genes, it was discovered that the 5' UTRs of those genes were about twice the length of the genome average and likely to form secondary structures [8].

A.

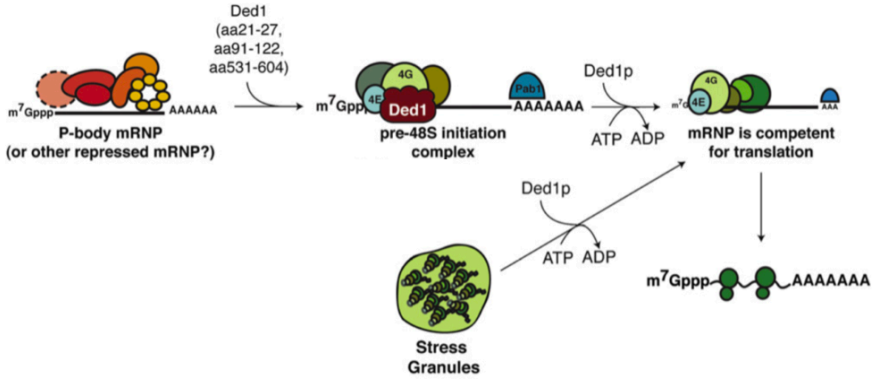

Figure 2. Proposed mechanism of Ded1's function. Ded1 acts as an RNA helicase that aids in the process of unraveling 5' UTR secondary structures and remodeling mRNPs in a ATP-dependent manner (Hilliker et. al. 2011).

C. Ded1 co-localizes with stress granule markers

Ded1 seems to be significant for translation initiation and translation efficiencies of genes with long 5' UTRs but it has also been shown to co-localize with several stress granule markers, during glucose deprivation and oxidative stress, suggesting it co-localizes to stress granules (Fig. 3A, B) [7]. Stress granules are a subset of stress-induced mRNP complexes that are mediated through a complex of networks of protein-protein interactions, protein-RNA interactions and RNA-RNA interactions (Fig. 3C) [9]. Nucleation of mRNPs causes the complexes to undergo LLPS due to the presence of multivalent scaffolding molecules with intrinsic disorder regions [10, 11]. mRNP complexes that phase separate form visible foci that can be visualized using microscopy. It is hypothesized that mRNPs influence gene expression during cellular stress response but further research needs to be conducted to understand their roles in gene regulation. Stress granules have been linked to neurodegenerative diseases and are implicated to having pathological implications [12]. Stress granules are composed of untranslated mRNAs, RNA binding proteins and translation factors. Ded1 is one of the translation factors responsible for stress granule formation [13]. Stress granules do have the ability to disassemble allowing the repressed mRNAs to be translated and Ded1 is believed to aid in the process of disassembly as well as their formation [7].

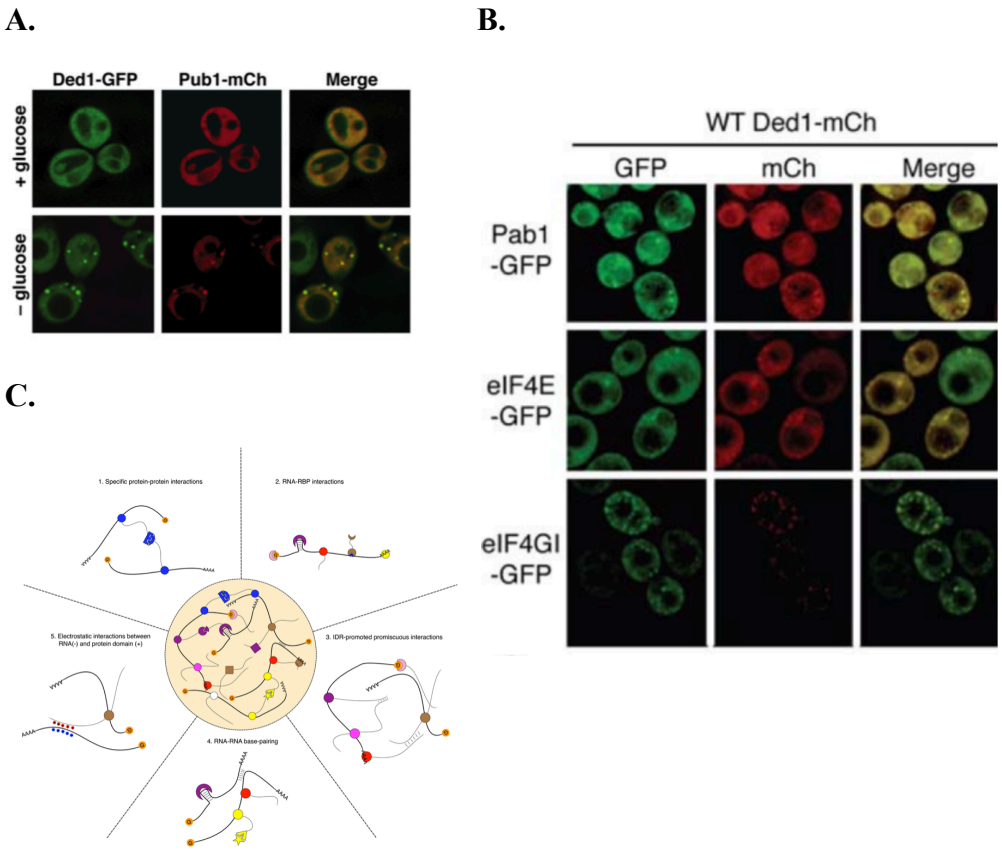

Figure 3. Ded1 is recruited to mRNPs. A) Ded1 co-localizes with Pub1, a stress granule marker, upon glucose deprivation (Hilliker et. al. 2011). B) Ded1 co-localizes with Pab1, eIF4E and eIF4GI, stress granule markers, upon sodium azide treatment (Hilliker et. al. 2011). C) Interactions that contribute to mRNP nucleation that lead to LLPS (Guzikowski et.al. 2019).

D. Ado1 gene deletion disrupts co-localization of Ded1 to stress granules

A recent discovery in yeast grown past their log phase growth, revealed Ded1 does not co-localize to stress granule markers when *Ado1* is deleted but co-localization is observed in the isogenic WT strain (Fig. 4A) [14]. Ado1 is an adenosine kinase that converts adenosine to AMP, a biomolecule that is a principal component in biological processes necessary for proper cellular functions such as gene expression, metabolism and enzyme regulation [15]. Ado1 is linked to the SAM cycle and coupled with the *de novo* purine biosynthesis pathway and purine salvage pathways it produces AMP [16]. It is unknown why this gene deletion hinders Ded1's co-localization to stress granules during respiratory and stationary phase, while other mRNP makers do not (Fig. 4A). The discovery proved to be conserved over a 120-hr growth period.

A.

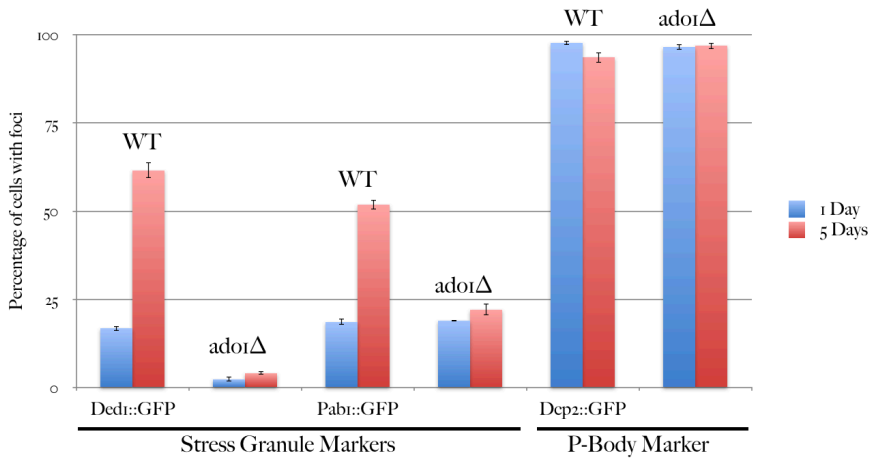

Figure 4. Ado1Δ disrupts Ded1 co-localization to stress granules. Yeast strains were grown for 120 hrs in YDP at 30˚C. Visualization of fluorescent foci were quantified using microscopy (Begovich et. al. unpublished)

E. Focus of study

Based on the current knowledge of Ded1 co-localization to stress granules during respiration after fermentative growth, we wanted to investigate how yeast cells would molecularly respond during the 5-day period in ado1Δ strain compared to WT. We were interested in genes that were Ded1 sensitive, meaning their translation efficiencies decreased when Ded1 was inactivated and also contained 5' UTRs longer than the genome average [8]. Additionally, gene expression needed to be unregulated during the 5-day growth period in the ado1Δ strain compared to the WT. Ribosome profiling of the WT and ado1Δ strains throughout the 120-hr growth assay suggests *Atp1* and *Atp2*'s mRNAs have slightly lower ribosome occupancy in the ado1Δ strain compared to WT, during log phase. But *Atp1* and *Atp2* have higher mRNAs and ribosome occupancy levels after log phase growth, specifically at Day 1 and Day 5 (Table 1). Both these genes met all three aforementioned specifications. Atp1 and Atp2 are the α- and β- subunits of the ATPase complex that generates ATP, the energy currency for most biological organisms.

We wanted to investigate if the endogenous proteins levels of Atp1 and Atp2 correlated with the ribosome profiling data. Analyzing the ribosome profiling data, I hypothesized that the protein levels of Atp1 and Atp2 would be higher in the WT strain during log phase but higher in the ado1Δ strain at Day 1 and Day 5 partly due to the availability of Ded1 in the cytosol since it is not co-localizing to stress granules, serving as a component for translation initiation and unraveling the 5' UTRs of both Atp1 and Atp2. I also re-introduced glucose to the cells after the 120-hr growth assay and observed Atp1 and Atp2 proteins levels. Presumably, after the cells respond to the re-addition of glucose, the proteins levels of Atp1 and Atp2 should be higher in

the WT strain than the ado1Δ strain because the stress granules would disassemble, because the stress, from glucose deprivation, hence immediately increasing the availability of Ded1 in the cytosol. Through the re-introduction of glucose and the subsequent increase of Ded1 in the cytosol, we can postulate that stress granules can function as a dock for important translation factors to harbor them until stress is alleviated or stress granules are disassembled.

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Table 1. Ribosome profiling data of WT and ado1Δ strains for *Atp1* and *Atp2*.

Gene	ΔTE *Ded1-cs*	5UTR	ΔG	RNA Levels Log Phase (Ado1Δ/WT)	Ribosome Occupancy	RNA Levels Day 1 (Ado1Δ/WT)	Ribosome Occupancy	RNA Levels Day 5 (Ado1Δ/WT)	Ribosome Occupancy
ATP1	**-1.4**	298	-35.77	-0.2	0.1	0.5	-0.0	1.3	**2.0**
ATP2	**-2.2**	193	-29.4	-0.3	-0.0	0.3	-0.1	1.6	**2.2**

II. Material and Methods

A. Tagging endogenous ATP1 and ATP2 with nanoLuc reporter

1. PCR amplification of nanoLuc cassettes

PCR amplification was accomplished by using Bio-Rad T100 thermal cycler and Phusion high-fidelity polymerase [17, 18]. The forward primers for the reaction were designed to have complementary overhangs with respect to either *Atp1* or *Atp2*'s 3' end, upstream of the stop codon. The overhangs for both forward primers were 43 bp long and the annealing sequence of the primers for amplification of the cassettes were 18 bp. On the other hand, the reverse primers were designed similar but the overhangs were complement to either *Atp1*'s or *Atp2*'s 3' end, downstream, of the stop codon. The long overhangs improve integration of the nanoLuc cassettes upstream of the stop codons of both *Atp1* and *Atp2*. The nanoLuc cassettes were amplified from a previously constructed plasmid harboring nanoLuc and a yeast selection marker, *His3*, for C-terminal tagging [19-21]. The predicted size of the cassettes were 2.5 kb, size confirmation of the amplified cassettes were verified using 1 % (w/v) agarose gel electrophoresis [22]. After confirmation, the PCR products were treated with Dpn1 for 1 hr at 37˚C and then purified using Zymo Research DNA Clean & Concentrator kit.

2. Yeast transformation of nanoLuc cassettes into BY4741

The nanoLuc cassettes for *Atp1* and *Atp2* was transformed into *BY4741* using LiAC/SS carrier and DNA/PEG method [23, 24]. The cassettes contained *His3*, a yeast selection marker, so the transformants were plated on bacteriological agar, containing YNB, 2% glucose and SC without histidine, and incubated at 30˚C.

3. Confirmation of proper integration via nanoLuc assay

Transformants were inoculated and grown overnight in liquid media, YNB, 2% glucose and SC without histidine, at 30˚C. The next morning, the OD_{600} was measured to ensure the cultures were still in log phase using Thermo Scientific Spectronic 200 Spectrophotometer. The cultures were diluted back to an OD_{600} of 0.1. When the cultures reached an OD_{600} of 0.3, using a white, flat bottom 96 well plate, an 90 µL aliquot from each culture was mixed with 9 µL of nanoLuc buffer and 1 µL of nanoLuc substrate. The samples were immediately read on a plate reader, Tecan Infinite 200Pro. The programmed method, for analyzing luminescence, subjected the plate to 5 s of orbital shaking at a frequency of 240 rpm and an integration time of 1000 ms.

B. Conjointly tagging endogenous ATP1 and ATP2 with eGFP and HSP12 with mCherry

1. PCR amplification of the DNA cassettes encoding the fluorescent reporters

Amplification of the DNA cassettes encoding the fluorescent reporters with overhangs complement to the 3' end of *Atp1*, *Atp2* or *Hsp12* was accomplished similar to the nanoLuc cassettes, stated above. The cassettes were amplified from a previously constructed plasmid harboring either yeast optimized *mCherry* or *eGFP* and a yeast selection marker, *Ura3* or *His3*, for C-terminal tagging [25, 26]. The predicted size of the cassettes were 2.5 kb, size confirmation of the amplified cassettes were verified, treated and purified following previously described methods.

2. Yeast transformation of two cassettes into BY4741

The DNA cassettes were transformed following a previously described method. First, the transformation of the cassette encoding *mCherry* with overhangs complement to the 3' end of *Hsp12* was completed. The mCherry cassette contained *Ura3, a* yeast selection marker, so the transformants were plated on bacteriological agar, containing YNB, 2% glucose and SC without uracil, and incubated at 30˚C. Upon confirmation of successful integration, a single colony from the transformants was isolated and grown in uracil drop-out liquid media at 30˚C for the transformation of the cassettes encoding *eGFP* with overhangs complement to the 3' end of either *Atp1* or *Atp2.* For the second transformation, the cassettes contained *His3*, a yeast selection marker, so the transformants were plated on bacteriological agar, containing YNB, 2% glucose and SC without uracil or histidine, and incubated at 30˚C.

3. Confirmation of proper integration via microscopy

The transformants with mCherry tagged HSP12 were grown following previously described methods but in uracil drop-out liquid media. Once the liquid cultures reached an OD_{600} of 0.3, for each culture, two 1 mL aliquots were made. One of the aliquots would be the positive control and the ladder would be glucose deprived. The glucose-deprived samples were pelleted at 12,000 rpm for 30 s with the supernatant being decanted and the pellets being washed with glucose drop-out media. Both positive control and glucose-deprived samples were incubated for 30 minutes at 30˚C. After, the cells were observed using Echo Revolve microscope, 60X objective and brightfield and TRITC channel [27]. The second set of transformants were grown to an OD_{600} of 0.3 and were observed under the microscope using 60X objective and brightfield and FITC channel, but instead they were grown in uracil and histidine drop-out liquid media [28].

C. Deletion of *Ado1*

1. PCR amplification of Hygromycin B resistance cassette for gene deletion

The primers used for amplification of the Hygromycin B resistance cassette for *Ado1* deletion contained 50 bp overhangs complement to the DNA sequences upstream and down stream of the gene encoding for Ado1 [29]. The cassette was amplified from a previously constructed plasmid containing the *Hph* gene. The predicted size of the cassette was 1.1 kb, size confirmation of the amplified cassette was verified, treated and purified following previously described methods.

2. Yeast transformation of Hygromycin B resistance cassette BY4741

The cassette was transformed following previously described method but the transformants were plated on YPD agar containing 100 µg/mL Hygromycin B and incubated at 30˚C [29].

3. Confirmation of proper integration via growth assay

Isolated transformants and a positive control were inoculated and grown overnight in liquid media, YPD, at 30˚C. The next morning, the OD_{600} was measured to ensure the cultures were still in log phase. The cultures were diluted back to an OD_{600} of 0.050. OD_{600} measurements were performed hourly until the cultures reached an OD_{600} of 1.0. Doubling time was then calculated.

D. Cloning of 5' UTR constructs with endogenous promoter and nanoLuc as the reporter

1. PCR amplification of inserts

The primers for the inserts, for the three DNA constructs, were designed with the intention of assembling the constructs via Gibson Assembly. Some of the primers had a 17 bp 5' overhang complement to the adjacent insert or linearized DNA vector. The endogenous promoter and 5' UTR of *Atp1* and *Atp2* were amplified from *BY4741* genomic DNA, 1000 bp upstream of the encoding gene. The reporter's amplicon included a destabilization domain, *PEST.* The predicted size of the endogenous prUTR fragment for both was 1.0 kb and nanoLucP is 700 bp, size confirmation of the amplified fragments were verified, treated and purified following previously described methods.

2. Gibson Assembly of constructs using pRS305

pRS305 was linearized using *SacII* and *NotI* restriction enzymes [30]. The linearized plasmid was purified using Zymo Research DNA Clean & Concentrator kit. Inserts and linearized plasmid were mixed at a ratio of 3:1 with respect to their DNA concentration and bp length, pmol/µL [31]. The volume of the reactions were 10 µL including NEB 2X Gibson Assembly Master Mix. They were incubated at 50˚C for 1 hr. 1 µL of each reactions was transformed into *E. coli*, DH5α. Recombinant plasmids were verified via colony PCR, restriction enzyme digest and DNA sequencing.

3. Yeast Transformation of recombinant plasmids into BY4741

The ligated plasmids were transformed following a previously described method. The transformants contained an integrated plasmid with *Leu2*, a yeast selection marker, so they were

plated on bacteriological agar, containing YNB, 2% glucose and SC without leucine, and incubated at 30˚C.

4. Confirmation of proper integration via nanoLuc assay

Confirmation of the three yeast strains generated, by integrating the three recombinant DNA plasmids, were verified following a previously described method.a

E. 5-Day growth assay

1. Log phase readings

The strains were either inoculated and grown in YP containing 2% glucose or YNB, 2% glucose or SC overnight at 30˚C. The next morning, the OD_{600} were measured to ensure the cultures were still in log phase. Then, the cultures were diluted back to an OD_{600} of 0.1 and allowed to grow to the desired OD_{600} of 0.3 for quantifying luminescence or 0.4 for quantifying fluorescence. Once the desired OD_{600} was reached, 1 mL aliquots were made of the cultures and, if needed, diluted, for standardization, to the exact desired OD_{600}. The samples were then added to, either a white or black, flat bottom 96-well plate for quantification of the reporters. Luminescences was recorded using a Tecan Infinite 200Pro. The programmed method subjected the plate to 5 s of orbital shaking at 240 rpm and an integration time of 1000 ms. Fluorescence was recorded using a Tecan Spark multimode plate reader. The programmed method exposed the samples to 485 nm light and recorded 530 nm emitted light followed by exposing the samples to 590 nm light and recording 640 nm emitted light. The excitation and emission bandwidth was 5.0 nm.

2. Day 1 readings

24 hrs after the log phase readings, the dilution factors for the all samples were quantified so the samples could be measured at an OD_{600} of 0.3 or 0.4. Then fresh aliquots of the samples were diluted and measured following previously mentioned methods.

3. Day 5 readings

96 hrs after the day 1 readings, dilution factors and samples were quantified following previously described methods. Strains containing the nanoLuc cassettes were re-introduced to glucose, the cultures were diluted with YPD to an OD_{600} of 0.3. Luciferase was measured at 0, 15, 45, 75 and 90 min, after re-addition of glucose.

Table 2. Sequences of the DNA oligonucleotides. The lower case sequences anneal to the template DNA for PCR amplification. The upper case sequences are the overhangs for proper integration or ligation of the DNA fragment for endogenously tagging genes or Gibson Assembly.

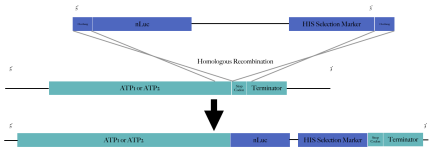
Purpose	Forward Primer	Reverse Primer
C-terminally tag *Atp1* with pKT linker	GCATCTCTAAAGAGTGCTACTGAATCATTTGTTGCCACTTTTggtgacggtgctggttta	AGACGTACCTTATATTCATTTTTATTTTTTTAGTTCACATTAtcgatgaattcgagctcg
C-terminally tag *Atp2* with pKT linker	GATGTTGTTGCTAAAGCTGAAAAGTTAGCCGCTGAAGCCAACggtgacggtgctggttta	TTTCAAATTTTGCTTCCCTTGGTTTAAGCTTTATTTCTTCTAtcgatgaattcgagctcg
C-terminally tag *Hsp12* with pKT linker	GTCGAATATGTTTCCGGTCGTGTCCACGGTGAAGAAGACCCAACCAAGAAGggtgacggtgctggttta	ACACATCATAAAGAAAAAACCATGTAACTACAAAGAGTTCCGAAAGATTTAtcgatgaattcgagctcg
Gene deletion of *Ado1* via Hygromyocin cassette	CTTGTGCAGGGTAGAACCAAAGATAACAGCAAAAGAAAGAGCAAGCAATTcggatcccgggttaattaaggcg	AGAGGGAACACGTAGAGTATATTTTATTTCTATTATATTGTAAGAAGAATgaattcgagctcgtttaaac
Atp1 5' prUTR for Gibson Assmebly into pRS305 (SacII & NotI)	AATTGGAGCTCCACCGCataggtctttaacacagcttc	TCTAAAGTAAAAACCATtattatatgtgcgtgttttttt
Atp2 5' prUTR for Gibson Assmebly into pRS305 (SacII & NotI)	AATTGGAGCTCCACCGCtgtctcttgggatgataatttttc	TCTAAAGTAAAAACCATtcttttttttatttttgttttctctc
nanoLucP for Gibson Assmebly into pRS305 (SacII & NotI)	atggttttactttagaagattttg	ATCCACTAGTTCTAGAGCttaaacattaatacgagcagaag

III. Results

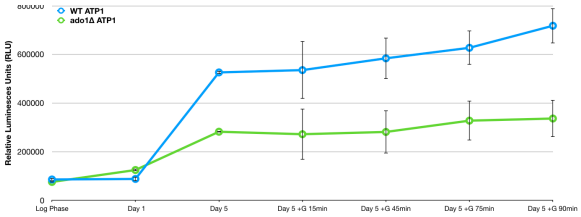
A. *In vivo* quantification of endogenous Atp1 and Atp2

BY4741 endogenous Atp1 and Atp2 were individually C-terminally tagged with nanoLuc to quantify their protein levels *in vivo* (Fig. 5A). Followed by gene deletion of *Ado1* generating 4 isogenic yeast strains. The yeast strains were grown over a 120 hr growth period with luciferase activity being quantified during log phase growth, Day 1, Day 5 and during glucose re-addition. All the readings were standardized to an OD_{600} of 0.3. During log phase of fermentative growth, proteins levels for Atp1 and Atp2 were slightly higher in the WT than ado1Δ strains, which was expected, according to the ribosome profiling data. 24 hrs later, there was a switch in protein levels of Atp1 and Atp2, such that proteins levels for both genes in ado1Δ strains were higher compared to the isogenic WT strains, which was expected. Surprisingly, 96 hrs later, the proteins levels, of Atp1 and Atp2, in the WT strains were much higher compared to the ado1Δ strains (Fig. 5B, C). The results for Day 5 for both genes was not expected and did not correlate with the ribosome profiling data.

A.



B.



C.

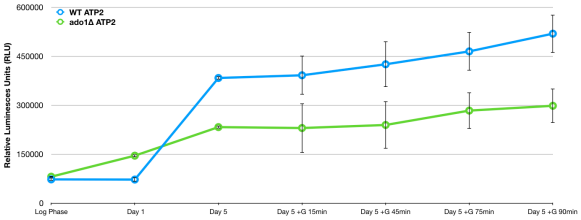


Figure 5. *In vivo* quantification of Atp1 and Atp2. A) Cartoon of nanoLuc cassettes' integration into yeast genome for C-terminal endogenous tagging, not to scale. B) Quantification of Atp1 protein levels during the 120-hr assay including glucose re-addition in WT and ado1Δ strains. C) Quantification of Atp2 protein levels during the 120-hr assay including glucose re-addition in WT and ado1Δ strains.

B. Dual i*n vivo* quantification of endogenous Atp1 or Atp2 and Hsp12

Confirmation of the quantification of protein levels for Atp1 and Atp2 using nanoLuc as a reporter, was accomplished by tagging endogenous *Atp1* and *Atp2* with eGFP cassettes. *Hsp12* was also tagged with a mCherry cassette. Hsp12 was chosen because its TE was not impacted by Ded1 inactivation and its 5' UTR is half the length of the genome average, so we expect protein levels to be similar in WT and ado1Δ strains during the growth assay [8]. Using two different fluorescent proteins with different excitation and emission wavelengths gave the ability to C-terminally tag two genes in a single yeast cell for dual *in vivo* qualification of protein levels. Integrating the cassettes into *BY4741* generated two isogenic yeast strains with either *Atp1* and *Hsp12* tagged or *Atp2* and *Hsp12*. (Fig. 6A). The *Ado1* deletion generated a total of four yeast strains. Similar to the yeast strains with the nanoLuc cassettes, fluorescence was measured over 120 hr growth period but glucose re-addition was not measured because the half-life of fluorescent proteins are much longer than nanoLuc [33, 34]. The quantified protein levels of Atp1 and Atp2, using eGFP as a reporter, in both WT and ado1Δ strains, had a similar trend compared to the luciferase data throughout the 120-hr growth assay (Figure 6B, C). Hsp12's protein levels were measured in WT and adoΔ1 strains (Fig. 6D). The reassurance of the luciferase data was exciting. The next question to address was could Ded1 be one of the translation factors influencing the change in protein levels in the ado1Δ strains during the 5-day assay.

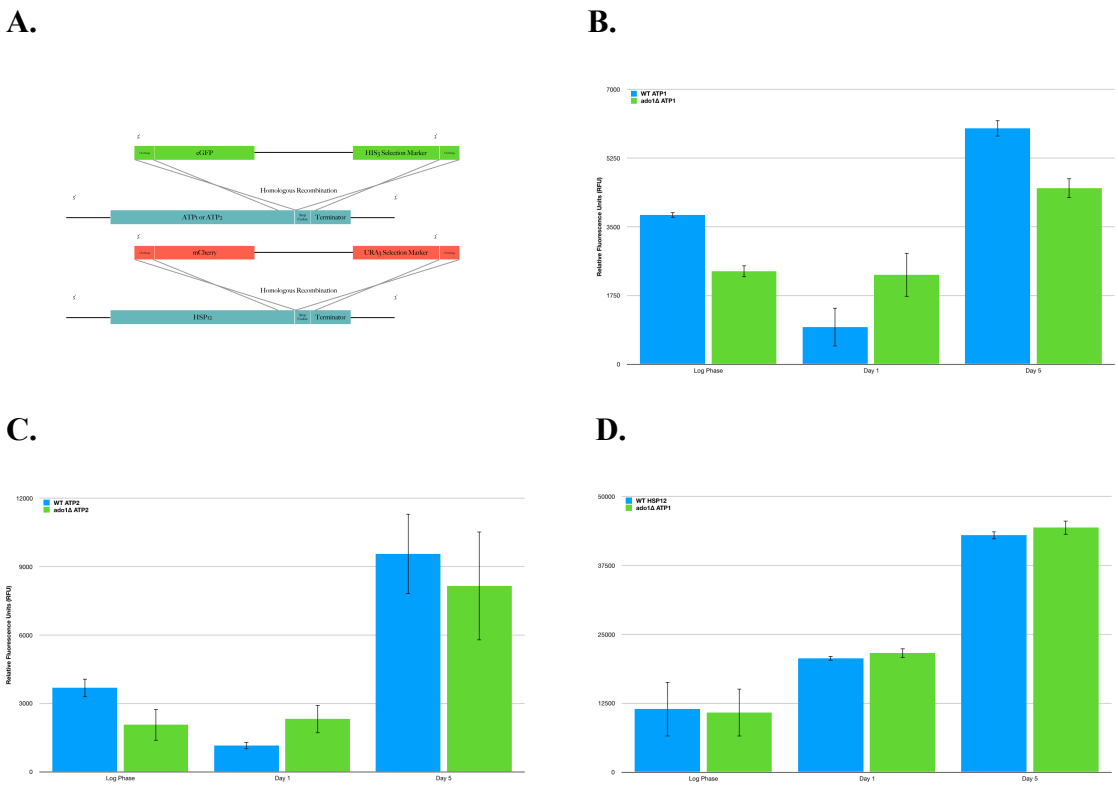
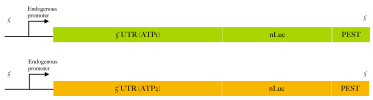


Figure 6. Dual *in vivo* quantification of Atp1 or Atp2 and Hsp12. A) Cartoon of fluorescent cassettes' integration into yeast genome for C-terminal endogenous tagging, not to scale. B) Quantification of Atp1 protein levels during the 120-hr assay in WT and ado1Δ strains. C) Quantification of Atp2 protein levels during the 120-hr assay in WT and ado1Δ strains. D) Quantification of Hsp12 protein levels during the 120-hr assay in WT and ado1Δ strains.

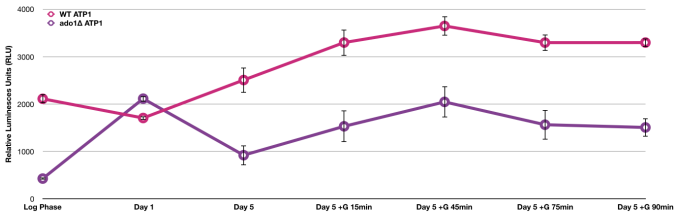
C. *In vivo* quantification of nanoLuc from 5' UTR integrated constructs

To investigate if Ded1 could be one of the translation factor influencing the changes in Atp1 and Atp2 protein levels in both WT and ado1Δ strains, DNA constructs containing 5' prUTRs of either gene with a downstream reporter were generated via Gibson Assembly and integrated into *BY4741*'s genome. Expression was driven by either Atp1 or Atp2's endogenous promoter (Fig. 7A). 5' prUTR of both genes and *nanoLucP* were cloned into pRS305. The destabilization domain, PEST, shortens the half life of nanoLuc, permitting real-time quantification of the reporter [21]. The PEST domain was not incorporated into the endogenous nanoLuc or fluorescent cassettes because it was lethal to the yeast cells. Recombinant plasmids were transformed into *BY4741* followed by the deletion of Ado1, generating four yeast strains. The yeast strains were grown over a 120-hr period and luciferase was quantified similar to previously described methods. The nanoLuc protein levels driven by either Atp1 or Atp2's 5' prUTR in both WT and ado1Δ strains had the same trend as the endogenous C-terminally tagged gene assays (Fig. 7B, C). Suggesting, Ded1 could be one of the translation factor influencing protein expression by promoting translation initiation and unraveling 5' UTR RNA secondary structures.

A.



B.



C.

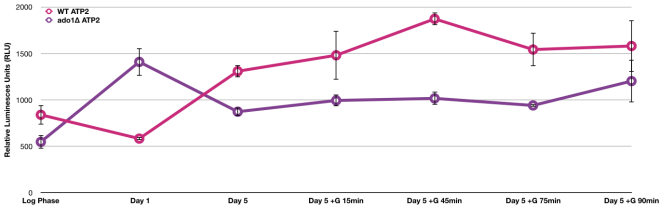


Figure 7. *In vivo* quantification of *Atp1* or *Atp2* 5' prUTR driven nanoLuc.
A) Schematic of reporter genes B) Quantification of luciferase driven by *Atp1* 5' prUTR during the 120-hr assay in WT and ado1Δ strains. C) Quantification of luciferase driven by *Atp2* 5' prUTR during the 120-hr assay in WT and ado1Δ strains.

D. Validation of 5-day growth assay

The readings for the 120-hr growth assays were standardized to an OD_{600} of 0.3 or 0.4. After the fermentative log phase readings, cultures were too concentrated, so dilution factor were quantified and aliquots from the cultures were diluted with YPD (Fig. 8A). Diluting the Day 1 and Day 5 aliquots would re-introduce glucose to the cells and potentially skew the results. The aliquots were diluted with either YPD or YP minus dextrose and immediately luciferase or fluorescence was quantified. The difference between the samples diluted with YPD or YP were negligible suggesting the re-addition of glucose would not skew the immediate results (Fig. 8B-C).

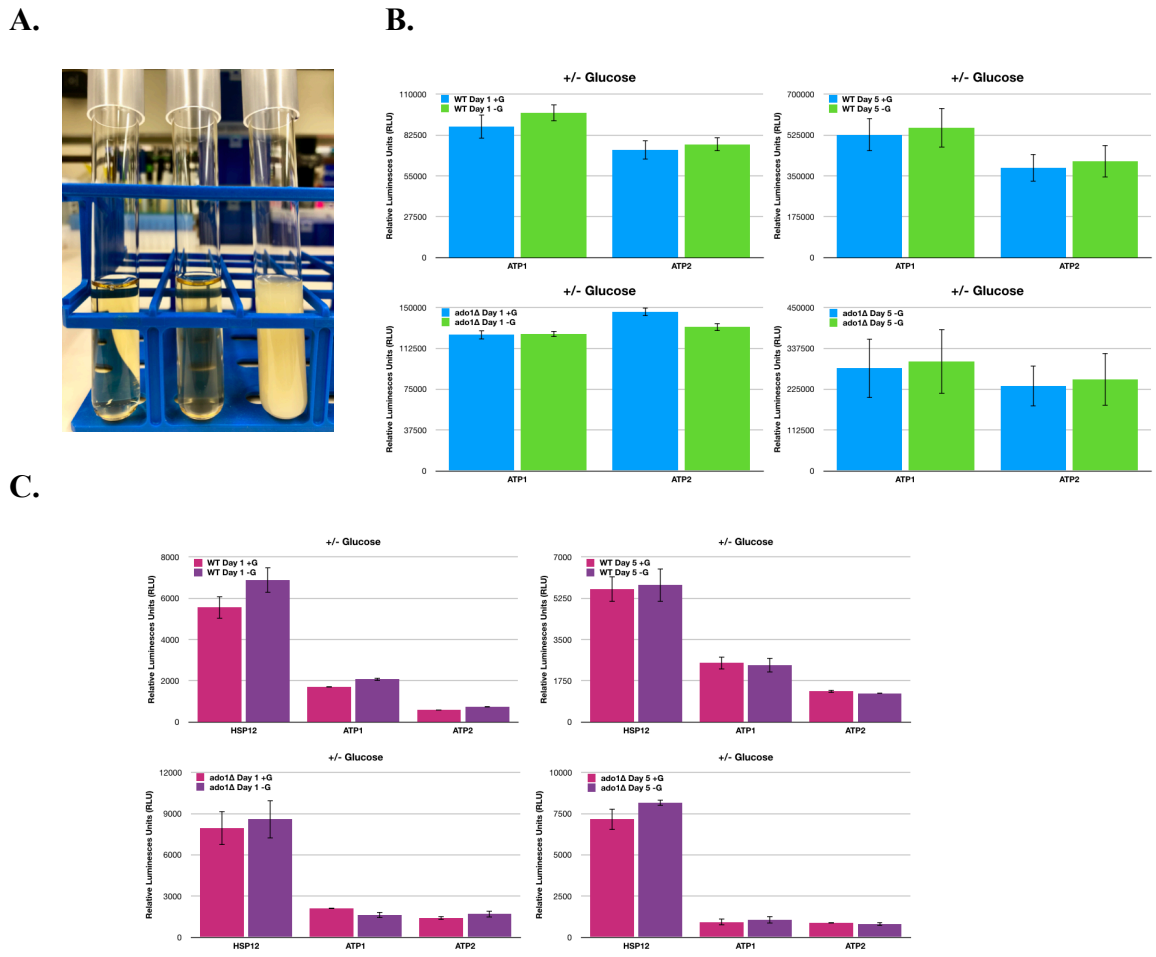


Figure 8. Validation of 5-day growth assays dilutions. A) Photograph of yeast cultures. From left to right, YPD, culture at OD_{600} of 0.3 and culture after 120-hr growth period. B) Validation of readings from Day 1 and Day 5 of yeast strains containing the integrated nanoLuc cassette. C) Validation of readings from Day 1 and Day 5 of yeast strains containing integrated nanoLuc driven by 5' prUTR DNA constructs.

IV. Discussion

A. Atp1 and Atp2 protein levels during 120-hr growth assay

Initially, analyzing the nanoLuc data for endogenously tagged Atp1 and Atp2, we noticed that both share a similar of trend (Fig. 5B, C). Endogenous protein levels of Atp1 and Atp2 are higher in WT at log phase during fermentative growth, then at Day 1 their levels in ado1Δ strain is higher than WT and finally at day 5 protein levels in WT are higher than ado1Δ strain. During glucose re-addition, protein levels do not seem to change during the 90 minute assay. The protein levels do not appear to correlate with the ribosome profiling data at day 5. Ribosome profiling suggest protein expression should be higher in ado1Δ strains than WT at day 5 but endogenously tagging *Atp1* and *Atp2* with nanoLuc and quantifying bioluminescence indicates the opposite. The contradiction was intriguing, however, because we had two conflicting data sets, we deemed it necessary to validate one of the results.

We chose to confirm the nanoLuc data. The experiment was repeated but instead of using nanoLuc, fluorescent proteins were used as the reporters. Curiosity led to endogenously tagging *Hsp12* because it had a 5' UTR half the length of the genome average and its translation efficiency did not decrease upon Ded1 inactivation, suggesting it may not be affected by ado1Δ disrupting Ded1 co-localization to stress granules during the 5-day growth assay [8]. To our surprise, the data validated our nanoLuc results and Hsp12 protein levels did not seem to change much between WT and ado1Δ strains during the 5-day period suggesting the tagging system could be reliable for quantifying *in vivo* protein levels (Fig. 6B-D). It's possible that ribosome profiling data is not a good

representation of *in vivo* protein levels because potentially ribosomes could be stalled or translation elongation rates can vary per mRNA during environmental changes such as transitioning from fermentative to respiratory growth. Validation of the sample dilutions for both experiments, quantifying *in vivo* protein levels, demonstrated the standardization of diluting the samples to a specific OD_{600} with YPD or YP did not show alarming differences suggesting the readings were not diluent-dependent (Fig 8B, C).

Further experiments need to be conducted to gain insight towards the day 5 readings such as polysome profiling to understand the association of mRNAs and ribosomes.

B. Atp1 and Atp2's protein levels during the 5-day assay could be Ded1-dependent.

The findings from endogenously tagging *Atp1* and *Atp2* were useful to understand how *Ado1* deletion, and the subsequent disruption of Ded1's co-localization to stress granules could increase protein level expression for genes that are negatively sensitive to Ded1 inactivation and have long 5' UTRs. Given Ded1's function of a RNA helicase and unraveling long, structured 5' UTRs, we individually cloned endogenous prUTR, of *Atp1* and *Atp2*, upstream of nanoLucP into pRS305 and integrated them into *BY4741*. The results from the 5-day growth assay were exciting. NanoLuc protein levels driven by endogenous prUTR of either *Atp1* or *Atp2* during the 120-hr period shared the similar trend as the endogenously tagged gene data (Fig. 7B, C) . It cannot be suggested that Ded1 is the actual culprit contributing to the change in protein levels during the 5-day assay because several other translational factors can be enhancing translation of Atp1 and Atp2 in combination to Ded1. Additional experiments need to be conducted to understand how protein levels of Atp1 and Atp2 are being influenced in ado1Δ strains during the 5-day growth assay. A potential experiment could use a Ded1 temperature sensitive mutant or a drug-induced inactivation mutant in the WT and ado1Δ strains to monitor the effects of inactivating Ded1 during the 5-day growth assay.

References

1. Pronk, J. T., Steensma, H. Y., & Dijken, J. P. (1996). Pyruvate Metabolism in Saccharomyces cerevisiae. *Yeast, 12*(16), 1607-1633. doi:10.1002/(sici)1097-0061(199612)12:163.0.co;2-4

2. Stahl, G., Salem, S. N., Chen, L., Zhao, B., & Farabaugh, P. J. (2004). Translational Accuracy during Exponential, Postdiauxic, and Stationary Growth Phases inSaccharomyces cerevisiae. *Eukaryotic Cell, 3*(2), 331-338. doi:10.1128/ec.3.2.331-338.2004

3. Werner-Washburne, M., Braun, E. L., Crawford, M. E., & Peck, V. M. (1996). Stationary phase in Saccharomyces cerevisiae. *Molecular Microbiology, 19*(6), 1159-1166. doi:10.1111/j.1365-2958.1996.tb02461.x

4. Derisi, J. L. (1997). Exploring the Metabolic and Genetic Control of Gene Expression on a Genomic Scale. *Science, 278*(5338), 680-686. doi:10.1126/science.278.5338.680

5. Senissar, M., Saux, A. L., Belgareh-Touzé, N., Adam, C., Banroques, J., & Tanner, N. K. (2014). The DEAD-box helicase Ded1 from yeast is an mRNP cap-associated protein that shuttles between the cytoplasm and nucleus. *Nucleic Acids Research, 42*(15), 10005-10022. doi:10.1093/nar/gku584

6. Yang, Q., & Jankowsky, E. (2006). The DEAD-box protein Ded1 unwinds RNA duplexes by a mode distinct from translocating helicases. *Nature Structural & Molecular Biology, 13*(11), 981-986. doi:10.1038/nsmb1165

7. Hilliker, A., Gao, Z., Jankowsky, E., & Parker, R. (2011). The DEAD-Box Protein Ded1 Modulates Translation by the Formation and Resolution of an eIF4F-mRNA Complex. *Molecular Cell, 43*(6), 962-972. doi:10.1016/j.molcel.2011.08.008

8. Sen, N. D., Zhou, F., Ingolia, N. T., & Hinnebusch, A. G. (2015). Genome-wide analysis of translational efficiency reveals distinct but overlapping functions of yeast DEAD-box RNA helicases Ded1 and eIF4A. *Genome Research, 25*(8), 1196-1205. doi:10.1101/gr.191601.115

9. Guzikowski, A. R., Chen, Y. S., & Zid, B. M. (2019). Stress−induced mRNP granules: Form and function of processing bodies and stress granules. *Wiley Interdisciplinary Reviews: RNA, 10*(3). doi:10.1002/wrna.1524

10. Banani, S. F., Rice, A. M., Peeples, W. B., Lin, Y., Jain, S., Parker, R., & Rosen, M. K. (2016). Compositional Control of Phase-Separated Cellular Bodies. *Cell, 166*(3), 651-663. doi:10.1016/j.cell.2016.06.010

11. Li, P., Banjade, S., Cheng, H., Kim, S., Chen, B., Guo, L., Rosen, M. K. (2012). Phase transitions in the assembly of multivalent signalling proteins. *Nature, 483*(7389), 336-340. doi:10.1038/nature10879

12. Dobra, I., Pankivskyi, S., Samsonova, A., Pastre, D., & Hamon, L. (2018). Relation Between Stress Granules and Cytoplasmic Protein Aggregates Linked to Neurodegenerative Diseases. *Current Neurology and Neuroscience Reports, 18*(12). doi:10.1007/s11910-018-0914-7

13. Protter, D. S., & Parker, R. (2016). Principles and Properties of Stress Granules. *Trends in Cell Biology, 26*(9), 668-679. doi:10.1016/j.tcb.2016.05.004

14. Begovich, Kyle (2019). Stress-Specific Recruitment of Metabolic Enzymes to RNA Stress Granules. *Unpublished*

15. Jauker, M., Griesser, H., & Richert, C. (2015). Spontaneous Formation of RNA Strands, Peptidyl RNA, and Cofactors. *Angewandte Chemie International Edition, 54*(48), 14564-14569. doi:10.1002/anie.201506593

16. Wierman, M. B., Matecic, M., Valsakumar, V., Li, M., Smith, D. L., Bekiranov, S., & Smith, J. S. (2015). Functional genomic analysis reveals overlapping and distinct features of chronologically long-lived yeast populations. *Aging, 7*(3), 177-194. doi:10.18632/aging.100729

17. Mullis, K. B., & Faloona, F. A. (1989). Specific Synthesis of DNA in Vitro via a Polymerase-Catalyzed Chain Reaction. *Recombinant DNA Methodology,* 189-204. doi:10.1016/b978-0-12-765560-4.50015-0

18. Dolgova, A. S., & Stukolova, O. A. (2017). High-fidelity PCR enzyme with DNA-binding domain facilitates de novo gene synthesis. *3 Biotech, 7*(2). doi:10.1007/s13205-017-0745-2

19. Imadeartika. (2009). The Use of HIS6 Gene as a Selectable Marker for Yeast Vector. *HAYATI Journal of Biosciences, 16*(1), 40-42. doi:10.4308/hjb.16.1.40

20. England, C. G., Ehlerding, E. B., & Cai, W. (2016). NanoLuc: A Small Luciferase Is Brightening Up the Field of Bioluminescence. *Bioconjugate Chemistry, 27*(5), 1175-1187. doi:10.1021/acs.bioconjchem.6b00112

21. Masser, A. E., Kandasamy, G., Kaimal, J. M., & Andréasson, C. (2016). Luciferase NanoLuc as a reporter for gene expression and protein levels in Saccharomyces cerevisiae. *Yeast, 33*(5), 191-200. doi:10.1002/yea.3155

22. Aaij, Cees, Piet Borst (1972). THE GEL ELECTROPHORESIS OF DNA. *Biochimica Et Biophysica Acta (BBA) - Nucleic Acids and Protein Synthesis*, 269, pp. 192–200., doi: 10.1016/0005-2787(72)90426-1.

23. Gietz, R. D. (2014). High Efficiency DNA Transformation of Saccharomyces cerevisiae with the LiAc/SS-DNA/PEG Method. *Fungal Biology Genetic Transformation Systems in Fungi, Volume 1,* 177-186. doi:10.1007/978-3-319-10142-2_17

24. Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., & Boeke, J. D. (1998). Designer deletion strains derived fromSaccharomyces cerevisiae S288C: A useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast, 14*(2), 115-132. doi:10.1002/(sici)1097-0061(19980130)14:23.0.co;2-2

25. Sheff, M. A., & Thorn, K. S. (2004). Optimized cassettes for fluorescent protein tagging inSaccharomyces cerevisiae. *Yeast, 21*(8), 661-670. doi:10.1002/yea.1130

26. Lee, S., Lim, W. A., & Thorn, K. S. (2013). Improved Blue, Green, and Red Fluorescent Protein Tagging Vectors for S. cerevisiae. *PLoS ONE, 8*(7). doi:10.1371/journal.pone. 0067902

27. Karreman, R. J., & Lindsey, G. G. (2005). A Rapid Method to Determine the Stress Status of Saccharomyces cerevisiae by Monitoring the Expression of a Hsp12:Green Fluorescent Protein (GFP) Construct under the Control of the Hsp12 Promoter. *Journal of Biomolecular Screening, 10*(3), 253-259. doi:10.1177/1087057104273485

28. Jimenez, L., Laporte, D., Duvezin-Caubet, S., Courtout, F., & Sagot, I. (2013). Mitochondrial ATP synthases cluster as discrete domains that reorganize with the cellular demand for oxidative phosphorylation. *Journal of Cell Science, 127*(4), 719-726. doi:10.1242/jcs.137141

29. Hentges, P., Driessche, B. V., Tafforeau, L., Vandenhaute, J., & Carr, A. M. (2005). Three novel antibiotic marker cassettes for gene disruption and marker switching inSchizosaccharomyces pombe. *Yeast, 22*(13), 1013-1019. doi:10.1002/yea.1291

30. Sikorski, Robert S., Philip Hieter (1989). A System of Shuttle Vectors and Yeast Host Strains Designed for Efficient Manipulation of DNA in Saccharomyces Ceravisiae. *Genetics*, *122 (1)*, pp. 19–27.

31. Gibson, D. G. (2011). Enzymatic Assembly of Overlapping DNA Fragments. Methods in Enzymology Synthetic Biology, Part B - Computer Aided Design and DNA Assembly, 349-361. doi:10.1016/b978-0-12-385120-8.00015-2

32. Hied, C. A., Stevens, J., Livak, K. J., & Williams, P. M. (n.d.). Real Time Quantitative PCR. *Genome Research, 6*, 986-994. doi:10.1101/gr.6.10.986

33. Snapp, E. L. (2009). Fluorescent proteins: A cell biologists user guide. *Trends in Cell Biology, 19*(11), 649-655. doi:10.1016/j.tcb.2009.08.002

34. Hall, M. P., Unch, J., Binkowski, B. F., Valley, M. P., Butler, B. L., Wood, M. G., Wood, K. V. (2012). Engineered Luciferase Reporter from a Deep Sea Shrimp Utilizing a Novel Imidazopyrazinone Substrate. *ACS Chemical Biology, 7*(11), 1848-1857. doi:10.1021/b3002478