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Factors affecting nuclear-encoded mitochondrial gene expression

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

in

Chemistry

by

Yuko Sugiyama

Committee in charge:

Professor Brian M. Zid, Chair
Professor Alexis Komor
Professor Jens Lykke-Andersen

2022

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The thesis of Yuko Sugiyama is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2022

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ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Professor Brian M. Zid for his invaluable feedback and patience during the whole master thesis program. I acquire the opportunities to conduct research projects which I am strongly interested in and with his precious consideration and direction and other predecessors, I accomplished my thesis and experiments successfully.

Especially, I am deeply indebted to Wanfu Hou, one of the Zid's lab member for mentoring me with his patience. He trained me to carry out the experiment methods and operate the experimental instruments. Without his assistance for the experiments, I could not accomplish the experiments.

Lastly, I would like to appreciate Molly Cohn, Vicky Chen and Zoey Litt for giving me assistance for the experiments as undergraduate research assistants. Without them, I would not finish my thesis experiments on time.

ABSTRACT OF THE THESIS

Factors affecting nuclear-encoded mitochondrial gene expression

by

Yuko Sugiyama

Master of Science in Chemistry

University of California San Diego, 2022

Professor Brian M. Zid, Chair

Protein production is tightly regulated by post transcription factors to control internal cellular elements and adapt themselves to the surrounding environment, such as the nutrient availability, temperature and oxygen absence. Translational elongation is one of the regulators for protein production. The mRNA localization to mitochondria during translational elongation has been reported to induce the increase in its protein production. The metabolic shift is a key factor for ATP synthase genes to localize its mRNAs to mitochondria during respiration to escalate ATP productions under oxidative phosphorylation. In the respiration, some ATP

synthase genes are reported to induce the localization switching metabolism from fermentation to respiration. We have been investigating if slowing translational elongation can improve the protein production in conditionally localized mRNAs. Here we found that the ATP2 and ATP4 in yeast cells have decent increases in mRNA levels upon translational elongation slowing. In addition to it, the promoter has a certain effect in protein induction under respiration, but promoter combined with ORF has the stronger effect in it. To identify genes involved in mRNA localization-dependent gene expression, we have constructed the design of CRISPRi screening with a group of gRNAs targets various promoters in yeast cells in combination with a MCP-MS2 system to target a reporter mRNA to the mitochondria.

Introduction

Regulating gene expression is a critical system in all living cells, which allows them to maintain their intercellular environment and adapt themselves to the surrounding nutrient availability. Sometimes, they even communicate with their neighbor cells to survive. Nutrient availability can also change a cell's metabolic states between fermentation and respiration. [30] Fermentation uses glucose and produces ethanol, CO2, and ATP in yeast cells, *Saccharomyces cerevisiae*, which is less dependent on ATP synthase gene expressions. The process of yeast respiration includes oxidative phosphorylation, which moves electrons to balance the intercellular charges with consuming oxygen and producing ATPs. While yeast respiration relies on mitochondrial activity and tends to synthesize more ATPs compared to fermentation, it is necessary for cells to be able to increase the expressions of ATP synthase genes. [20] The central dogma of gene expression - replication, transcription, and translation - gives various possible steps to regulate the DNA, RNA, and protein production in cells. Some regulations in gene expressions take place in the process of translation, which produces proteins from mRNAs. [16] Translation can be categorized into three main steps: initiation, elongation, and termination, with each step carrying multiple opportunities for regulation. Initiation is the recruiting step for a ribosome and an aminoacyl-tRNA for fMET. Elongation is a process for adding peptides by reading codons, which tells which anti-codons are complementary to, binding to aminoacyl-tRNA, forming peptide bonds, and translocating to P-site in the ribosome. [25] In yeast, in addition to EF-1a and EF2 as translation factors, the third factor YEF3 is required to proceed with translation in the proper manner. [23] It hydrolyzes ATP and stimulates EF-1a to release the deacyl-tRNA from the E-site. The complete disruption of this gene is lethal to yeast cells.

[21] Comparing the two steps, initiation is the rate-limiting step in the process of translation, while elongation is considered to be less rate-limiting. However, our lab has found that genes that utilize co-translational translocation, which translocate a nascent peptide sequence to the targeted organelles while the mRNAs are being translated, are dependent on translational elongation rate, which induces mRNA localization to membranes on organelles and leads to an increase in protein production. [29]

There are certain benefits when the cell performs translation with co-translational translocation. [26][6] For one, it saves a lot of energy because the polypeptides are produced after they reach organelles. Mechanically, the mRNAs locate to an organelle's membrane before translational termination, via translocase, which allows them to avoid flowing in the cytosol as polypeptides and preserves their unfolded structure until they are transported and eventually folded in the organelle. The channel also prevents the degradation of polypeptides since they are protected from interacting with ubiquitination elements. Compared to post-translational translocation, the co-translational translocation mechanism is more efficient because it targets the organelles while translation occurs and requires a smaller number of mRNAs because the produced proteins are less likely to be degraded. For those reasons, co-translational translocation is very sufficient and efficient. ATP synthase genes are a type of those genes which use co-translational translocation while in their respiratory metabolic state. [4][5][31]

Our lab has been investigating how mRNA localization induces protein production in conditionally localized ATP synthase genes to the mitochondria. [29] The ATP3 gene showed significant increases in the mRNA localization when we switched cellular metabolism from

fermentation to respiration, which resulted in an increase in the ATP3 protein synthesis upon the metabolic shift. Compared to ATP3, the TIM50 gene, which is constitutively localized to mitochondria, did not show any significant changes in the mRNA localization or in protein production. When reporter mRNAs were manually tethered to the mitochondria, they also showed an increase in protein production. These studies indicated that induced mRNA localization for co-translationally translocated mRNAs increases the possibility of forwarding the synthesis of protein expression for nuclear-encoded mitochondrial genes.

Considering that many cancer cells are active in fermentation and inactive in respiration [18], one question of interest is whether it is possible to switch from fermentative to the respiratory metabolic state by slowing down the translational elongation rate; this would induce mRNA localization of ATP synthase genes while the mRNA undergoes co-translational translocation. One way to accomplish this is via cycloheximide, a translational elongation inhibitor that binds at the E site of the ribosome and stalls deacyl tRNA removal. Testing cycloheximide with the ATP3 and TIM50 mRNAs has shown that only ATP3 mRNA increases its localization to mitochondria upon cycloheximide treatment. [29] When ATP2 mRNA is in respiration, it showed a similar level of mRNA localization to mitochondria with the one of ATP2 mRNA treated with cycloheximide. The proximity ribosomal profiling data that showed ATP1, ATP2, ATP3, ATP4, and ATP5 mRNAs induced localization when the elongation rate is slowed by cycloheximide [31], which could imply that those additional conditionally localized mRNA are also sensitive to changes in elongation rate.

In my thesis, we use a fused methionine-repressible promoter upstream of YEF3, the elongation factor 3 for translation, to slow down translational elongation by adding methionine.

Incorporating this promoter allows us to determine if elongation slowing during translation affects protein expressions of the ATP synthase genes and shifts the cell's metabolism from fermentation to respiration. In addition to it, FACS-based CRISPRi screening on tethered mRNAs to mitochondria will give us insight as to which genes are involved in the regulation of mitochondrial mRNA localization to dependent-gene expression.

Chapter 1 Methionine sensitive elongation inhibition on translation

1.1 Translational elongation inhibition system by methionine addition

Methioine25 (MET25) is found in *Y. lipolytica*, homologous to MET15 in *S. cerevisiae*. The gene encodes for O-acetyl homoserine sulphydrylase and is an important catalyst for various reactions related to sulfur metabolism. [2] Mumberg et al found that the promoter for the sulphydrylase was highly sensitive to methionine concentrations. [17] The MET25 promoter can catalyze the repression in transcription level by regulating the methionine concentrations. [24] Addition of exogenous methionine to the yeast strain with this MET25 promoter leads to inhibition in the downstream genes.

Kasari et al inserted this methionine-repressible MET25 promoter (pMET25) upstream of the YEF3 gene (Figure 1.1.1) and tested its deficiency upon methionine addition by western-blot. Also, their ribo-seq data found that methionine addition on this strain caused a large decrease in ribosome reads along mRNA, which indicated the YEF3 gene had been repressed and was less chance of translation for other genes. [10]

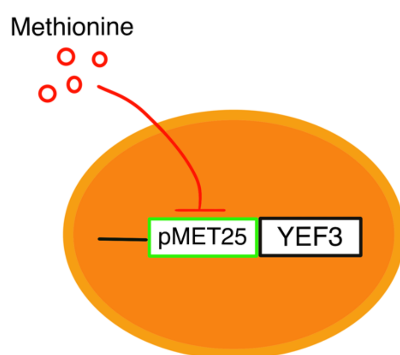

Figure 1.1.1 Design of pMET25 strain
pMET25 is activated when there is no methionine added and transcribes the YEF3 gene. Upon the methionine addition, pMET25 is repressed, and the YEF3 gene is not transcribed, which results in the inhibition of translation.

The methionine concentration is a key component to optimize YEF3 inhibition. The previous literature applied four different concentrations to their pMET25 strain: 0.1, 0.5, 1.0 and 2.0 mM. In 0.1mM methionine addition, there was no significant change in the cell growth, but YEF3 protein expression was inhibited on western blot. The 0.5mM methionine is considered to be moderately effective, as it slowed down the cell growth and inhibited the YEF3 protein expression manifestly. The 1.0 and 2.0mM did not allow the cells to grow and thoroughly depleted YEF3 protein expression.

1.2 Methionine effect on yeast growth

The first step to initiating our project was to make the pMET25 embedded strains the upstream of the YEF3 gene. We amplified the pMET25 gene and transformed it into the wild-type strain (WT). The cell growth was tested in three different concentrations of methionine: 0, 0.5, and 2mM. As Kasari et al indicated, the 2.0mM methionine inhibited cell growth severely. The 0.5mM methionine has significant inhibition in cell growth (Figure 1.2.1).

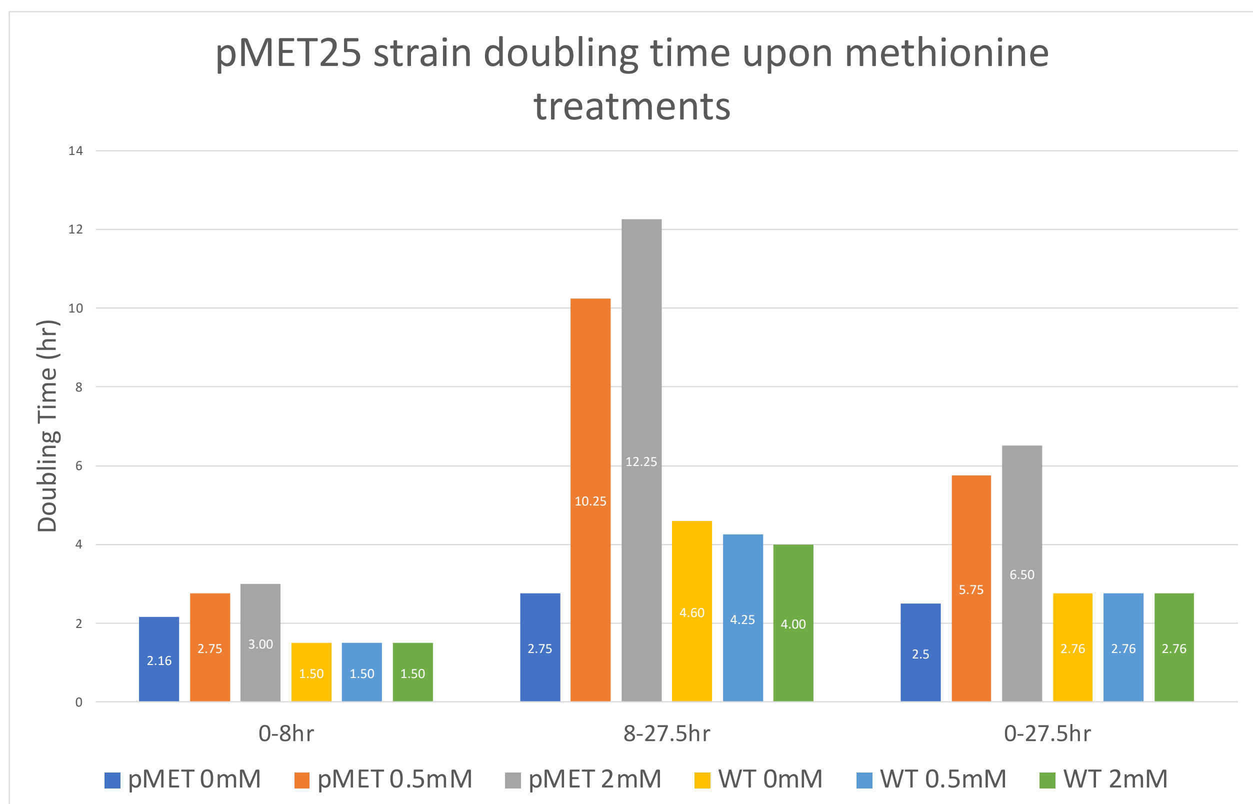

Figure 1.2.1 pMET strain doubling time upon methionine treatments

Growth is measured in optical density. Doubling time was converted from ODs between 0, 8 and 27.5 hours. Upon the methionine treatments, 0.5mM and 2mM, the pMET25 strain shows the longer doubling time compared to 0mM. The pMET25 strain without methionine has the similar doubling time as the WT strain with 0, 0.5 and 2mM methionine.

1.3 Optimizing experimental conditions

The six pMET25 strains we made were tested to measure the growth rate with 0, 0.5, and 2mM methionine while comparing it to the WT. The pMET25 #6 strain with no methionine gave the closest growth rate compared to the WT, but with methionine, its growth rate significantly slowed down. Therefore, this pMET25 #6 stain was used for all the following experiments.

To see changes in the YEF3 RNA transcripts upon methionine addition, we have tested 3 different time points to compare to 0hr: 1, 6, and 24 hours (Figure 1.3.1). After an hour of the methionine addition, the qPCR data shows decent decreases in a YEF3 RNA transcript. Between 6-hour and 24-hour time points, there is no significant difference in the depletion of the YEF3 RNA transcripts (Figure 1.3.2). Summarizing them, we decided to collect the pellets at 1 and 6 hours after methionine addition.

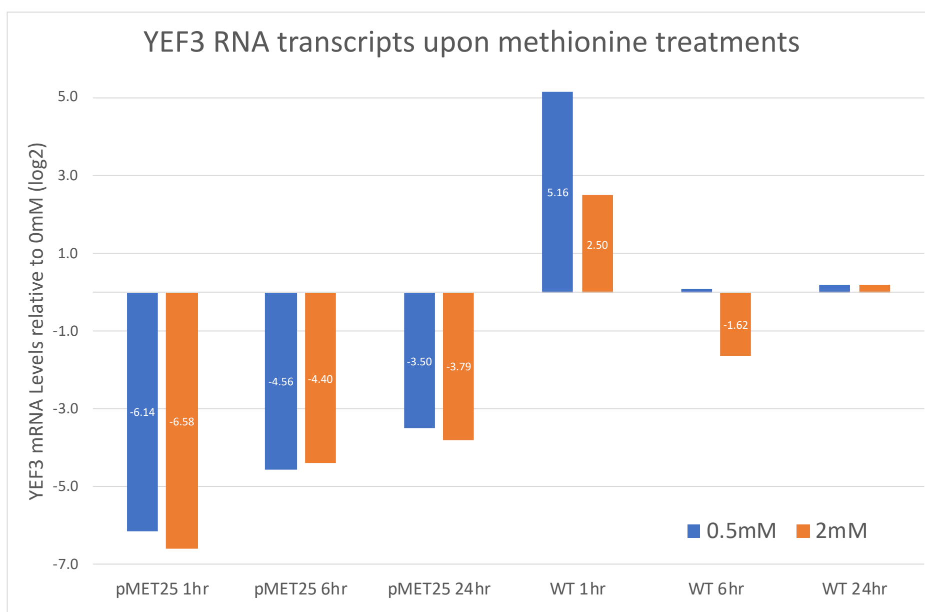

Figure 1.3.1 YEF3 mRNA level changes upon methionine treatments
YEF3 RNA transcripts are measured at four timpoints: 0, 1, 6 and 24 hours. They are tested under three different concentrations of methionine: 0, 0.5 ane 2mM. The pMET25 strain is grown in SC-MET-CYS media for overnight before the methionine treatments. All YEF3 CT values are compared to Actin, contol, CT values and normalized to 0mM at each timepoint.

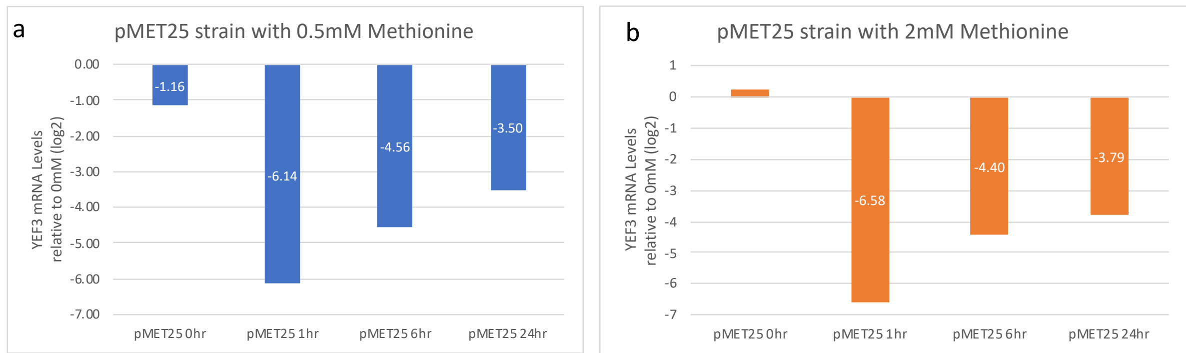

Figure 1.3.2 YEF3 mRNA levels compared by timepoints with 0.5mM and 2mM

a) YEF3 RNA expression is compared by 0, 1, 6 and 24-hour timepoint with 0.5mM methionine relative to 0mM.

b) YEF3 RNA expression is compared by 0, 1, 6 and 24-hour timepoint with 2mM methionine relative to 0mM.

1.4 YEF3 inhibition effect on RNA transcripts of ATP synthase genes

YEF3 is known for translational elongation factor and stimulates EF1-a via ATP and GTP hydrolysis. Inhibiting this YEF3 gene will slow translational elongation by disrupting ATP hydrolysis. ATP synthase genes are genes we hypothesize to be sensitive to translation elongation inhibition. While the experimental design regarding the optimal concentration of methionine and harvesting timepoint was being solidified, the qPCR for the pMET25 strain and the WT strain was performed.

The qPCR data has shown that there are increases between different concentrations of methionine in some ATP synthase genes.

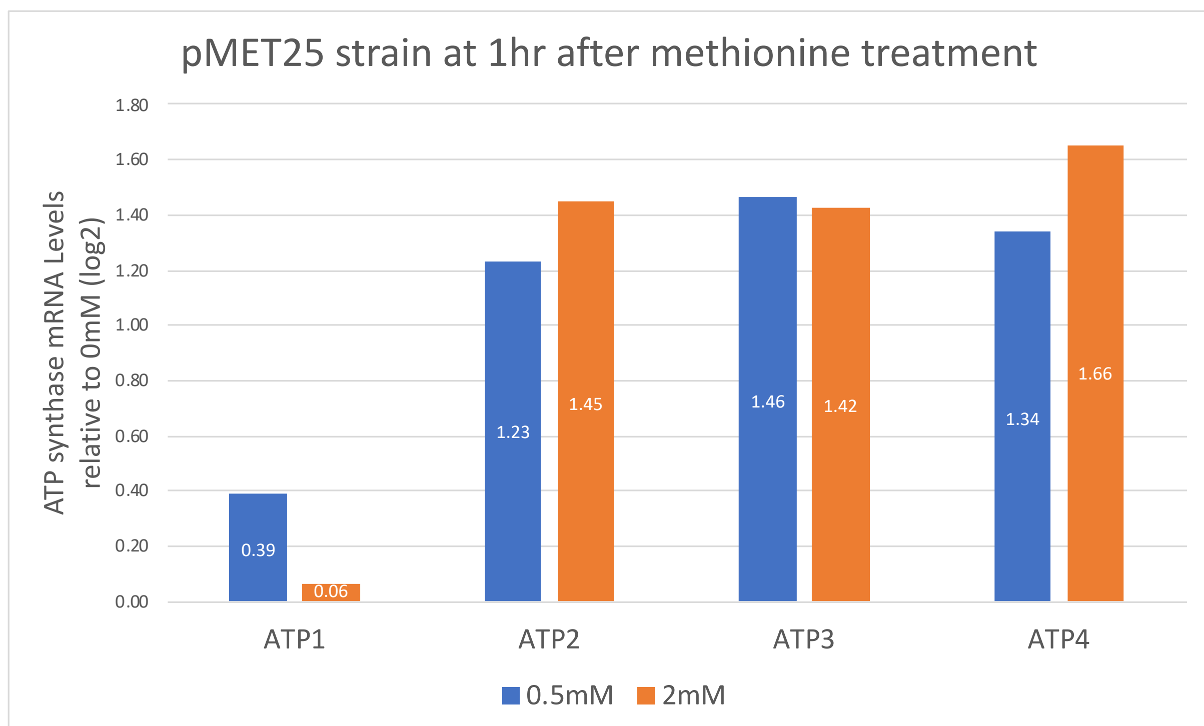

Figure 1.4.1 ATP synthase mRNA level increases upon 1 hour methionine treatments
The RNA transcripts in the ATP synthase genes are measured in three different methionine concentrations, 0, 0.5 and 2mM, at the 1-hour timepoint. All YEF3 CT values are compared to Actin, contol, CT values and normalized to 0mM at each timepoint.

At the 1-hour timepoint, ATP2, ATP3, and ATP4 showed increases in the RNA transcripts of each ATP synthase gene as the concentration of methionine added was increased

(Figure 1.4.1).

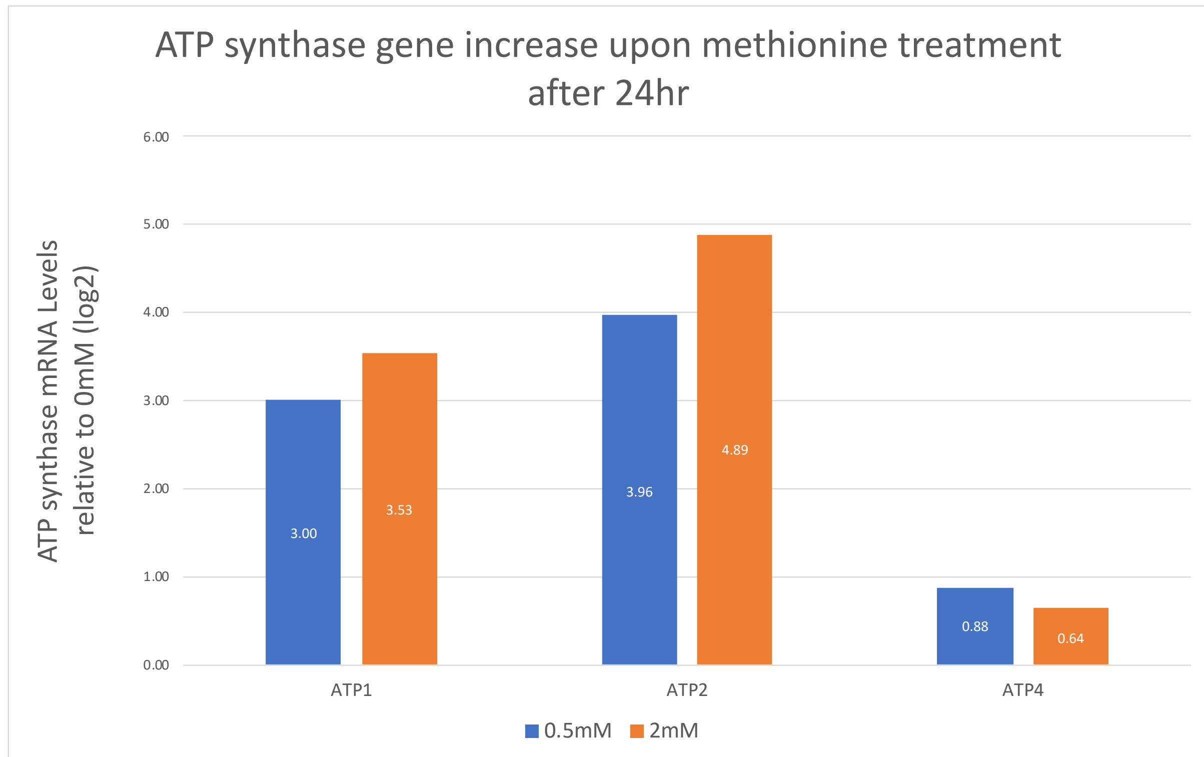

Figure 1.4.2 ATP synthase mRNA level increase after 24 hours of methionine treatments
The RNA transcripts in the ATP synthase genes are measured in three different methionine concentrations, 0, 0.5 and 2mM at the 24-hour timepoint. All YEF3 CT values are compared to Actin, contol, CT values and normalized to 0mM at each timepoint..

We tested the 24-hour timepoint as we tested YEF3 RNA transcripts (Figure 1.4.2). ATP1 and ATP2 genes at the 24-hour time point are transcribed more highly than those at the 1-hour time point. Also, those genes more significantly show the increases in their RNA transcripts by increasing the methionine concentration compared to the 1-hour time point. One possible explanation could be that the yeast cells have enough time to show a down-regulation effect upon adopting their condition to the respiratory state.

These results support our hypothesis that slowing down elongation increases the mRNA stability due to the induced mitochondrial mRNA localization by observing the increase in ATP synthase mRNAs. Compared to the other ATP synthase genes, ATP3 did not show

significant increases in its RNA transcripts upon slowing the translation elongation. More biological replicates are required to verify these results.

1.5 Conclusion and future direction

We have confirmed that the slow translational elongation affects the respiratory-related mRNAs' production, particularly during mRNAs localization to the mitochondria. However, we have not confirmed if those increase in RNA production induces protein production or if the protein increase for those genes is caused by the increases in mRNA localization to mitochondria. In terms of those undetermined factors, it will be important to investigate if the protein productions of those genes are induced by the slow translational elongation and if the slow elongation with modifying the translation elongation factor causes the mRNA localization rate to mitochondria. Elongation rate measurement will be one of the principal things to examine in order to determine whether the partial YEF3 gene deletion under pMET25 regulation slows down elongation rate rather than determining YEF3 gene depletion.

In addition, because this project is based on the question if it is possible to shift the metabolic state by mimicking the respiratory metabolic state and changing the protein expressions of respiratory-related genes, it is necessary to determine if the yeast cells are in respiration by measuring either oxygen consumption or ethanol production upon methionine addition.

Chapter 2 Driving force of nutrient-dependent changes in mitochondrial gene expression

2.1 ORF vs promoter

Nuclear-encoded mitochondrial genes contains the mitochondria targeting sequence (MTS), which guides its mRNAs to mitochondrial outer membrane (Figure 2.1.1). The localized mRNAs are transported into the organelle via the mitochondrial outer membrane translocases (TOMs). [4]

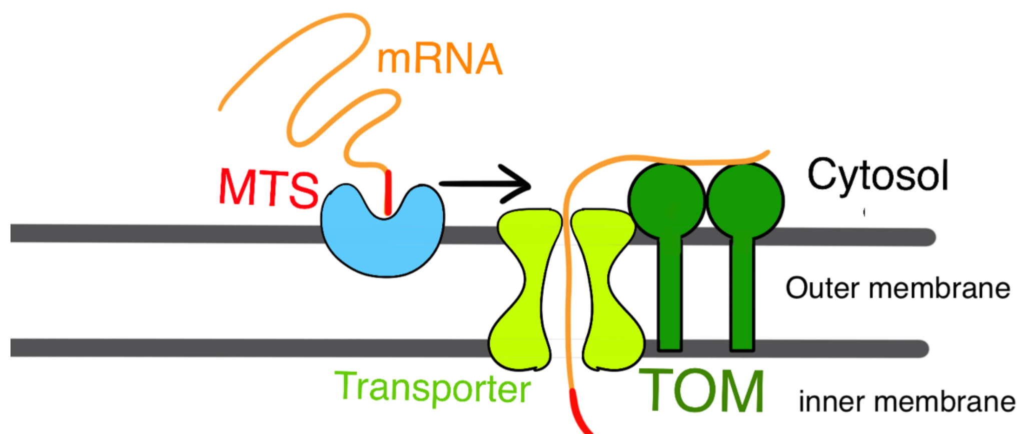

Figure 2.1.1 mRNAs with mitochondria targeting sequence leading translocation to mitochondria
The MTS is c-terminus peptide targeting to mitochondria to translocate mRNAs to mitochondria. It cooperates with TOM proteins and leads the mRNAs via outer mitochondrial outer membrane.

This MTS is in the open reading frame (ORF) of the mRNA and out of the downstream coding region (CDS). The deletions of the MTS regions have shown significant deficiency in terms of mitochondrial localization [13]. Tsuboi et al compared the MTS and CDS for responsibility in the protein production. They found that the CDS which indicates for what it is encoded for is responsible for responding to changes in metabolism regardless of the type of MTS. [29] Here, we wonder if the gene expression changes caused by metabolic states is driven solely by transcriptional control through the promoter or some combination of ORF and promoter region upstream of the ORF.

2.2 FACS data for fermentation vs respiration

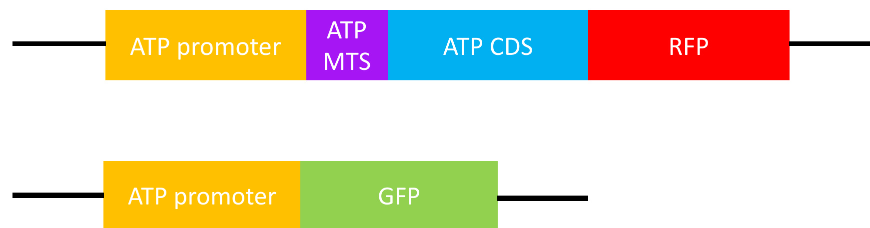

Figure 2.2.1 Experimental yeast design
To test the ATP promoter and ATP ORF including MTS driving force, two plasmids are made: one has the promoter and ORF upstream of a red fluorescent maker, and the other has only the promoter upstream of a green fluorescent marker without the ORF.

ATP1, 2, 3, 4 and 5 are targeted to measure the protein expression differences upon the metabolic shift using two different types of media: YPD keeps yeast cells in fermentation below the optical density (OD) 0.8 and YPG keeps the yeast cells in respiration. ATP1 promoter was successfully tagged with a green fluorescent marker (GFP) and transformed into the W303 yeast strain, which does not include the MTS. ATP1 ORF that includes its promoter was also tagged with mRuby, a red fluorescent maker (RFP), in the yeast strains, which includes the MTS (Figure 2.2.1). This yeast strain was grown in YPD and YPG liquid media overnight. After the overnight culture, the fluorescent expressions were measured on FACS.

In addition, the OD differences between samples possibly cause fluorescent measurement errors due to the cell size differences. To correct for this FACS can measure Forward-scattered light (FSC), which is proportional to cell surface area, and side scatter (SSC), which measures the complexity inside of the cell. Dividing the fluorescent expressions by FSC or SSC, allows us to normalize the fluorescent expression in the two different concentrations of cells between samples.

(Normalized fluorescent level) = (Raw Fluorescent level log area) / (FSC or SSC area)

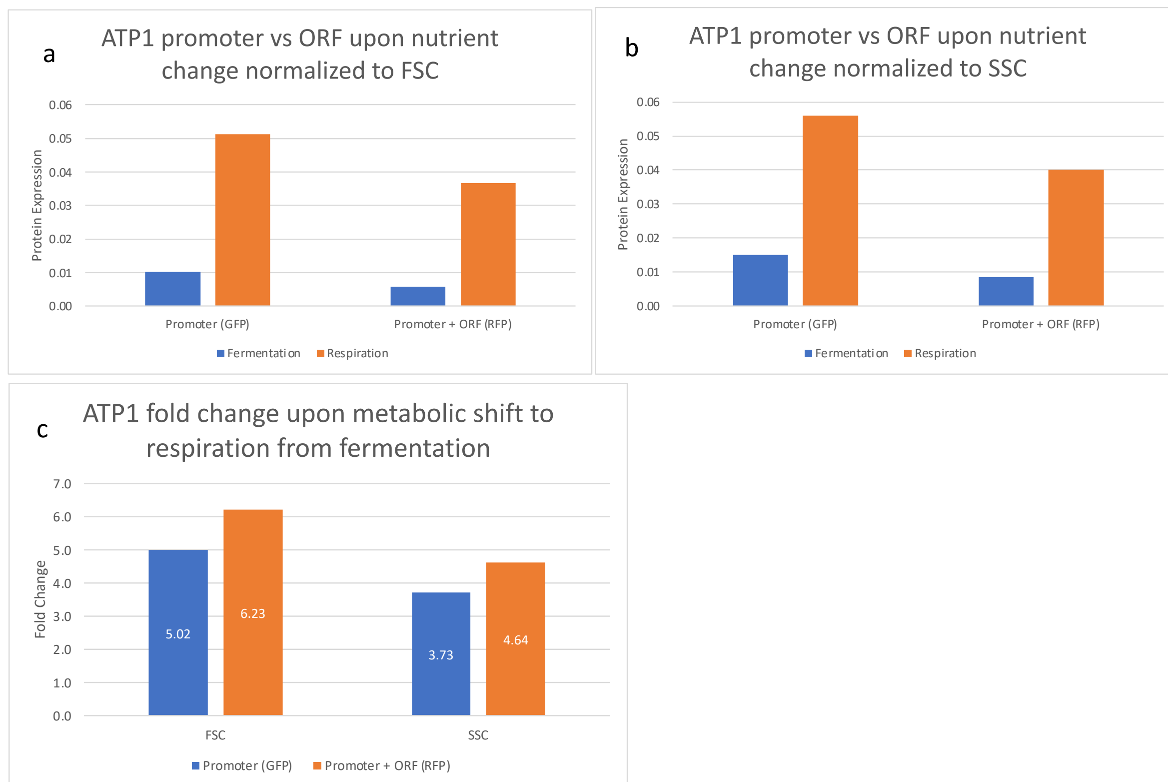

Figure 2.2.2 ATP1 promoter and ORF driving force to mRNA localization related gene expression
ATP1 mRNA protein expression changes are measured on FACS using the green and red fluorescence. The fluorescent levels are normalized to the cell size and complexity, FSC and SSC correspondingly.

a) Protein expression in ATP1 promoter and ATP1 ORF combined promoter upon metabolic shift normalized to cell size
b) Protein expression in ATP1 promoter and ATP1 ORF combined promoter upon metabolic shift normalized to cell complexity
c) Fold change in protein expressions in ATP1 promoter and ATP1 ORF combined promoter upon metabolic shift normalized to either cell size or cell complexity

The protein expression changes in GFP indicate the promoter driven gene expression changes caused by mRNA localization effect upon metabolic shift from fermentation to respiration. In contrast, the protein expression changes in RFP indicate the combined promoter and ORF driven gene expression changes caused by mRNA localization effect (Figure 2.2.2). For the ATP1 synthase gene, there was a 5.02-fold change in the protein expression upon metabolic shift for the only promoter strain when it was normalized to FSC, and there was a 6.23-fold change for the promoter combined ORF strain. The results of data indicate that the

combined ATP1 promoter and ATP1 ORF strain has larger effect on gene expression changes caused by the mRNA localization upon the metabolic shift. The fold changes normalized to SSC also showed a similar trend.

The increase in the only promoter strain possibly is caused by the transcriptional regulators. HAP4 is one of the well-studied transcriptional activators and is reported to be repressed in nutrients including glucose. [11][12][14] Due to the media switch to YPG from YPD in my experiment, the HAP4 expression could be induced by removing the repression factor for HAP4, which means there would be more transcription in respiration than in fermentation for all respiratory targets of HAP4, including ATP1. This media-based transcriptional increase from YPD to YPG would be the reason why the only promoter strain has a certain level of increase in the GFP expression. It means that the increase in protein expression of the only promoter strain is possibly caused by the transcriptional level, not caused by the mRNA localization. While the potential transcriptional induction of ATP1 is substantial, it is not the sole means of regulating ATP1 expression during the transition from YPD to YPG.

2.3 Conclusion and future direction

Here, we have found that ATP1 ORF strain including ATP1 promoter has more impact on protein production compared to the only ATP1 promoter strain. ATP3, 4 and 5 will be tested for the comparison of promoter and ORF. It is important to test ATP ORF strains which contains non-ATP promoters instead of ATP promoters.

Moreover, these strains we tested are made to test driving factors for the mRNA localization by manipulating nutrient conditions, and it will be interesting to test if the changes in translational elongation rates will have the same results as the nutrient changes. To perform this, we will apply the MET25 promoter into these strains to slow down the translational elongation.

Chapter 3 FACS-based CRIPSRi Screening

3.1 CRISPR system

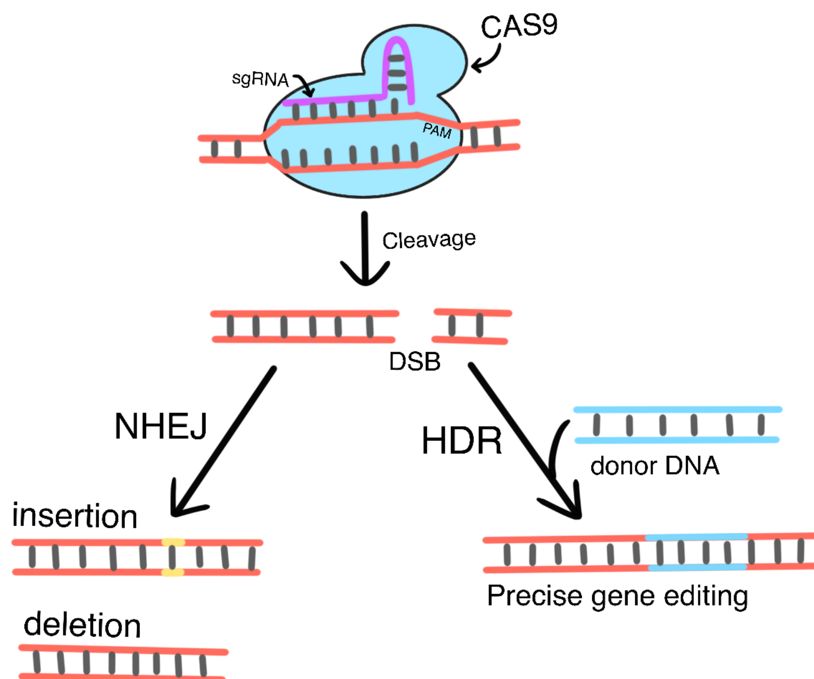

Figure 3.1.1 The CRISPR mechanism
CRISPR system requires Cas9 protein and single guide RNA which targets the DNA sequence to cleave the cut site on the target DNA sequence. After the cleavage, there are two methods to ligate the DNA sequence: non-homologous end joining and homology-directed repair. They result in either insertion, deletion or precise gene editing.

CRISPR, clustered regularly interspaced short palindromic repeat, was first discovered by Ishino et al and published in 1987. [9] In 2005, Emmanuelle Charpentier and Jennifer Doudna were awarded the Nobel Prize for developing CRISPR as a genome editing tool combining with the Cas9 protein. The mechanism has three steps: recognition, cleavage and repair. The first step is that the Cas9 proteins bind to sgRNAs. The PAM sequence near the target sequence is recognized by the Cas9 protein while the Cas9 screens the DNA helix. Once the PAM sequence is recognized by Cas9, the protospacer region on the sgRNA binds to the target sequence once the DNA helix is uncoiled. When the protospacer has the efficient

complementary region to the target sequence, the Cas9 protein cleaves the cut site on the target DNA sequence. The repair step has two methods to introduce mutations. One strategy is that during the repair process, the cell itself tends to introduce a base pair at the cut site, which causes the frame shift mutation, causing the following genes to not be expressed due to the frame shift. Also, this strategy tends to introduce deletion of a base pair and disrupts the following genes. The second method requires the designed donor DNA fragment to mutate itself to become the desired sequence that the DNA fragment is bound to in order to add the desired DNA sequence to the cut site (Figure 3.1.1). [22]

CRISPR interference, a genomic knockdown method using CRISPR system, is commonly used as a genetic perturbation technique, and it is commonly used for CRISPR screening (Figure 3.1.2). CRISPRi screening allows scientists to perform a large-scale screening for loss of function by applying differently designed gRNAs which delete different genes.

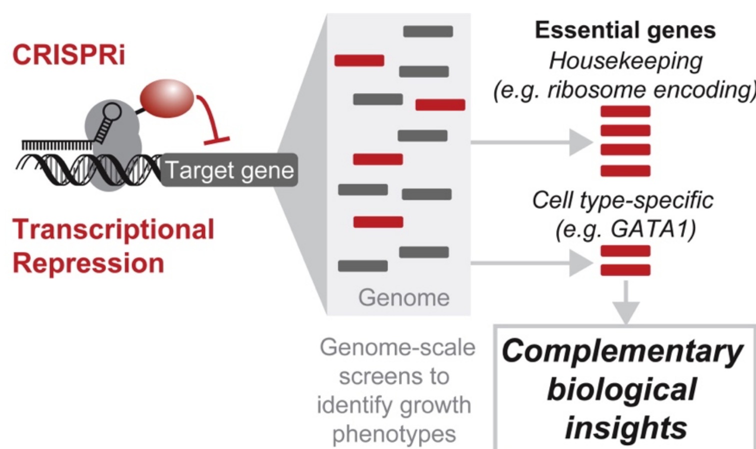

Figure 3.1.2 CRISPRi Screening flow
Cited from: [7]
CRISPRi is the gene editing pointing out specifically deletion. A group of gRNAs are inserted into cells that each gRNA targets different sequences. By comparing the deletions to phenotypes corresponding to the deletions, it identifies genes having specific responsibilities to cell features.

3.2 Inducible gRNA system

McGincy et al, Ingolia's lab, developed inducible gRNAs to add to the CRISPR interference guide library. [15] They introduced the RPR1 promoter and embedded two tetracycline operator sites (Figure 3.2.1.a). The operators allow the promoter to repress the downstream gene expression in the absence of tetracycline. On the other hand, in the presence of tetracycline, the operators will de-repress the downstream gene expression of the promoter (Figure 3.2.1.b). As the downstream gene, the guide RNAs are added after the promoter and expressed in the constant sgRNA scaffold. The various sgRNA sequences were introduced to perform CRISPRi screening. Under the presence of tetracycline, the dCas9-Mxi1 proteins are bound to the expressed gRNAs and target the complementary sequences in the targeted genome.

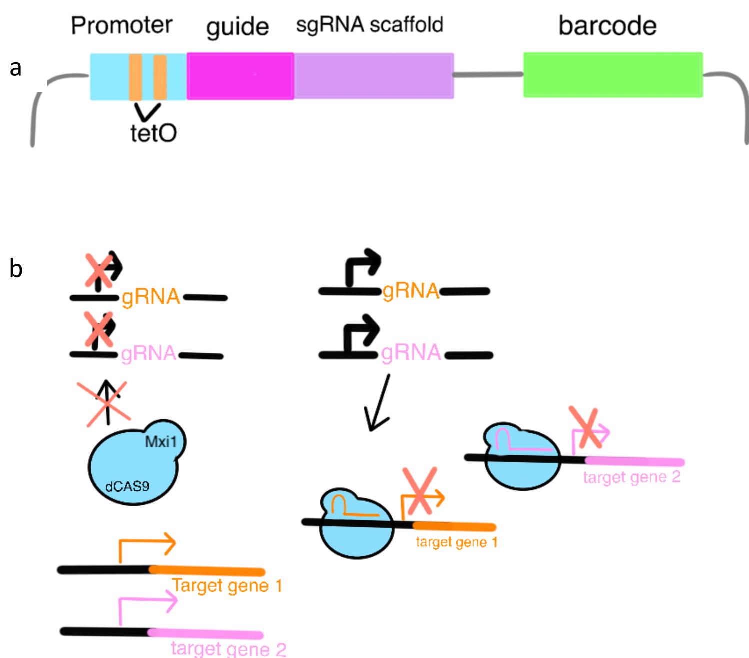

Figure 3.2.1 The tetracycline inducible gRNA construct and its inducing process

a) The construct of gRNA plasmids obtained from Ingolia's lab has tetracycline inducible operators embedded in promoter region.
b) The tetracycline presence initiates the gRNA transcriptions and the dCas9-Mxi1 protein inhibits the target DNA sequence to inhibit the downstream gene expression.

3.3 Mechanism of MCP-MS2 system

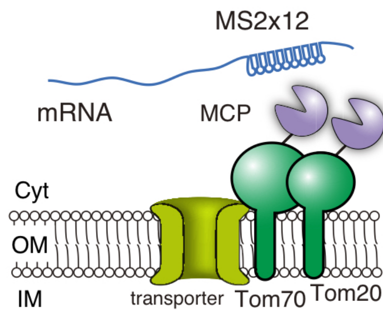

Figure 3.3.1 The MCP-MS2 system to tether mRNAs to mitochondria
Cited from [29]
MS2 coating protein (MCP) is bound to MS2 loop region on the mRNA in cytosol and tethers the mRNA to the mitochondrial membrane.

MS2 was found in bacteriophage and has a stem loop shape. Applying the system the bacteriophage uses, scientists introduced the MS2-tagging system into yeast and human cells (Figure 3.3.1). [28][8] It has been used for the research of TOM20 to manually tether the mRNAs to the mitochondria. [3] The MS2 coat protein can be fused on a surface of membrane to lead MS2 loop mRNAs to be localized to the membrane and translocated into the organelle. Beach et al has developed a system to track the mRNA localization and dynamics of its movement using MS2-MCP system. They introduced green fluorescent protein on MS2 coating protein, which allows mRNAs to be visualized upon the translation for MS2 loop mRNAs. [1]

3.4 Experimental design

Using the MS2-MCP system, the MS2 loop is introduced after yoGFP (green fluorescent protein), and the MS2 coating protein is introduced to the TOM70 protein. TOM70 is a protein which is embedded on the outer membrane facing the cytosol and helps with mRNA translocation from cytosol to the mitochondria by cooperating with Hsp proteins. As the negative control, Ruby (red fluorescent protein) is transformed into the yeast strain, which is not affected by the MS2-MCP system.

The tetracycline-inducible gRNA library obtained from the Ingolia Lab targets some genes which are involved in the mRNA localization of nuclear-encoded mitochondrial genes. We are aiming to determine which genes are involved in the gene expression changes due to mRNA localization of nuclear encoded mitochondrial genes compared to others by observing the GFP expression. FACS, fluorescent activation cell sorter, measures the GFP and RFP expression.

3.5 Optimizing promoter and FACS condition

To observe the GFP expression differences upon gene editing by CRISPRi, the GFP expression level before the gRNA introduction must be high enough to show the inhibition in the expression. The TIM50 promoter was embedded at the first time of the GFP expression and was checked on FACS; the GFP expression was low compared to what we expected. It led us to replace the promoter to other promoters, which give us the higher GFP expression. Peng et al performed the comparison experiment to figure out which promoters have higher gene expression downstream of the promoter, out of the twenty different promoters in seven different nutrient conditions. [19] They used a fluorescent marker to visualize the promoter effects on gene expression. In their results, the TDH3 promoter emitted the highest GFP expression in six out of the seven conditions, yet there were expression differences between the different nutrient conditions, which is not appropriate for our research. Compared to the TDH3 being steadily dependent on the nutrients, the TEF promoters similarly express the GFP protein in various nutrient conditions and placed second highest in terms of the expression experiments. Therefore, we replaced the TIM50 promoter to TEF1 and 2 promoters in our constructs.

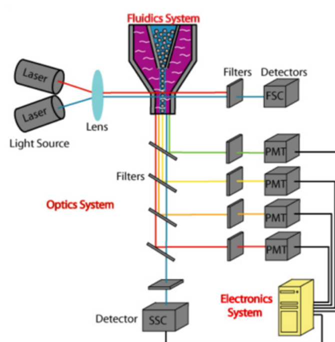

Figure 3.5.1 The FACS detecting sensitivity mechanism
Cited from: https://www.flowjo.com/learn/flowjo-university/flowjo/before-flowjo/58
The sample drop goes through different filters to distinguish the emission wavelengths after the lasers hit the drop particles. After the filters distinguish the wavelength, the PMT can multiply the intensity of the filtered fluorescence. This system allows it to control the detection sensitivity of each fluorescent marker.

Due to the replacement of the promoter that expresses the downstream genes particularly well, the FACS lasers caused measurement bleed-through from the GFP emission range to RFP mission range. To fix this interference into the RFP measurement, it was necessary to change the sensitivity in the fluorescent detector, photomultiplier tubes (PMT) voltage, against both the RFP and GFP emission (Figure 3.5.1). By decreasing the GFP PMT voltage, it lowered the sensitivity to detect the fluorescent to allow it to get rid of the GFP bleed-through to the RFP measurement.

3.6 Expression increase upon MCP addition on FACS

Two yeast stains were made: one has TEFp-yomGFP-MS2, TEFp-mRuby and TOM70-MCP, and the other one has TEFp-yomGFP-MS2 and TEFp-mRuby. The difference between these two strains is that the one with TOM70-MCP tethers the TEFp-yomGFP-MS2 mRNA to mitochondria manually. They were grown in YPD media to OD below 0.8 to keep them in the fermentative metabolic state.

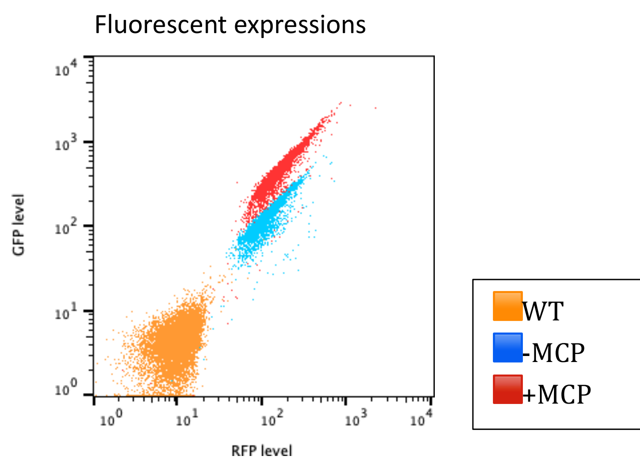

Figure 3.6.1 The increase in protein expression upon the mRNA tethering to mitochondria
The x-axis is the RFP measurement for the Ruby marker, and the y-axis is the GFP measurement for the yomGFP marker. The data is measured on FACS. The orange is the wild type, the blue is the population of -MCP strain and the red is the population of +MCP strain.

After normalizing the fluorescent expression based on the cell size and internal complexity, the data showed 4.09-fold increase upon the TOM70-MCP addition to the protein expression with MS2 loop (Figure 3.6.2.a and b). The data shows 1.57-fold change in the protein expression without MS2 loop upon MCP addition. Those data indicates that MS2 loops help mRNAs with tethering them to mitochondria, which induces the protein expressions.

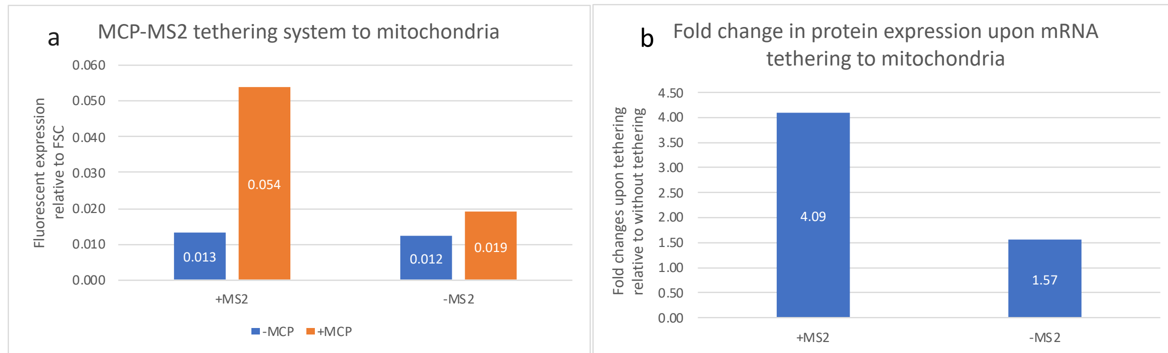

Figure 3.6.2 The average of fluorescent protein expression upon tethering to mitochondria with or without MS2 loop

a) Fluorescent expression levels with or without MCP, for mRNAs with and without MS2 loop.
b) Fold change in the fluorescent expression levels upon the MCP addition for mRNA with or without MS2 loop relative to -MCP

3.7 Conclusion and future direction

We confirmed that the MS2 tethering system allows us to have induced protein production on its mRNA, which is not the case for mRNAs without MS2 loops. It is likely caused by the increased localization to mitochondria surface by the MS2 coating protein tagged on TOM70.

The first step for the CRISPRi screening is to optimize the cell harvest timepoint after applying tetracycline on FACS using the yeast strain which has induced mRNA localization with the MCP. We will use the TOM70 targeted gRNA to deplete the TOM70, which is upstream of MCP, and we suspect that the point deletions on TOM70 sequence cause the MCP interruption and inhibit the GFP expression upon the TOM70 gRNA application. It will determine how long the strain takes to show the gRNA effects on the GFP expression.

Once figuring out the cell harvest timepoint, the full screening with the gRNAs obtained from the Ingolia Lab will be performed, and the yeast cells will be cell sorted based on the GFP expression level on FACS to distinguish between low, middle and high expression. After the cell sorting, the harvested cells will go through treatments to be sent to next-generation sequencing.

We will compare the sequencing data to the phenotype, GFP expression, which will give us insight as to which genes are involved in gene expression changes caused by the mRNA localization since the lowered GFP expression is expected to be caused by an mRNA localization deficiency.

Chapter 4 Conclusion

In my thesis, we are interested in the factors which are involved in the post transcriptional regulations affecting the respiratory related gene expression upon metabolic changes. In chapter 1, we designed experiments to determine how slowing the translational elongation rate affects protein productions for conditionally mitochondrial localized respiratory-related genes. Our qPCR data shows that the deletion of YEF3 gene using the MET25 promoter increases in some ATP synthase mRNA production as the concentration of methionine was increased. This result suggests that slowing the translational elongation rate has an indirect effect on the increase in ATP synthase RNA transcripts and implies there is the possibility of a metabolic switch to respiration.

In chapter 2, the promoter is found to carry certain responsibility of gene expression changes by localizing the mRNAs of ATP synthase genes to mitochondria upon the metabolic shift to respiration. However, the ORF including the promoter has the stronger effect on gene expression by the mRNA localization of those genes compared to the only promoter effect.

In the chapter 3, I introduced the CRIPSRi screening to identify the genes which are involved in the gene expression changes caused by the mRNA localization using the inducible gRNAs. MS2 tagging to the mitochondria allows us to have constitutively tethered mRNAs to the mitochondria in order to run the CRISPRi screening, which is found by the strains with or without MCP having 5-fold change upon MCP added. Combining this screening to the fluorescent activation cell sorter that measures fluorescent emissions, the FACS-based CRISPRi screening will be performed to analyze which genes are involved in gene expression changes resulting from the mRNA localization to mitochondria.

To confirm the metabolic state is switched to respiration, it will be important to measure the O2 consumption or ethanol production using those experimental designs and strains. This confirmation will provide us with a decent idea of the relations between translational elongation rate and control of the metabolism in yeast cells.

Chapter 5 Materials and methods

5.1 Yeasts and Plasmids

The Saccharomyces cerevisiae strains in those projects is called "S288C MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0" "S288C MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ" and "W303-1a MATa ade2-1 can1-100 his3-11 leu2-3 trp1-1 ura3". The Yeast strains made for that research are listed on the following table:

Table 5.1.1 Yeast strains required in thesis

Name of Yeasts	Functions	Source
ZY846:VKY8-pMET25	pMET25 strain	Lab Stock
ZY847: ZY10-ATP1p-GFP, ATP1ORF-mRuby2	Promoter vs ORF	Lab Stock
ZY8-TEF2p-eGFP-MS2, TEF2p-mRuby, TOM70-MCP, dCas9-Mxi1	MCP-MS2	Lab Stock
ZY8-TEF2p-eGFP-MS2, TEF2p-mRuby, dCas9-Mxi1	MCP-MS2 control	Lab Stock

5.2 MET25 promoter methionine treatments

Methionine aliquot was made as 0.25M to use it for 0.5mM and 2mM in liquid cultures.

All cultures' pre-treatments should be in log phase, OD00.3-0.8, to avoid the glucose starvation shifting the metabolic state from fermentation to respiration. The methionine treatments were proceeded by the following steps for the growth curve. (Chapter 1)

1. Culture is incubated at 30C for overnight, keeping them in either lag or log phase without methionine treatments.
2. Methionine is added into the cultures at concentrations 0, 0.5 and 2mM.
3. The post-treatment cultures are incubated overnight, keeping them in either lag or log phase.
4. The post-treated cultures are diluted to nearly OD0.05 and the OD is measured and monitored for next 24 hours.

For strains to measure the RNA transcripts, the RNA effects upon methionine treatments are considered to take up to 4 hours after the treatment (Chapter 1):

1. Culture is incubated at 30C for overnight with keeping them in either lag or log phase without methionine treatments.
2. Methionine is added into the cultures, 0, 0.5 and 2mM.
3. The cells are harvested at 0, 1, 6 and 24 hours by being centrifuged down and freezing in liquid nitrogen.

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