UNIVERSITY OF CALIFORNIA SAN DIEGO

# Effects on Protein Production in *S. cerevisiae* by Localization to Mitochondria

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by

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The thesis of Jordan Leff 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

2020

# DEDICATION

This work is dedicated to my family and friends, near and far, who have supported and encouraged me throughout this entire process.

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I would like to acknowledge Professor Brian Zid for all of his support over the past year. Working in the Zid lab has been one of the most rewarding experiences of my academic career, and it would not have been possible without his guidance and understanding.

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ABSTRACT OF THE THESIS

Effects on Protein Production in *S. cerevisiae* by Localization to Mitochondria

by

Jordan Leff

Master of Science in Chemistry

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Professor Brian Zid, Chair

Mitochondrial-localized mRNAs have implications in various neurodegenerative diseases including Parkinson's Disease. Recent findings show that in yeast, certain mRNAs exhibit different localization to the mitochondria that is dependent on the switch from fermentative growth conditions to respiratory growth conditions. This localization is accompanied by an increase in both mRNA number and protein expression levels. We investigated if these increased levels of mRNA and protein expression were due to transcription or translation through RT-qPCR and FACS. We also explored genes that are essential to protein expression at the outer membrane of the mitochondria through a genetic screen that utilized microscopy and image analysis. We found that the increased mRNA and protein expression levels are mostly due to

transcription of mRNAs. Furthermore, we identified three genes that are essential for protein expression at the mitochondria, including *fzo1*, *om45*, and *rps26b*.

# 1. Introduction

### 1.1 Cells recognize and adapt to changing environmental conditions

The cell is the smallest unit of life, and one of the most basic survival techniques conserved across all organisms is the maintenance of cell conditions in order to overcome the effects of stressful surroundings. One such example of cells recognizing and adapting to environmental changes is the switch from fermentative to respiratory growth in budding yeast *Saccharomyces cerevisiae*. This switch in growth type depends on the depletion of nutrients including oxygen and glucose. Fermentative growth occurs when glucose is available for use as a carbon source, and yeast is able to convert carbohydrates to ATP, with ethanol and carbon dioxide produced as byproducts. When glucose supply runs out, yeast will switch into respiratory growth, in which they will consume the ethanol in order to maintain this second, slower growth phase (Stahl et al. 2004).

The switch in growth condition has also been shown to effect size and morphology of cells and their organelles, specifically the mitochondria. When *S. cerevisae* switch from fermentative to respiratory conditions, the volume of the mitochondria increases as it functions to produce ATP through oxidative phosphorylation. In addition, the overall cytoplasmic volume of the cell decreases. This leads to an increase in mitochondrial volume fraction, which could effect localization of mRNAs and translation of nuclear-encoded proteins (Tsuboi et al. 2020).

### 1.2 Mitochondrial size and translational regulation within disease

Parkinson's Disease (PD) has been linked to mutations in two separate genes, PRKN and PINK1 (Kitada et al. 1998, Valente et al. 2004). Decreases in mitochondrial membrane potential stabilize levels of mitochondrial PINK1 (PTEN-induced kinase 1) which then recruit and activate the ligase activity of Parkin (E3 ubiquitin ligase), which is inactive while in the cytoplasm (Narendra et al. 2008, Matsuda et al. 2010). Mutations consistent with PD have been shown to disrupt the role of these factors in mitochondrial quality control, highlighting the importance of mitochondrial regulation in the prevention of disease (Geisler et al. 2010, Lee et al. 2010).

PINK1 and Parkin have also been linked to localization of nuclear mRNAs that encode proteins of the five respiratory chain complexes (RCCs) which are responsible for OXPHOS (Gehrke et al. 2015). This study focused on nuclear encoded mRNAs for RCC components (nRCC mRNAs), which were found to be bound to the outer membrane of the mitochondria in an active state while being translationally inactive in the cytoplasm, suggesting localized translation. In Drosophila, PINK1 was shown to regulate nRCC mRNA localization in a tissue dependent manner, as PINK1 mutation led to a reduction in nRCC mRNAs bound to the mitochondria in neuromuscular tissues, but not intestinal tissues. Disruption of components of the TIM/TOM complex further amplified the effects of PINK1 mutation on nRCC mRNA localization, showing that localization is linked to co-translational import of proteins. Furthermore, PINK1 or Tom20 over-expression reciprocally rescued the deletion of the other, suggesting that PINK1 and Tom20 could work together in the protein import pathway, possibly via PINK1 recruitment of mRNAs, with subsequent docking to the mitochondrial outer membrane by Tom20 (Gehrke 2015).

### 1.3 mRNA localization facilitates protein translation and vice versa

Functions of mRNA localization to the targeted area of the encoded protein include the regulation of further protein synthesis, aid in the assembly of organelle complexes, and specific localized function of proteins; and is an important mechanism that is conserved from yeast to humans. It is also speculated that by restricting translation to the target site, the cell is able to save energy that would otherwise be spent on the active transport of large proteins along the cytoskeleton (Holt and Bullock 2009). The transportation of a single mRNA would be more energy efficient, especially if it can remain at the target site to provide multiple copies of a protein. Localization of mRNAs can also allow for a quickened production of proteins that are necessary to keep up with the constantly changing conditions of the cell, and local translation reduces the risks of unwanted interactions or folding as the protein travels from the cytosol to its final destination.

Localization that occurs before an mRNA is translated into a protein is dependent on RNA binding proteins (RBPs) which recognize and bind to a specific portion of the newly transcribed mRNA's sequence, known as a zip code. The mRNA is held in a translationally inactive state until it reaches its target destination, in which case it is able to begin translation of its encoded protein (Besse & Ephrussi 2008). Once the RBP is bound to an mRNA, the messenger ribonucleoprotein particle (mRNP) may localize in several ways. The localization methods we will focus on include active-transport and facilitated diffusion (Eliscovich et al. 2013). Active transport for mRNA localization involves mRNPs that contain motor proteins which travel along the cytoskeleton in a specific direction, fueled by the hydrolysis of ATP.

Motor proteins include myosins that “walk” along the actin cables of the cytoskeleton, as well as kinesins and dyneins which travel along microtubules. (Depina and Langford1999). While active transport is a more costly effort in terms of energy, it is also a quicker method than passive transport. Myo4p in yeast has been shown to move at speeds of 0.20-0.44 μm/sec (Bertrand et al. 1998), whereas mRNA diffusion coefficients can range from 0.02 - 0.2 $\mu m^2$/sec, in organisms ranging from yeast to chicken and human cells (Ben-Ari et al. 2010, Yan et al. 2016).

In budding yeast, ASH1 is a well studied transcription factor that undergoes localized translation, facilitated by RBPs and active transport. ASH1 (asymmetric synthesis of homothallic switching (HO)) represses the transcription of the HO endonuclease in order to prevent mate type switching in daughter cells (Bobola et al. 1996). This cell type specific mechanism is due to the localization of the ASH1 mRNA to the bud tip of yeast cells during the latter part of anaphase (Takizawa et al. 1997). The She2 RBP recognizes multiple stem-loop regions in the coding sequence and 3’UTR of ASH1. Further association of She3 adaptor protein and molecular motor Myo4p allows for active transport along the actin cytoskeleton to the bud nucleus, where ASH1 is translated. (Shepard et al. 2003).

Another example of cytoskeleton traversing mRNAs is osk (oskar) mRNA in Drosophila oocytes, which localizes to the posterior pole of oocytes and is essential for development of germ plasm and a positive feedback loop that regulates microtubule polarity for further mRNA localization (Zimyanin et al. 2007). Localization of osk utilizes plus-end directed kinesin motor proteins to walk along microtubules, which bind to oskar via localization elements found in the 3’ UTR (Brendza 2000). Upon reaching the posterior pole, osk maintains localization through association with myosin V, which anchors osk to the actin cytoskeleton (Krauss et al. 2009).

Passive transport via diffusion is another process of mRNA localization. Instead of using ATP, or any energy source for that matter, this mechanism sees mRNPs randomly diffuse into the cytoplasm. A localized protein receptor is necessary in order to "trap" the mRNP complex at the site of intended translation. While this method saves energy for the cell, it is a much slower and less direct process than to active transport.

Localization of mRNAs to the Endoplasmic Reticulum (ER) is a well studied phenomenon which is worth exploring in the context of mitochondrial localization, as the mechanisms of ER localization draw parallels to that of the mitochondria. Furthermore, the two organelles make contact in multiple sites and work together to regulate various cellular functions including calcium transfer and the biogenesis of phagophores and inflammasomes, possibly explaining the similarities they share in methods of mRNA localization and protein import (Marchi et al. 2014). Localization of mRNAs to the ER exists in both a nascent peptide chain dependent manner as well as a RNA dependent manner, similar to the mechanisms proposed for the mitochondria. The co-translational model relies on the signal recognition particle (SRP) ribonucleoprotein that recognizes hydrophobic signal sequences in the N-terminus of nascent secretory protein. The SRP halts further elongation of the polypeptide, and then targets the ribosome-polypeptide complex toward the ER. The local slowdown of ribosomes increases the chance that the ER will recognize the SRP and consequently promote its association with the ER surface (Zhang and Shan, 2012). The SRP then docks to an SRP receptor on the ER membrane, and the nascent chain is fed into the Sec61p channel, known as the translocon, while polypeptide elongation resumes as the SRP dissociates from the complex (Blobel et al. 1982, Gorlich and Rapoport 1993). While other methods of mRNA localization to the ER exist, the co-translational

localization model has been widely accepted within the field for decades, and certainly draws some parallels to the emerging idea of mitochondrial co-translational localization and protein import.

Nascent polypeptide chains have also been implicated in mRNA localization to the mitochondria. Certain polypeptides have been found to contain an N-terminal mitochondrial signal (MTS) that is sufficient to direct the newly synthesized protein to the mitochondria, and is cleaved once localization is complete, similar to that of secreted proteins. This signal is recognized by protein receptors on the outer membrane of the mitochondria, namely the translocase of the outer membrane (TOM) complex, which, as later described, is the initial contact point for mitochondrial protein import. It is suggested that the association of nascent polypeptides with these receptors lead to an enrichment of mRNAs encoding for mitochondrial proteins at the outer membrane. Deletion of Tom20, a key subunit of the TOM complex, has been shown to decrease mitochondrial association of many mRNAs that are known to normally localize to the mitochondria, suggesting that this protein receptor plays an important role in anchoring mRNAs as they are being translated (Eliyahu et al. 2010).

Another example is Puf3 mediated localization. Puf3 is a well-known pumilio family RBP, conserved from yeast to humans, that is associated with the outer membrane of the mitochondria and plays a key role in mitochondrial biogenesis. Puf3 binds to specific motifs in the 3' UTR of mRNAs encoding for mitochondrial proteins, and regulates both translation through deadenylation, and mRNA stability. In yeast, Puf3 has been specifically shown to regulate deadenylation rate of Cox17 (cytochrome c oxidase copper chaperone 17), which encodes for a protein that may be involved in the recruitment of copper to mitochondria for use

in the COX mitochondrial respiratory chain complex, suggesting a role for Puf3 in the realm of mitochondrial biogenesis (Olivas 2000). Further study identified a class of mRNAs that contain specific Puf3 motifs (including COX assembly factors) that are localized to the mitochondria under the presence of Puf3, and mostly dissociated upon Puf3 deletion. The majority of these mRNAs encode for mitochondrial proteins that are essential for early mitochondrial biogenesis (Saint-Georges et al. 2008).

A further study explored a yeast cell strain in which both Tom20 and Puf3 were deleted, as well as strains in which only one gene was deleted. Under fermentative conditions, all strains grew well, with no significant defects. When switched to respiratory conditions, results indicated that while single deletion strains grow normally, the double deletion strain suffers severe growth defects. Adding back endogenously expressed Puf3 and Tom20 proteins to the double deletion strain led to rescue of growth, suggesting that both of these proteins are necessary for cells under stressful respiratory conditions. This suggests that both post translational and pre-translation localization of mRNAs are essential for cell function under conditions that require highly functioning mitochondria (Eliyahu et al. 2010). Once localization and translation have begun, proteins must be further organized for maintenance of organelles and management of functioning cells. In the case of mitochondria, two distinct methods exist for processing of proteins.

### 1.4 Mitochondrial proteins can be imported post- and co-translationally

Of all the approximately 900 proteins necessary for mitochondrial biogenesis, only 13 are encoded from mitochondrial DNA. Therefore, the vast majority of necessary nuclear encoded proteins must be translated in the cytosol and imported to the mitochondria. There are currently

two methods proposed for the creation, transportation, and subsequent import of mitochondrial proteins – a co-translational pathway and a non-co-translational pathway. Until fairly recently, the most popular theory is that mitochondrial proteins are translated in the cytosol and then translocated and imported to the inner membrane, intermembrane space, or outer membrane to serve their intended purpose.

While post-translational import is a well characterized and widely accepted mechanism, it is a complex sequence of events that is briefly summarized here. In order for these proteins to enter the mitochondria, they must first interact with the Translocase of the Outer Membrane (TOM) complex. This complex includes protein receptors Tom20 which recognizes an N-terminal MTS of pre-proteins, and Tom70 which recognizes hydrophobic pre-proteins containing internal signals. Cytosolic chaperones, such as HSP70 (heat shock protein 70), play a role in stabilizing the pre-protein and guiding it toward the TOM complex (Stan et al. 2003). After recognition, the pre-protein travels through the outer membrane-spanning translocase pore and begins interaction with the Translocase of the Inner Membrane (TIM) complex. The Tim23 complex specifically, which is composed of proteins including Tim50, Tim23, and Tim 21, sorts proteins for transport to the inner membrane or to the mitochondrial matrix. The membrane potential is important for inner membrane import, as a negative matrix-side potential drives pre-proteins with positively charged MTSs toward the TIM complex. Tim23 also undergoes a voltage-mediated conformational change necessary for protein import. Mitochondrial membrane potential is sufficient to sort proteins to the mitochondrial inner membrane, but pre-proteins destined for the mitochondrial matrix require the further aid of HSP70 to form an ATP driven

motor that can shuttle the pre-protein into the matrix, where it undergoes further folding in order to reach maturity (Wiedemann and Pfanner 2017).

While post-translational import has been well characterized for decades, the co-translational model of importing a protein that is being synthesized at the outer mitochondrial membrane is a relatively new idea. This process utilizes the nascent chain associated complex (NAC), a heterodimeric protein which associates with ribosome nascent chain complexes (RNCs), consisting of a nascent polypeptide chain with its C terminus still attached to its translating ribosome. NAC binds to ribosomes near the protein exit tunnel and has been shown to target ribosomes to the mitochondria. Eliminating NAC and Mft52p, a cytosolic targeting factor that further helps it localize to the mitochondria, is shown to lead to mitochondrial defects, loss of mitochondrial DNA, and disturbance of organelle morphology, suggesting that NAC is essential to mitochondria biogenesis (George et al. 1998). While the receptor for NAC was unknown for quite a while, a 2014 study identified outer mitochondrial membrane protein OM14 as a NAC receptor, as well as an important factor for co-translational import (Lesnik et al. 2014).

An even more recent study, in which yeast mitochondria were purified from cells treated with magnesium ions and cycloheximide-- in order to stabilize RNCs, and stall the ribosomes, respectively-- was able to visualize ribosomes bound to the outer mitochondrial membrane through electron cryo-tomography (cryoET). Furthermore, these ribosomes were oriented such that the polypeptide exit tunnel points toward the outer membrane, suggesting that the nascent chains will be imported to the mitochondria as they emerge from the ribosome. The association between ribosomes and the TOM complex was seen to be reversible with release of nascent

chains from the ribosome, strongly suggesting that mitochondrial imports are occurring as these ribosomes locally synthesize proteins (Gold et al. 2017).

### 1.5 Condition Dependent Localization and Translation

This thesis is heavily influenced by the work of Dr. Tatsuhisa Tsuboi, a post doctoral fellow of the Zid Lab, and his findings are used as control factors in various experiments. One of his most interesting findings is that certain mRNAs experience different localization to the mitochondria depending on growth condition (Tsuboi et al. 2020). Three mRNAs were studied in fermentative and respiratory growth conditions, including TIM50, ATP3, and. TOM22. TIM50 mRNA contains an MTS and has been previously shown to be localized to the mitochondria. Both TIM50 and ATP3 mRNAs contain a mitochondrial targeting signal, while TOM22 does not. It was found that TIM50 mRNA is always localized to the mitochondria, regardless of growth condition. TOM22 mRNA was shown to never be localized to the mitochondria, regardless of growth condition. However, while ATP3 mRNA was shown to not be localized to the mitochondria under fermentative conditions, under respiratory conditions this mRNA does localize to the mitochondria (Figure 1).

It was also shown that ATP3 experiences an increase in mRNA number and protein expression when localized to the mitochondria under respiratory conditions. When localized to the mitochondria, ATP3 sees about a 2 fold increase in mRNA number, and about a 4 fold increase in protein expression levels. In comparison, TIM50, the mRNA that is always localized to the mitochondria, experiences no change in mRNA number or protein expression levels when growth conditions are switched (Figure 2).

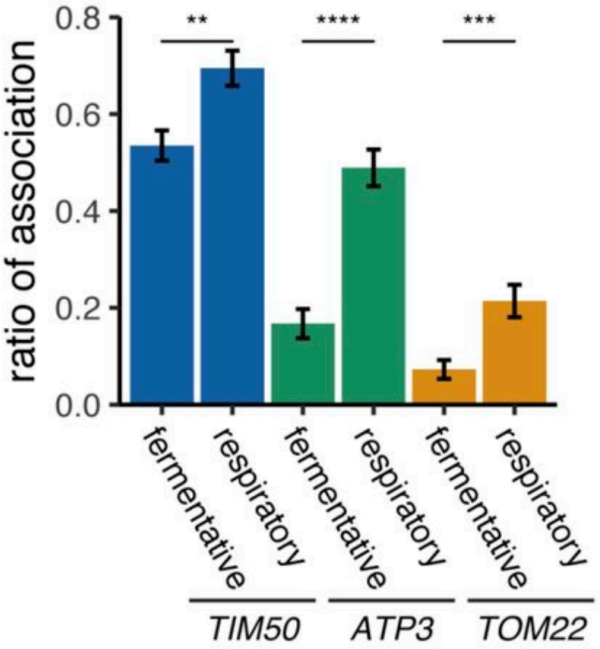



**Figure 1. ATP3 undergoes condition dependent localization.** Ratio of mitochondrial associated mRNAs under fermentative and respiratory conditions (Tsuboi et al 2020).

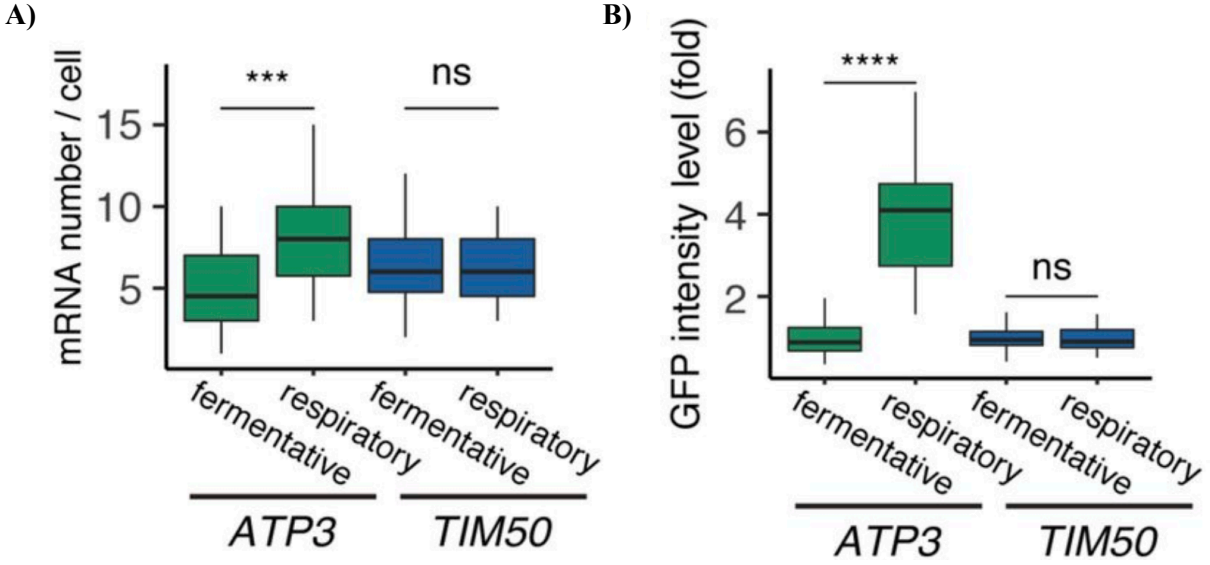



**Figure 2. ATP3 sees increase in mRNA number and protein expression levels with growth condition switch.** A) mRNA number per cell for ATP3 and TIM50 in fermentative and respiratory conditions. B) GFP protein expression for ATP3 and TIM50 in fermentative and respiratory conditions (Tsuboi et al 2020).

### 1.6 MS2 Coat Protein Tagging System

In order to tether mRNAs to the mitochondria for further study, a tagging system dependent on the MS2 Coat Protein (MCP) was utilized. In this system MCP was used to tag TOM70 and TOM20 protein receptors on the outer membrane of the mitochondria (Tsuboi et al 2020). MCP recognizes and binds to the MS2 sequence in the 3' UTR of certain mRNAs, effectively tethering them to the mitochondria for localized translation (Figure 3). It was found that tethering TIM50-flag-GFP, ATP3-flag-GFP, flag-GFP, and Tub1 (control) mRNAs to the mitochondria using MCP tags on either TOM70 or TOM20 yielded a significant increase in protein expression, as compared to strains not expressing an MCP (Figure 4).

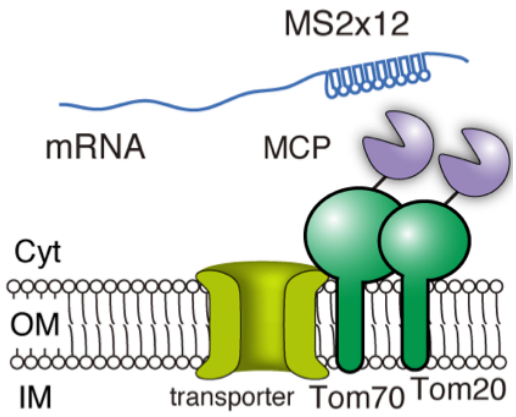



**Figure 3. MCP Tagging System.** Cartoon of MCP tagging system, in which TOM Complex protein receptors are tagged with MCP, which binds MS2 sequence on mRNAs (Tsuboi et al 2020).

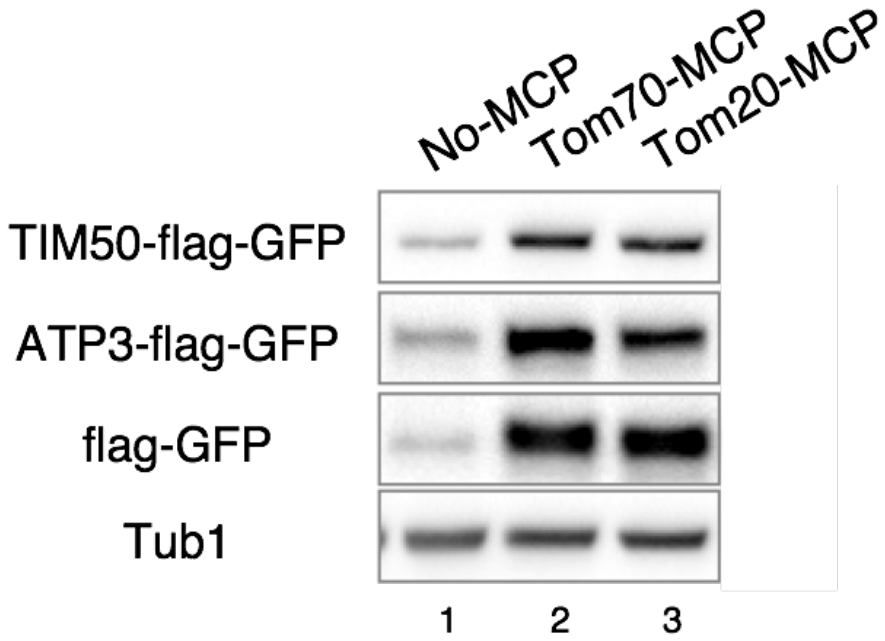



**Figure 4. Tethering mRNAs to the mitochondria increases protein expression.** Protein expression of TIM50-flag-GFP, ATP3-flag-GFP, and flag-GFP increases when tethered to the mitochondria with MCP tagging (Tsuboi et al 2020).

### 1.7 Focus of project

The idea behind these experiments was to further explore some of Dr. Tsuboi's findings. Previous data showed that ATP3 experiences an increase in both mRNA number and protein expression when growth condition is switched from fermentation to respiration. It was assumed that this increase was due to ATP3 mRNAs localizing to nearby mitochondria and inducing increased translation. One thing that we wanted to explore was if this increase is more so due to transcription of genes or translation at the mitochondrial outer membrane. In order to study this, we developed a strain that contains only the promoter sequence of ATP3 driving GFP, thus eliminating the ATP3 open reading frame (ORF). By comparing results to the previously studied ATP3 mRNA, we aim to study what contribution the coding sequence has to mRNA number and protein expression when cells experience a switch in growth condition from fermentation to respiration, thereby facilitating the localization of ATP3 mRNAs to the mitochondria.

The second goal of this work was to determine what genes are essential for protein expression at the outer membrane of the mitochondria. Previous data showed that when TIM50 and ATP3 mRNAs are tethered to the mitochondria with TOM70-MCP and TOM2-MCP, there is a significant increase in protein expression. In order to elucidate what genes are most important for this localized protein expression, we studied thirteen strains of *S. cerevisiae* with single gene mutations that we predict could effect mitochondrial protein production. In order to determine what genes are "essential" or not, we looked for deletions which caused an overall decrease in GFP expression when TIM50-GFP-MS2 is localized to the mitochondria via MCP tagged TOM70.

## 1.8 Acknowledgment

Chapter 1, in part is currently being prepared for submission for publication of the material as it may appear in Biochemical Society Transactions, 2020, Leff, Jordan; Tsuboi, Tatsuhisa, Portland Press, 2020. The thesis author was one of the primary authors of this paper.

# 2. Materials and Methods

## 2.1 Cloning of ATP3pr-GFP

### 2.1.1 PCR amplification

Cloning of the ATP3pr-GFP strain began with PCR amplification of the promoter sequence of the ATP3 gene as well as the green fluorescent protein (GFP) sequence. These cassettes were amplified from plasmids and primers that were made available from other members of the Zid Lab. Cassettes were verified by comparing predicted size to results of a 1% gel electrophoresis analysis. PCR fragments were treated with Dpn1 restriction enzyme for 1 hour at 37˚C and cleaned using a Zymo Research DNA Clean and Concentrator kit.

### 2.1.2 Gibson assembly

Following purification, Gibson Assembly was used to orient PCR fragments into a pRS403 vector that was linearized using SacI and AflII restriction enzymes. A 3:1 fragment to vector ratio was used. NEB 2X Gibson Assembly Master Mix was used in the reaction, which was carried out for 1 hour at 50˚C, and then transformed into DH5α *E. coli.* Following bacterial transformation, restriction enzyme digestion and sequencing was used to verify plasmids.

### 2.1.3 Yeast transformation

The ATP3pr-GFP plasmid was then transformed into the W303 wild type strain of *S. Cerevisiae* using a High Efficiency Transformation protocol (Gietz, R.D. and R.A. Woods. 2002). Transformants were then plated on an agar plate containing YNB, glucose, and SC without histidine, as the vector contained the *His3* yeast selection marker. The plates were then incubated at 30˚C and allowed to grow for 2 days.

## 2.2 Transformation of mutant strains

### 2.2.1 Transformation of TIM50-GFP/RFP plasmid into 13 strains of *S. cerevisiae*

Thirteen strains of *S. cerevisiae* with single gene mutations were obtained from fellow lab member, Dr. Tatsuhisa Tsuboi. Mutations included deletion of *clu1, fzo1, om14, om45, rpl1, rpl2, rps26b, slf1, sro9, tom5, tom20*, and *tom70*. A W303 strain with no mutation was used as the control strain. A plasmid containing sequences for TIM50pr-GFP-MS2 and TIM50pr-RFP was also obtained from Dr. Tsuboi. This plasmid was transformed into all thirteen strains.

### 2.2.2 Colony Selection

Two colonies of cells were selected based on visual appearance. Colonies were selected based on shape, size, color, and growth rate. The two colonies were then grown in a 96 well plate and visualized under a Revolve microscope in order to determine which colony would be selected for further analysis. “Healthiest” colonies were selected based on size, shape, and absence of vacuoles in cells and were grown in an overnight culture of YPD media. Two cultures of each strain were then saved, one to be used as the non-localized control in the GFP intensity experiment, and one for further transformation of a plasmid containing TOM70-MCP, as described below.

### 2.2.3 Transformation of TOM70 plasmid into 13 strains of *S. cerevisiae*

Using the healthiest colony selected in the previous step, a plasmid containing TOM70-MCP, which was also obtained from Dr. Tsuboi, was transformed into each of the thirteen strains, now also containing GFP and RFP.

### 2.3 qPCR of ATP3pr-GFP

Quantitative Reverse Transcription PCR (RT-qPCR) was used in order to analyze changes in mRNA number in cells that are switched from fermentative to respiratory conditions. Two sets of ATP3pr-GFP cells were grown in parallel in YPAD, a complex media containing glucose, and YPAGE, a complex media containing glycerol in order to facilitate fermentative and respiratory growth, respectively. Once reaching OD of ~0.6, cells were frozen with liquid nitrogen, and RNA was extracted. Next, cDNA was synthesized in order to perform RT-qPCR. Primers amplifying the GFP region of cells were used, as well as a primer amplifying B-actin to be used as a control in the experiment. RT-qPCR results were measured and analyzed, with GFP values for both glucose and glycerol containing cells normalized by B-actin control values.

### 2.4 FACS analysis of ATP3-P2A-GFP and ATP3pr-GFP

To examine the change in protein expression from fermentative to respiratory conditions, Fluorescence-activated Cell Sorting (FACS) was used. The ATP3pr-GFP strain was used again as the experimental strain. For control, a strain that was already available in the lab of ATP3-P2A-GFP was used. Cells were again grown in parallel in both YPAD (glucose) and YPAGE (glycerol) media. Cells were analyzed using a Bio-Rad FACS machine. Data was expressed as dot plots showing clusters of similar events, and as a bar graph of geometric mean fluorescence intensity

### 2.5 Microscopy of mutant strains

After transformation of plasmids containing TIM50pr-GFP-MS2, TIM50pr-RFP, and TOM70-MCP, it was noticed that colonies of varying sizes and appearance were growing on each plate. One large colony that appeared to be growing well was selected for analysis and labelled as the “Fast Growing” Colony. One small colony that appeared to be lagging behind in growth was selected for analysis and labelled as the “Slow Growing” Colony. It was expected that there was some additional mutation in this strain that was leading to slower growth. Both types of colonies, as well as a control colony, not containing TOM70-MCP were imaged under the Revolve microscope (Figure 5). In general, the fast growing colony has an increased number of cells and overall higher visual GFP intensity compared to the slow growing colony.

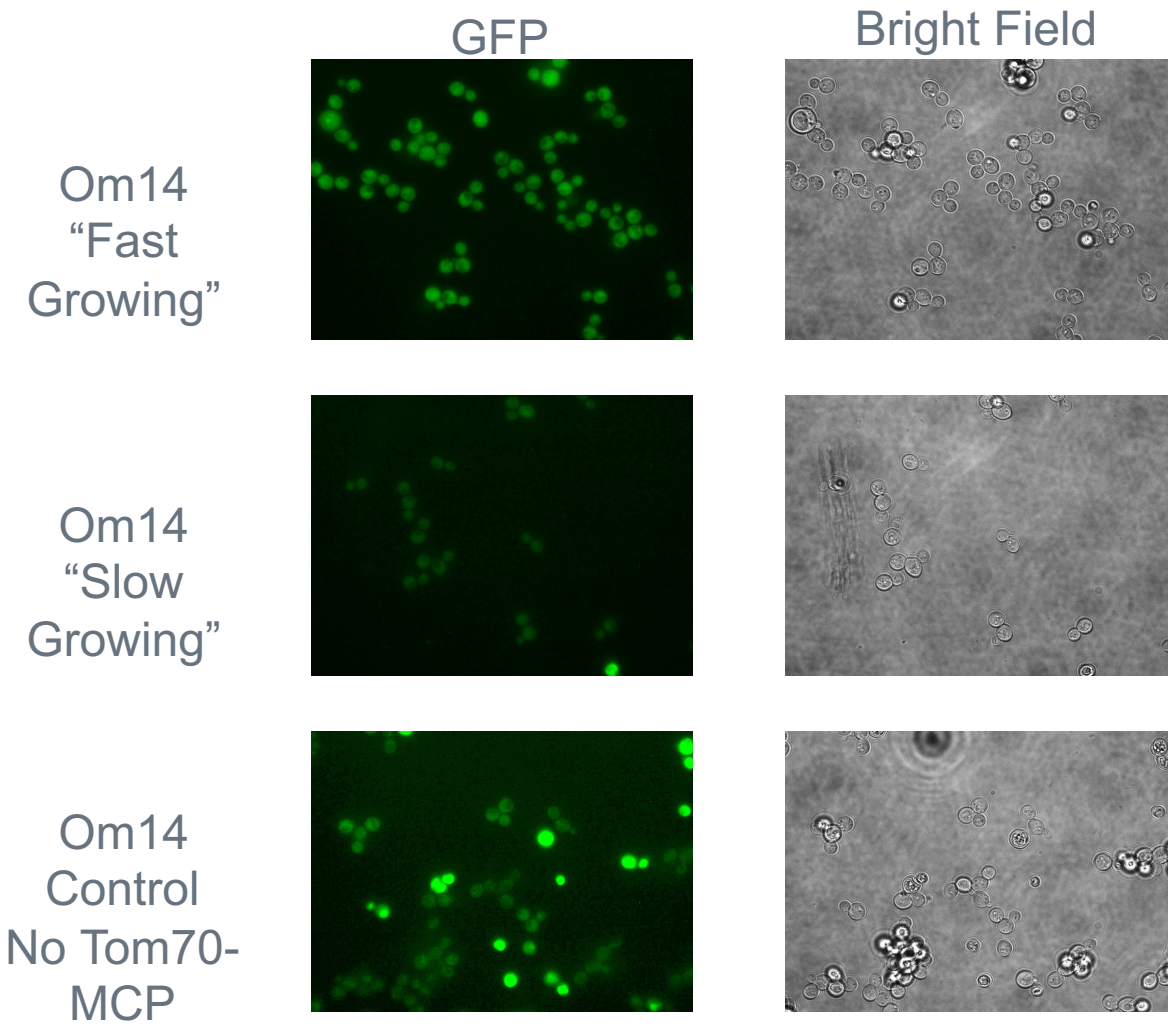
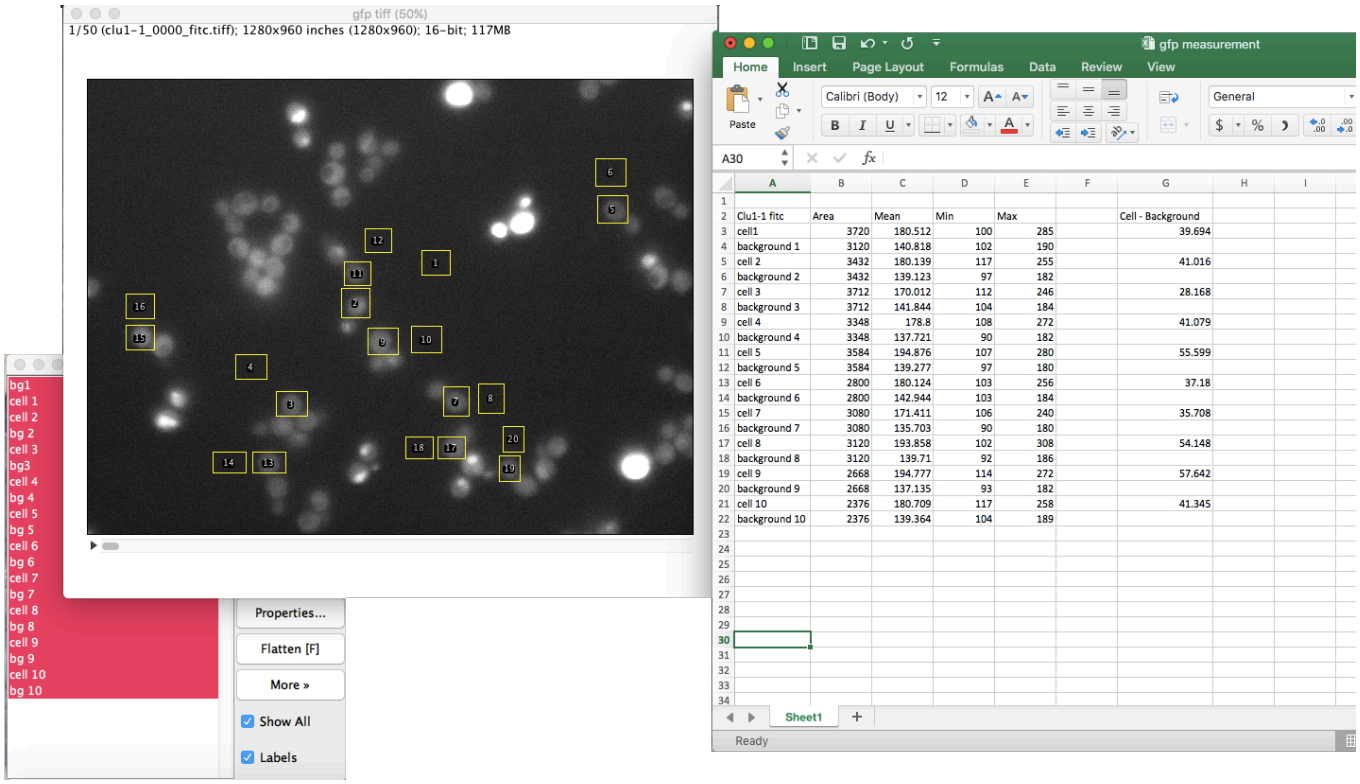



**Figure 5. Differences in cell number and fluorescence based on phenotype** Microscopy images for the fast growing, slow growing, and control colonies selected to represent the *om14*Δ strain.

### 2.7 Image analysis with FIJI to look at GFP and RFP intensity

Image analysis of microscopy was conducted using FIJI software. Stacks of GFP and RFP images in .TIFF file format were separately imported into the FIJI software. Using the ROI (region of interest) Manager tool, circular regions of interest were drawn around cells that would be measured. Only cells that appeared to be healthy were selected for measurement. Cells of abnormally large size, and abnormal shape, brightness, or appearance of visible vacuoles were excluded, as these cells were most likely not healthy or indicative of average cell. Regions of interest of the same size were selected on the background next to the cell being measured. As many measurements as possible were recorded for each image based on amount of cells present, up to a maximum of 30 cells. Intensity measurements of both the cell and the background region were recorded and exported to excel. Intensity of background area was subtracted from intensity of cell region (Figure 6). This measurement was then divided by the area of the regions for that respective measurement in order to provide intensity concentration. These concentrations were averaged for each slow growing, fast growing, and control strain of all mutants in order to compare relative GFP and RFP intensities.




| | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| 1 | | | | | | | |
| 2 | Clu1-1 fitc | Area | Mean | Min | Max | | Cell - Background |
| 3 | cell1 | 3720 | 180.512 | 100 | 285 | | 39.694 |
| 4 | background 1 | 3120 | 140.818 | 102 | 190 | | |
| 5 | cell 2 | 3432 | 180.139 | 117 | 255 | | 41.016 |
| 6 | background 2 | 3432 | 139.123 | 97 | 182 | | |
| 7 | cell 3 | 3712 | 170.012 | 112 | 246 | | 28.168 |
| 8 | background 3 | 3712 | 141.844 | 104 | 184 | | |
| 9 | cell 4 | 3348 | 178.8 | 108 | 272 | | 41.079 |
| 10 | background 4 | 3348 | 137.721 | 90 | 182 | | |
| 11 | cell 5 | 3584 | 194.876 | 107 | 280 | | 55.599 |
| 12 | background 5 | 3584 | 139.277 | 97 | 180 | | |
| 13 | cell 6 | 2800 | 180.124 | 103 | 256 | | 37.18 |
| 14 | background 6 | 2800 | 142.944 | 103 | 184 | | |
| 15 | cell 7 | 3080 | 171.411 | 106 | 240 | | 35.708 |
| 16 | background 7 | 3080 | 135.703 | 90 | 180 | | |
| 17 | cell 8 | 3120 | 193.858 | 102 | 308 | | 54.148 |
| 18 | background 8 | 3120 | 139.71 | 92 | 186 | | |
| 19 | cell 9 | 2668 | 194.777 | 114 | 272 | | 57.642 |
| 20 | background 9 | 2668 | 137.135 | 93 | 182 | | |
| 21 | cell 10 | 2376 | 180.709 | 117 | 258 | | 41.345 |
| 22 | background 10 | 2376 | 139.364 | 104 | 189 | | |
| 23 | | | | | | | |
| 24 | | | | | | | |
| 25 | | | | | | | |
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**Figure 6. Image Analysis Process** FIJI region of interest (ROI) manager was used to measure fluorescence concentration of cells, by subtracting area of background from area of measured cell and dividing by ROI area.

# 3. Results

### 3.1 mRNA number increases with switch from fermentative to respiratory conditions

RT-qPCR was performed on ATP3pr-GFP expressing cells that were grown in glucose and glycerol based media to facilitate fermentative and respiratory conditions, respectively. Values were normalized by the B-actin housekeeping gene control values. Results show that in the switch from fermentative to respiratory growth, ATP3pr-GFP cells sees a slightly less than 2 fold increase in mRNA number.

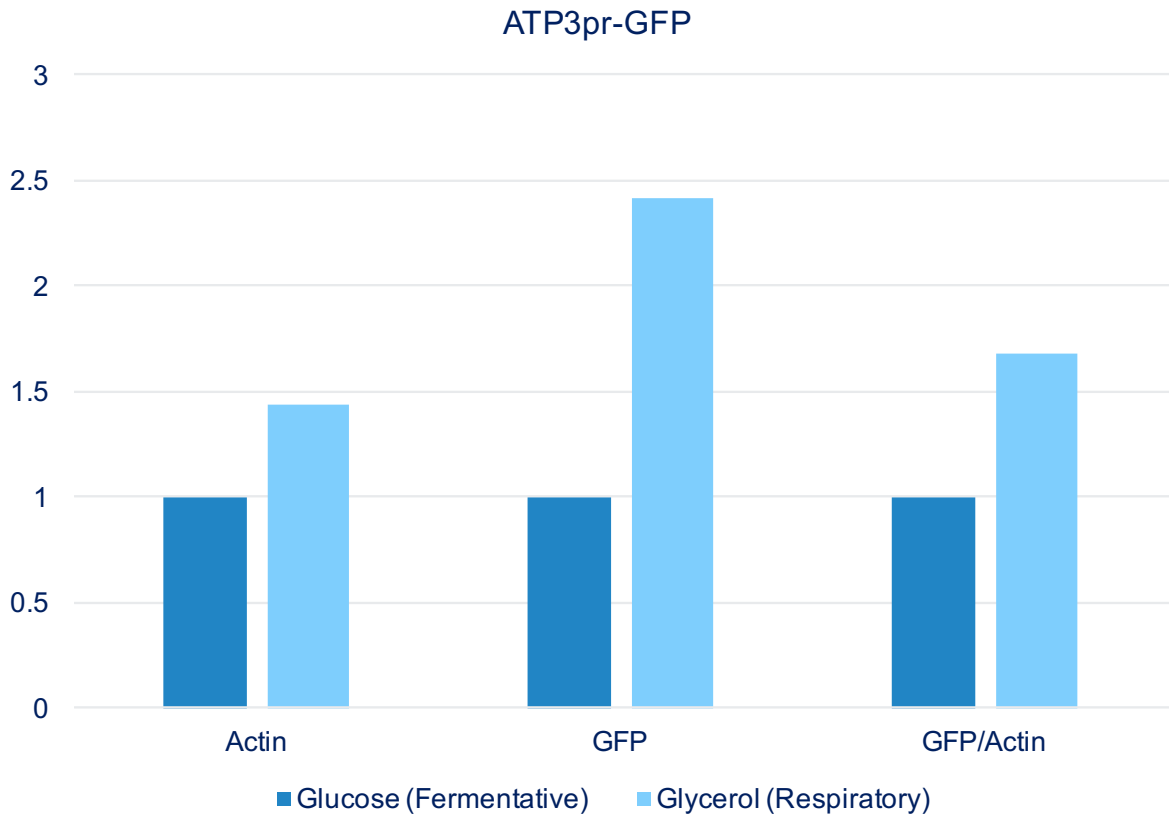


**Figure 7. mRNA Number Increases with Switch from Fermentative to Respiratory Conditions** RT-qPCR data of ATP3pr-GFP strain using primers that amplify GFP, as well as B-actin housekeeping gene for control. GFP values are normalized by B-actin

### 3.2 Protein Expression increases with switch from fermentative to respiratory conditions

FACS analysis was performed on the ATP3pr-GFP strain in both glucose and glycerol media in order to facilitate fermentative and respiratory growth conditions, respectively. Data shows that in the switch from fermentative to respiratory conditions, the ATP3pr-GFP strain undergoes about a 3 fold increase in protein expression. The ATP3-P2A-GFP control strain undergoes about a 2 fold increase in protein expression.

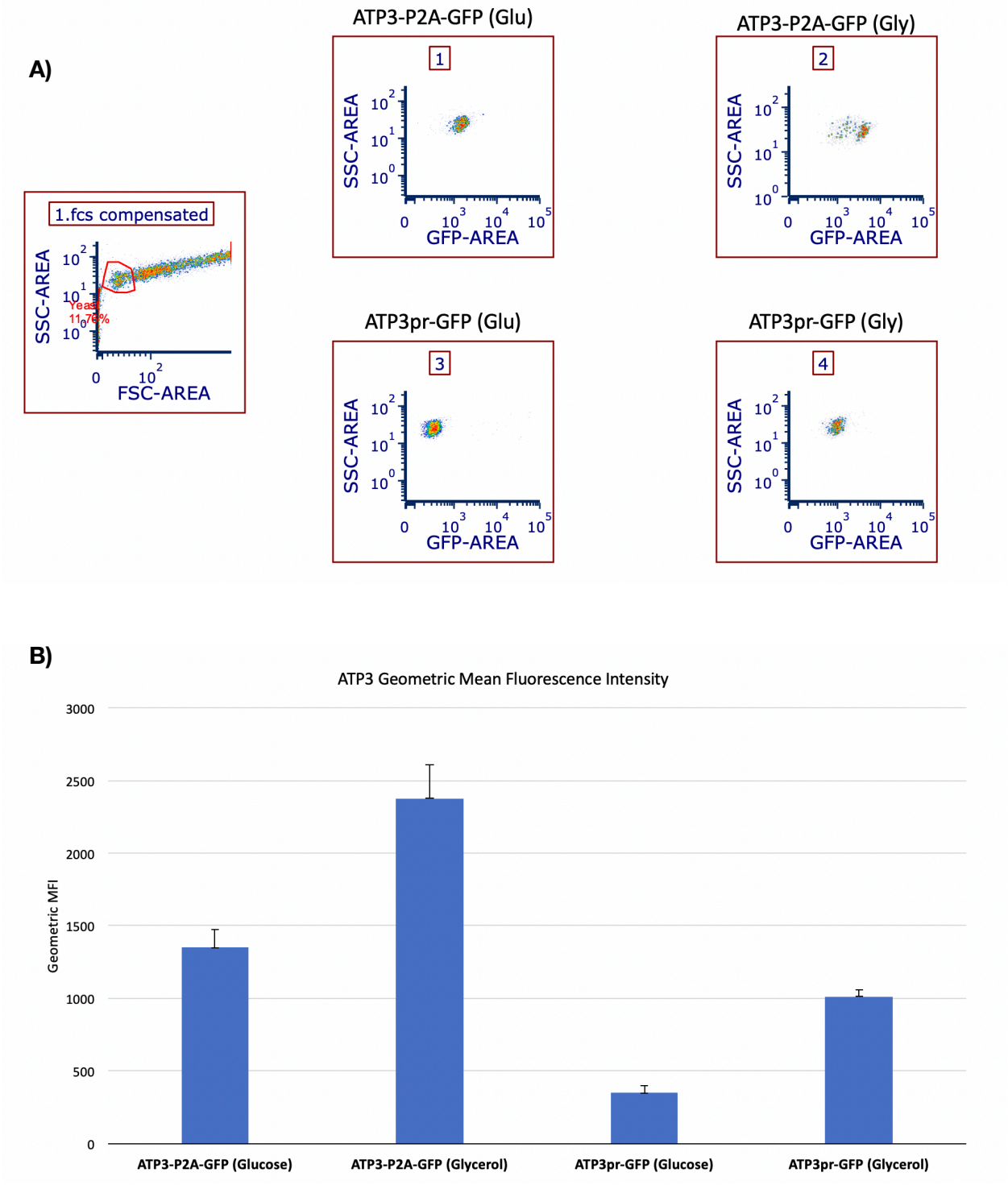


**Figure 8. Protein Expression Increases with Switch from Fermentative to Respiratory Conditions** A) Dot plot of FACS data for ATP3pr-GFP cells and ATP3-P2A-GFP cells in both glucose and glycerol. First box on the left shows how the data was gated based on initial reading. B) Geometric Mean Fluorescence Intensity for ATP3pr-GFP cells and ATP3-P2A-GFP cells in both glucose and glycerol.

### 3.3 Gene deletion effects protein expression at the outer membrane of the mitochondria

Fluorescence concentration was analyzed to determine protein expression in strains of *S. cerevisiae* containing various single gene deletions. “Fast growing” and “slow growing” colonies see TIM50pr-GFP-MS2 localized to the mitochondria via TOM70-MCP. TIM50pr-RFP serves as a control. The control strains do not contain TOM7-MCP, and see no localization of GFP or RFP. Mutated strains were organized and compared to each other based on the protein’s function. Groupings include mitochondrial morphology proteins, Translocase of the Outer Membrane (TOM) Complex proteins, outer membrane proteins, RNA binding proteins, and ribosomal subunit proteins. No data is available for *rpl1*Δ*, rpl2*Δ*,* and *tom20*Δ. Because of this, the ribosomal subunit protein *rps26b* is included in the RNA binding protein group. Data is compared to previous data from another member of the Zid lab, which suggests that if GFP is localized to the mitochondria, there should be a 10 fold increase in protein expression (Tsuboi et al 2020). Deletion of *clu1* shows a ~4 fold change in mitochondria-localized GFP expression for the the fast growing colony, and an overall decrease GFP expression for the slow growing colony. Deletion of *fzo1* shows a drastic decrease in GFP expression for both the fast and slow growing colonies with localization to the mitochondria. Deletion of *tom5* shows a ~5 fold increase in mitochondria-localized GFP expression for the the fast growing colony. No slow growing colony of *tom5*Δ was available, as only one colony grew on the plate. Deletion of *tom70* shows a ~7 fold and a less than 2 fold increase in localized protein expression for the fast and slow growing colonies, respectively. Deletion of *om14* shows a ~6 fold increase in protein expression for the fast growing colony, and a decrease in GFP expression in the slow growing

colony. Deletion of *om45* shows no change in localized GFP expression for either the fast or slow growing colonies. Deletion of *slf1* shows a ~5 fold change in mitochondria-localized GFP expression for the the fast growing colony, and a decrease in GFP expression for the slow growing colony. Deletion of *Sro9* shows a ~5 fold change in mitochondria-localized GFP expression for the the fast growing colony, and a decrease in GFP expression for the slow growing colony. Deletion of *Rps26b* shows a decrease in mitochondria-localized GFP expression for the the fast growing colony. No slow growing colony of *rps26b*Δ was available, as only one colony grew on the plate.

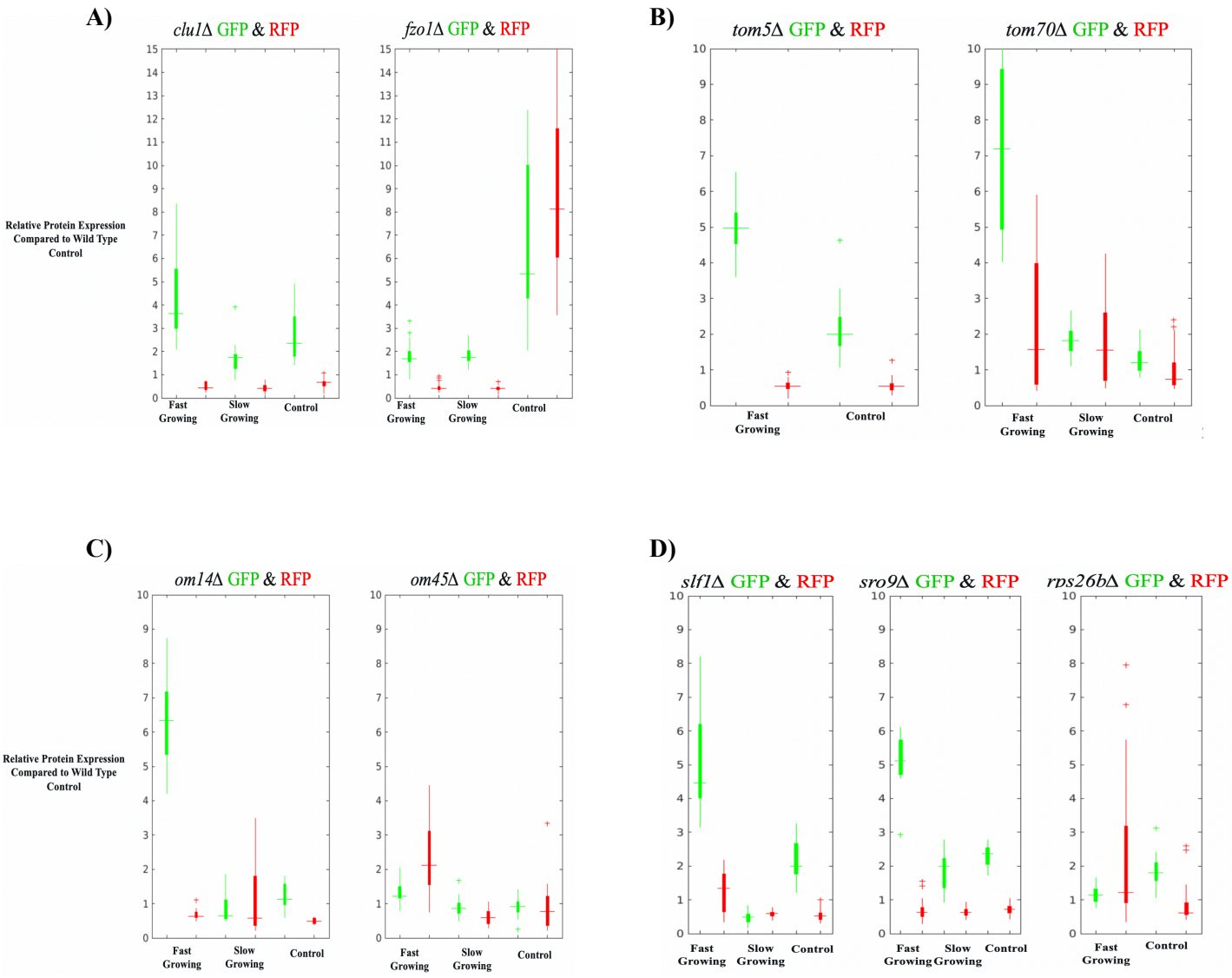


**Figure 9. Gene Deletion Effects Protein Expression at the Outer Membrane of the Mitochondria** Relative mitochondria-localized protein expression for various single gene deletions normalized by wild type control, for both fast and slow growing colonies, as well as non-localized control. A) Mutations of mitochondrial morphology proteins clu1 and fzo1. B) Mutations of Translocase of the outer Membrane Proteins tom5 and tom70. C) Mutations of outer membrane proteins om14 and om45. D) Mutations of RNA binding proteins slf1 and sro9, and ribosomal subunit protein rps26b.

# 4. Discussion

### 4.1 Increase in mRNA number and protein expression with switch in growth type

Overall, we found that most of the increase in mRNA number and protein expression that occurs when yeast switch from fermentative to respiratory growth is mostly due to transcription. In order to determine this, we looked at mRNA levels from RT-qPCR and protein expression levels from FACS of an ATP3 promoter only strain. This data was compared to previous findings that with the switch from fermentation to respiration, ATP3 mRNA will localize to the mitochondria and see an approximately 2 fold increase in mRNA number and a 4 fold increase in protein expression levels (Tsuboi et al 2020).

Our results show the promoter itself can produce a 2 fold change in mRNA number, which is very similar to the previous data. In addition, we saw that the ATP3pr strain sees a 3 fold increase in protein expression with growth condition switch. This was compared to an ATP3-P2A-GFP control strain that exhibited a 2 fold increase in protein expression, as well as the ATP3-GFP strain from previous data that shows a 4 fold increase in protein expression. Overall, this leads us to conclude that the increase is most likely due to effects of transcription, while translation of proteins may have some effect on further localization of mRNAs and translation of proteins at the mitochondria.

Due to the COVID-19 pandemic, the RT-qPCR data was only performed once. Reproducing the data in triplicate is an important future step in order to verify the results of this experiment. Additionally, the ATP3-P2A-GFP strain was only used as control because it was the best option available during the lab shutdown. Cloning of an ATP3-UTR-ORF-GFP strain to use as a true control for FACS analysis would be helpful in the future.

### 4.2 Gene deletions effect protein expression at the mitochondria

#### 4.2.1 Previous data used as control

Protein expression analysis of mutant strains was grouped together based on gene function. Groupings include mitochondrial morphology proteins, mitochondrial outer membrane proteins, RNA binding proteins and ribosomal subunit proteins, and translocase of the outer membrane proteins, as well the wild type strain that was used as control. Previous data shows that when GFP is localized to the mitochondria with TOM70-MCP, there is a 10 fold increase in protein expression levels (Figure X). For this experiment, if a gene deletion shows a 5 to 10 fold increase in GFP expression when GFP is localized to the mitochondria, we will conclude that this gene is non-essential for protein expression at the outer membrane of the mitochondria. In contrast, if the gene deletion causes a decrease in GFP expression, even when GFP is localized to the mitochondria, we will conclude that this gene is essential for protein expression at the outer membrane.

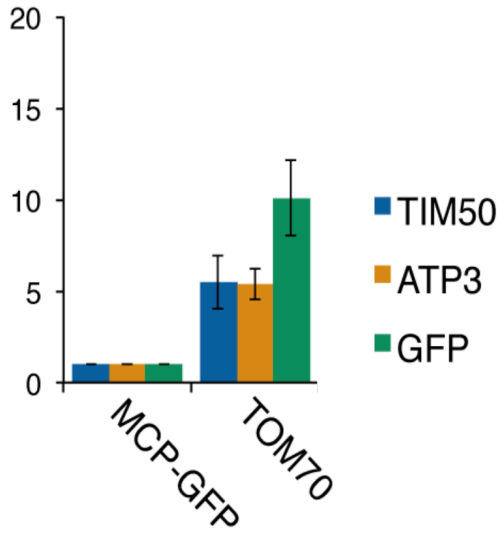


**Figure 10. GFP experiences 10 fold increase in protein expression when localized to the mitochondria.** Protein expression levels of TIM50, ATP3, and GFP when not localized (MCP-GFP), and when localized to the mitochondria (TOM70) (Adapted from Tsuboi et al. 2020).

### 4.2.1 Mitochondrial morphology proteins

The mitochondrial morphology group includes *clu1*Δ and f*zo1Δ* strains. Clu1 is a subunit of eukaryotic translation initiation factor 3 (eIF3) and is related to mitochondrial organization and morphology, but is not necessarily essential for growth, translation initiation or respiratory functions (Fields 1998). Fzo1 is involved in mitochondrial outer membrane fusion and mitochondrial genome maintenance. Deletion of *fzo1* leads to small, fragmented mitochondria and loss of mitochondrial DNA (Rapaport et al. 1998).

Results of the experiment show that *clu1*Δ shows about a 4 fold increase in the fast growing strain. While this was not as high as the expected 10 fold increase in the control, it is close enough to our 5 to 10 fold threshold that we can say *clu1* is probably not essential for protein expression at the outer membrane. Deletion of *fzo1* caused a drastic decrease in protein expression when GFP was localized to the mitochondria. This was a surprising result, and leads us to conclude that *fzo1* is indeed essential for protein expression. At this time, we cannot explain why *fzo1*Δ shows such surprising decrease in protein expression, but mitochondrial morphology and its effects on mitochondrial protein expression is a topic that is currently being explored by other members of the lab.

### 4.2.2 Translocase of the Outer Membrane Complex proteins

The translocase of outer membrane (TOM) complex group includes *tom5*Δ*, tom20*Δ*,* and *tom70*Δ. These genes encode for proteins that are essential components of the TOM complex, which is involved in the recognition and initial import steps for mitochondrial directed proteins. Tom20 and Tom70 are the key protein receptors on the outer membrane of the mitochondria, and

are the first contact a protein will make before import (Muto et al. 2001,Wu et al. 2006). Tom5 is part of the membrane pore that allows for protein import (Dekker et al. 1998).

We found that both *tom5*Δ and *tom70*Δ strains showed increases in localized protein expression that are significant enough to determine they are non-essential. While this finding makes sense for deletion of *tom5*, which plays a structural role in the TOM complex, it was surprising to find that *tom70*Δ shows the closest effect to that of the non-mutated control. However, even though endogenous *tom70* is deleted in this strain, we essentially added functional Tom70 back with the transformation of the TOM70-MCP system. Therefore, this strain almost acts like a control in itself, explaining why deletion of what is understood to be a very important gene yields little change. A further experiment would be to use a TOM20-MCP system to study the effects of *tom70* deletion. No data was available for *tom20*Δ, as this strain was incomplete at the time of the experiments. However, this would be an interesting gene to study with the TOM70-MCP system, as it is the other main protein receptor of the TOM complex.

### 4.2.3 Mitochondrial outer membrane proteins

The grouping of outer mitochondrial membrane proteins include *om14*Δ and *om45*Δ. Om14, an essential protein of the mitochondrial outer membrane that acts as a receptor for cytosolic ribosomes, is involved in co-translational mitochondrial import through its interaction with NACs and helps dock cytosolic ribosomes to the mitochondria. Works together with Om45 (Lesnik et al. 2014). Om45 is another mitochondrial outer membrane protein, that extends to inter-membrane space. Its function is not well known, but it has been shown to associate with

Om14 and Por1, which forms the porin pore that allows small molecules to travel into the mitochondria (Lauffer et al. 2012).

Deletion of *om14* was shown to induce an approxintley 6 fold increase in protein expression with localization to the mitochondria in the fast growing strain. Therefore, we conclude in the context of this experiment that *om14* is not essential for localized protein expression. In contrast, *om45*Δ showed almost no change in protein expression with localization to the mitochondria. This was an interesting finding, as Om45's function is generally unknown, but we can conclude that the gene is indeed essential for protein expression at the mitochondria. Additionally, this was one of the few strains that had similar protein expression for the fast and slow growing colonies. At this time we cannot explain these results, but this gene is certainly one that should be explored further.

### 4.2.4 Ribosomal subunit and RNA binding proteins

The Ribosomal protein group includes *rpl1*Δ*, rpl2*Δ*,* and *rps26b*Δ, which include proteins of the large and small ribosomal subunits, respectively. Rpl1 and Rp12 are part of the large 60S ribosome subunit which is responsible for protein translation (Lee et al. 2002). Rps26b is a component of the small 40S ribosomal subunit, and plays a role in subunit assembly in yeast (Belyy et al. 2016). Because data for *rpl1*Δ and *rpl2*Δ was not available, *rps26b*Δ data has been included in the RNA binding protein group, as these proteins interact with translating ribosomes.

The RNA binding protein group includes *slf1*Δ and *sro9*Δ. Slf1 is an RNA binding protein that associates with polysomes and could be involved in regulating mRNA translation. Sro9, another cytoplasmic RNA binding protein, is a paralog of Slf1. These genes evolved by

duplication and code for proteins with similar functions. This is evident by Sro9's involvement in mRNA binding via association to translating ribosomes. (Yu et al. 1996, Sobel et al. 1999)

Results show that both *slf1*Δ and *sro9*Δ lead to a 5 fold increase in protein expression with localization in the fast growing strains. Therefore, we can conclude that these genes are not essential for protein expression at the mitochondria. However, *rps26b*Δ led to a slight decrease in protein expression even with localization in the fast growing colony. No slow growing colony was available for analysis, but based on the fast growing colony we can conclude that Rps26b is essential for protein expression at the mitochondria. This finding makes sense, as Rps26b assists in assembly of ribosomes that are needed for protein translation.

### 4.2.5 Slow growing colonies

An additional finding of this experiment was that all of the slow growing colonies exhibited less protein expression compared to the fast growing colonies. Most also had decreased or equal protein expression to the non-localized control colonies. At this time we cannot say why this is, but we hypothesize that there is some additional mutation present in these colonies that further hinders protein expression at the mitochondria. Perhaps this mutation, when coupled with various gene deletions, is harmful enough to effect not only mitochondrial protein expression, but cell growth in general. An important future direction for this project would be to sequence and compare the fast and slow growing colonies in order to determine what confounding mutations could be occurring.

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