Ribosome Processivity in NIH 3T3 Cells and *Saccharomyces cerevisiae*

Abstract

Translation, a life-sustaining activity, is the process by which proteins are produced in the cell. The site for biological protein synthesis, the ribosome, consists of two main subunits. Ribosome processivity is probability that a ribosome that has already initiated translation on an open reading frame of a messenger RNA, or mRNA, will complete elongation and terminate at the stop codon. In simpler terms, processivity is the probability that the ribosome will translate a full-length protein. When a ribosome produces a truncated protein and falls off the mRNA before the stop codon, it is defined as non-processive. When a ribosome produces the full-length protein, however, it is defined as processive. We know that processivity plays a critical role in cellular development and growth, as cells would not be able to survive without the production of full-length proteins. Nonetheless, ribosome processivity has yet to be studied in-depth. Using proteins derived from a green fluorescent protein family, we created and used a plasmid to quantify ribosome processivity. We used mammalian, wildtype yeast, and mutant yeast cells to study processivity. Thus far, we have seen evidence of varying processivity in wild type yeast during the diauxic shift of growth. Moreover, different mutant forms of yeast and varying growth conditions have affected translation and prompted additional questions.

Introduction

Processivity describes the probability that once a ribosome has initiated translation, it will fully complete translation and produce a full-length protein. The process of translation begins when the ribosome identifies a start codon on the mRNA. The ribosome consists of two subunits, a large and small subunit. When translation initiates, these subunits come together and remain

together until termination. When a ribosome is non-processive and terminates before the stop codon, this means that the two subunits of the ribosome prematurely separate.

Translation is a complex process, and the creation of a full-length polypeptide chain involves more proteins in addition to just the ribosome. A processive ribosome requires many proteins and protein complexes, including initiation factors, eIFs, and elongation factors, eEFs. Previous research has demonstrated that certain eIFs and eEFs, like eIF5A, may be important in promoting translation and preventing ribosome release from the mRNA strand (Saini et al., 2009). The ribosome itself is also important in controlling processivity, and when certain components of the ribosome are mutated, processivity decreases significantly (Dong & Kurland 1995). Despite our limited understanding, very little research has been conducted on the protein complexes involved in processivity.

Previous research highlights evidence of non-processive ribosomes. Researchers have used deep sequencing techniques to determine that 5' regions of mRNA are bound by more ribosomes than 3' regions of mRNA (Ingolia et al., 2009). This means that some of the ribosomes may be falling off the mRNA strand prematurely, before reaching the 3' end. Likewise, ribosome density mapping has also demonstrated that ribosome density is higher on mRNA strands with shorter open reading frames than on those with longer open reading frames (Arava et al., 2005).

In order to assess processivity, we constructed a plasmid that once transcribed and translated, produces four different colored variants of a fluorescent protein in tandem. During translation, the proteins are produced sequentially in the following order: mCerulean3 (blue), Ypet (yellow), mOrange2 (orange), and mCardinal (red). We used the order of the proteins to determine the relative processivity of the ribosomes. If a ribosome was non-processive, it would

not produce all four fluorescent colors. Therefore, the production of the red fluorescent protein serves as a signal for a processive ribosome that terminated successfully. Other studies have used similar concepts in the past. For example, firefly luciferase, another fluorescent reporter, has also be used to quantify ribosome processivity (Bonderoff & Lloyd 2010).

Methods

NIH 3T3 Cells

Cell Culture: NIH 3T3 cells were cultured in 25 cm^2 flasks containing DMEM and 10% newborn calf serum (NBS) with penicillin and streptomycin. When the cells reached a confluence above 70%, they were washed with 4mL of sterile PBS and Trypsin-EDTA was applied as a dissociation reagent. When the cells were prepared for transfection, they were transferred into a 24-well plate and incubated overnight. The cells were transfected after reaching a confluence of about 60%.

Transfection: Before the cells were transfected, stock solutions DNA and Lipofectamine 2000 Reagent were made. The stock solution of DNA consisted of 6.6µL processivity construct DNA (2µg/µL) to 165µL of Opti-MEM, reduced serum medium. At the same time, 13.2 µL of Lipofectamine 2000 Reagent was added to 165µL of Opti-MEM. The two solutions were incubated at room temperature in the tissue culture hood for 5 minutes. The two solutions were then combined and incubated for another 20 minutes at room temperature. While the solution was incubated, the DMEM was removed from the cells in the 24-well plate; the cells were washed with 500µL of sterile PBS, and 500µL of new DMEM with 10% NBS without antibiotics was added to each well. Then, 50µL of the DNA and Lipofectamine stock was added to each well. The cells were incubated for 24 hours at 37°C. The following morning, 50µL of diluted

Torin1, an mTOR inhibitor, was added to half of the wells containing cells. 7 hours later, the cells were imaged using confocal microscopy.

Confocal Microscopy: The fluorescence of the NIH 3T3 cells was analyzed on a confocal microscope. The microscope was set to excite each protein at its excitation wavelength and measured each protein's emission at its corresponding emission wavelength. The excitation wavelengths were as follows: ~440nm (mCerulean3), ~513nm (Ypet), ~600nm (mCardinal). Images were taken with mCerulean3 and mCardinal, while Ypet signals were more useful for simply locating fluorescent cells before imaging.

Saccharomyces cerevisiae **Cells**

Plating: Mutant yeast strains were plated onto YPD (yeast extract peptone dextrose) plates and incubated overnight at 30°C. One colony was removed from the YPD plate and spread into a 2 cm^2 patch on another YPD plate.

Transformation: To make the T-mix we used 1200µL of PEG 3500 (polyethylene glycol), 180µL of lithium acetate, 50µL of single stranded carrier DNA, 25µL of plasmid DNA, and 345µL of water. The single stranded DNA (ssDNA) was denatured on a hot plate at 96°C for 5 minutes and immediately placed on ice. A ~50µL blob of the yeast was suspended in 1mL of sterile water in a 1.5mL Eppendorf tube. The cells were spun down in a centrifuge for 30 seconds and supernatant was removed. 360µL of T-mix was added to each Eppendorf tube, and the tube was vortexed vigorously to resuspend the pelleted yeast. The Eppendorf tubes were incubated in a 42°C water bath for 40 minutes to allow the cells to take up the external DNA. After removal from the water bath, the tubes were spun down in a centrifuge for 30 seconds again, and the supernatant of T-mix was removed. 1mL of water was pipetted into the

tube, stirred, and vortexed to resuspend the pellet. 100µL of the cell suspension was pipetted onto Ura$^-$ plates. Only successfully transformed yeast would grow on this selective media. To spread the cell suspension across the entire surface of the plates, 3-5 sterile glass balls were added onto the plates and swished around for about 15 seconds. The Ura$^-$ plates were then incubated at 30°C for 3-4 days until colonies appeared.

TECAN plate Measurements: The fluorescence of the yeast cells and the growth of the yeast cells was measured over time by a TECAN plate reader. Each fluorescent protein: blue, yellow, orange, and red was measured separately.

Trizol Reagent: Trizol reagent was used to separate RNA from yeast cells. 400µL of Trizol reagent and 50µL of glass beads were added to centrifuged yeast cells. The solutions were vortexed for 60 seconds. The solutions were incubated at room temperature for 5 minutes before 200µL of chloroform was added. The solutions then were incubated on ice for 2 minutes and was centrifuged at 4°C for 15 minutes. The centrifuge spun at 14,000rpm. Supernatant was removed. 100µL of 70% ethanol solution was added to the remaining pellet and the solution was spun down again in the cooled centrifuge at 4°C for 1 minute. Supernatant was removed and the pellets were vacuum dried until no liquid remained. 20µL of TE buffer was added to all of the tubes. The PCR tubes were stored in the freezer overnight for later analysis.

RT-qPCR (Reverse Transcription Quantitative Polymerase Chain Reaction): In a PCR tube, we added 1µL of 200ng/µL random primers, 1µL of RNA (from yeast of varying densities), 1µL of 10mM dNTP, and 7µL of water. The tubes were incubated at 65°C for 5 minutes and were immediately placed on ice afterwards. 4µL of 5x M-Mu-LV buffer, 1µL M-Mu-RT, 6.5µL of water, and 0.5µL of RNAse inhibitor were added to each PCR tube. The solutions were incubated for 1 hour at 42°C. Next, 0.5µL of RNAse-H was added to the tubes. The tubes were

then incubated at 37°C for 15 minutes. The resultant product was cDNA. Forward and reverse primers were diluted to 1mM, vortexed, and centrifuged. Forward primers and reverse primers were combined. In a 96-well plate, 5µL of cDNA, 16µL of the diluted primer solution, and 10µL of the SYBR universal super mix were added together. cDNA originating from multiple densities of yeast was used. The plate was spun down in a large centrifuge before being placed into the thermocycler. The "Friendlab qPCR" protocol was run. The quantities of mRNA and rRNA were normalized and then divided. Ratios of around one would demonstrate that the mRNA levels were not changing, relative to the rRNA, as the densities of the yeast increased.

Western Blot: Our most recent investigation focused on analysis of two TIF mutants: TIF4631 and TIF4632. These mutants generate altered forms of a the eukaryotic initiation factor, eIF4G, which serve as important scaffolding proteins and subunits of the cap-binding protein complex in mRNA translation initiation. The TIF mutant yeast and control YBK P06 and YBK P05 yeast were plated and grown on plates, made from 0.154g Ura-, 1g sugar, 1.34g nitrogen base without amino acids, and 5g agar. In an attempt to continue previous analyses, we included plates made with both dextrose and galactose as the sugar. The plates were then incubated at 30°C for 3-4 days until colonies appeared. After colonies grew, we made Ura- solution for the yeast to grow in. The yeast grew in this medium with constant stirring for ~4 days, or until the optical densities reach levels above 6 or 7, as measured by the spectrophotometer. These optical densities indicate a high quantities of yeast. A total of 2mL of each yeast variant were spun down for 1 minute at 10,000rpm, and the supernatenant was removed. All yeast were stored in their 1.5mL Eppendorf tubes in the freezer for later use.

Before running our SDS Page gel, we added 500µL of loading dye to each sample of yeast. The samples were heated at 96°C for 5 minutes. Then, we loaded 10µL of the yeast

samples into the wells of our SDS page gel. Additionally, we added 2µL of protein ladder to the far end of the gel for comparison in imaging. We ran the gel is SDS running buffer at 120V for 60 minutes, checking the gel every 15 minutes to ensure there were no errors. Once completed, the gel was carefully removed from the glass plates and placed on a piece of filter paper. The gel and filter paper were placed on top of a sponge and submerged in the SDS transfer buffer. While only touching the edges, a piece of nitrocellulose membrane was placed on top of the gel, followed by another piece of filter paper. A glass rod was pressed and rolled over the top of the filter paper to remove any air bubbles. Then, the final sponge was placed on top of the filter paper. The combination of the sponges, filter paper, nitrocellulose membrane, and gel were placed into the gel holder cassette and into the tank for transfer. We placed the holder in the tank so that the gel faced the cathode and the membrane faced the anode. The set up was moved into the cold room, and the transfer ran at 35V over night for ~ 15 hours.

The following day, the transfer apparatus was disassembled, and the membrane was removed from the transfer stack and holder. The membrane was placed in a 5% milk, TBS Tween solution (2.5g milk power, 50mL TBS Tween) and refrigerated for an hour. This step is referred to as blocking and prevents antibodies from binding to the membrane nonspecifically. After a minimum of 1 hour, the membrane was rolled up and placed in in a 15mL conical tube. We added 3.3µL of primary antibody to 10mL of our 5% milk TBS Tween solution ($\frac{1}{3000}$ diluation). We incubated the membrane in this solution for 1 hour in the cold room. Then, we washed the membrane in TBS Tween 3 times for 8 minutes. In a new 15mL conical tube, we rolled up the membrane. This time, we added 2µL of secondary antibody, Alexa Fluor 488, to 10mL of our 5% milk TBS Tween solution. Again, we incubated the membrane in this solution

for one hour in the cold room. After incubation, we washed the membrane with TBS Tween 5 times for 8 minutes. The results were imaged and analyzed.

Results

Figure 1: Cerulean and Ypet Production

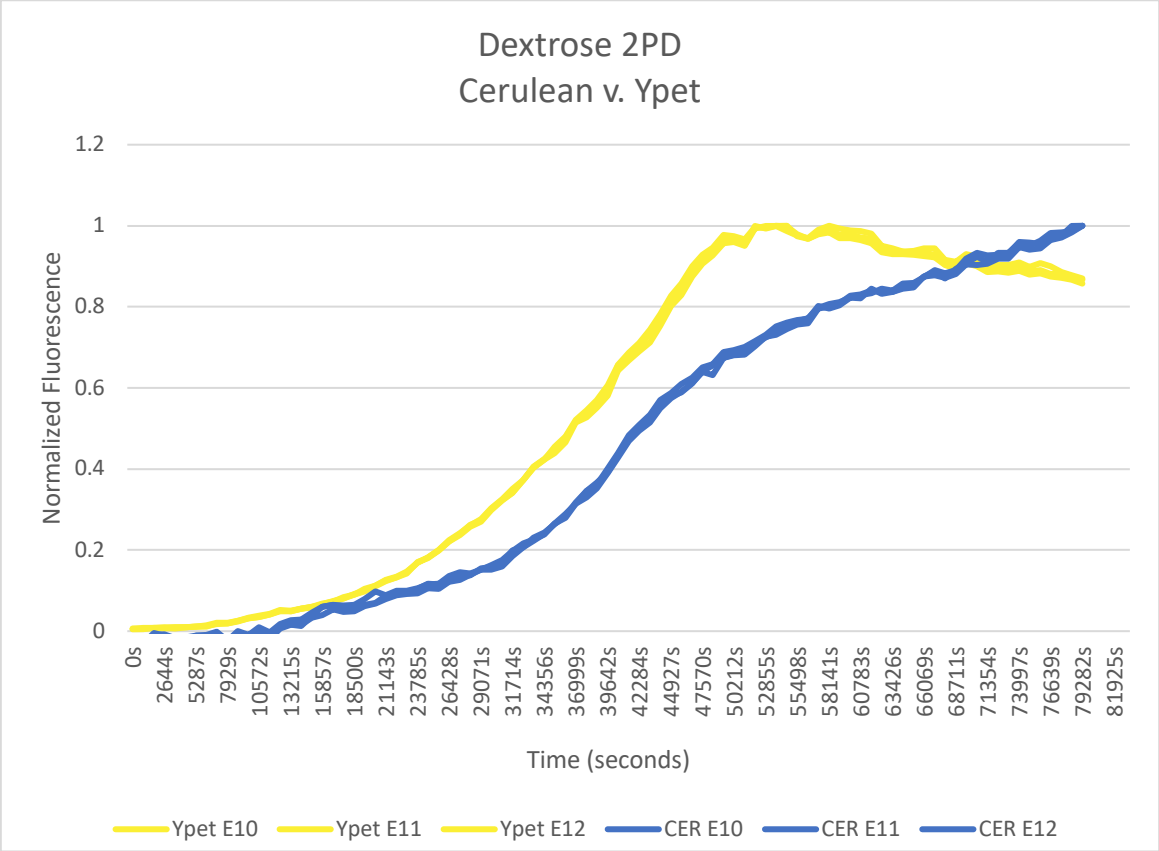
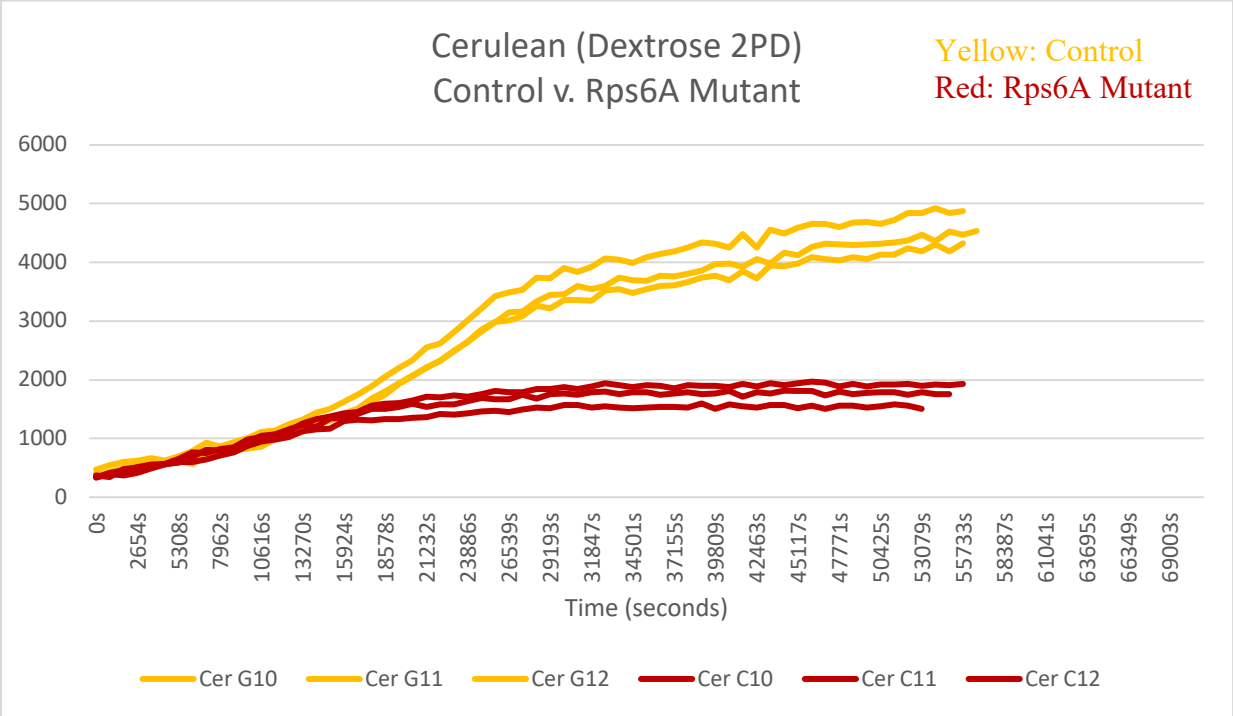


Figure 2: Cerulean and Ypet Production in Rps6A Mutant



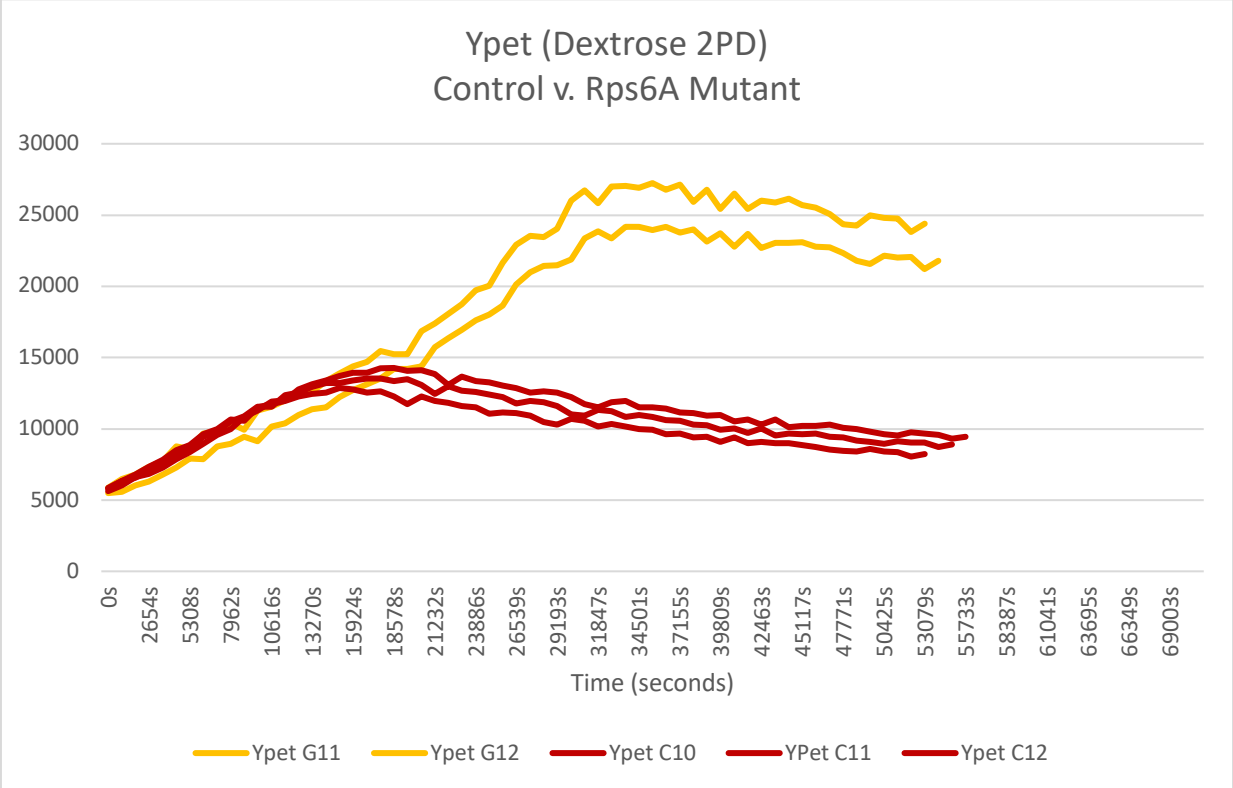
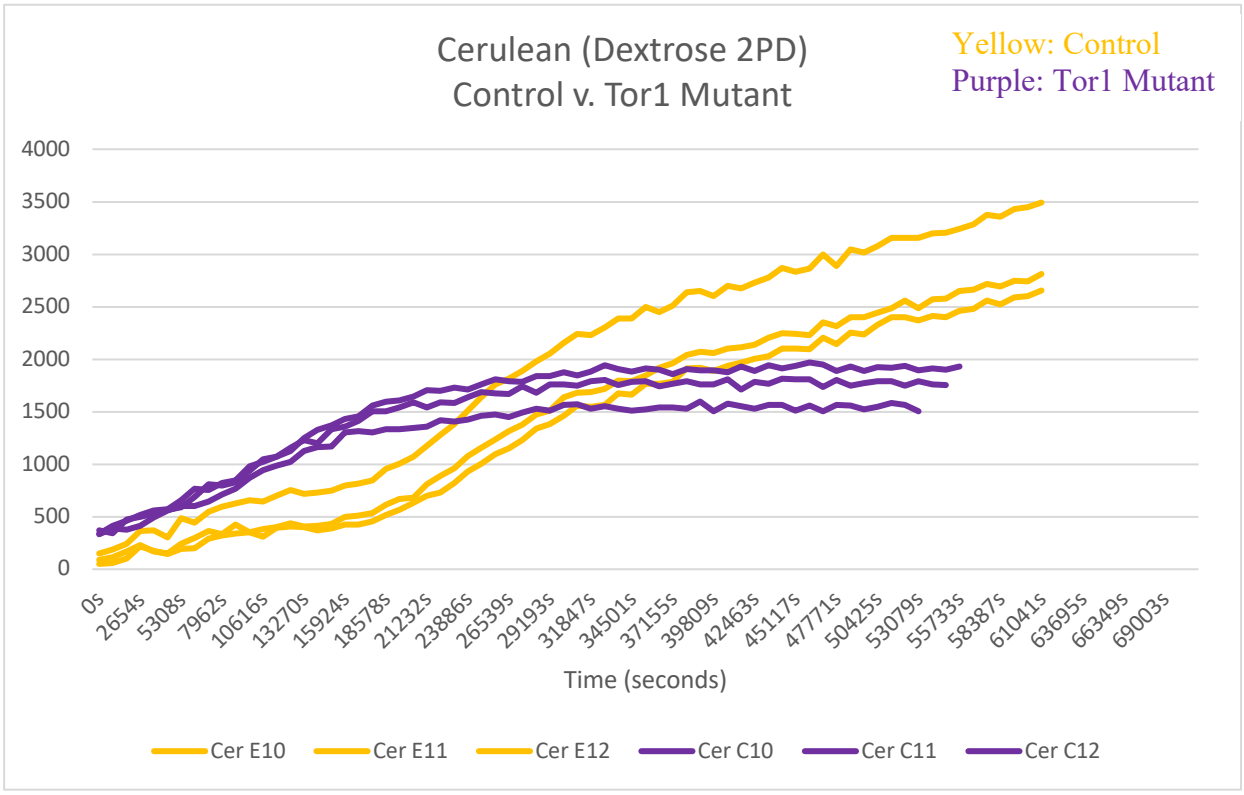


Figure 3: Cerulean and Ypet Production in Tor1 Mutant



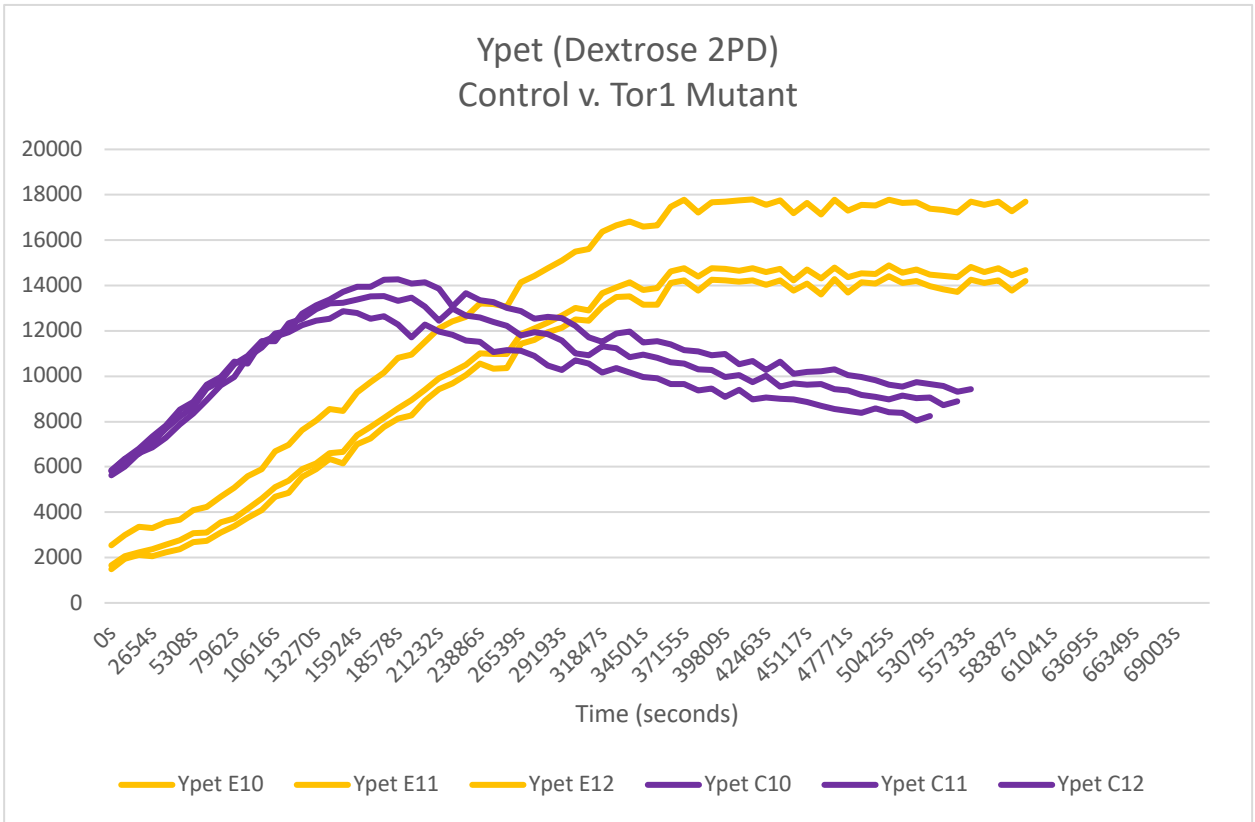
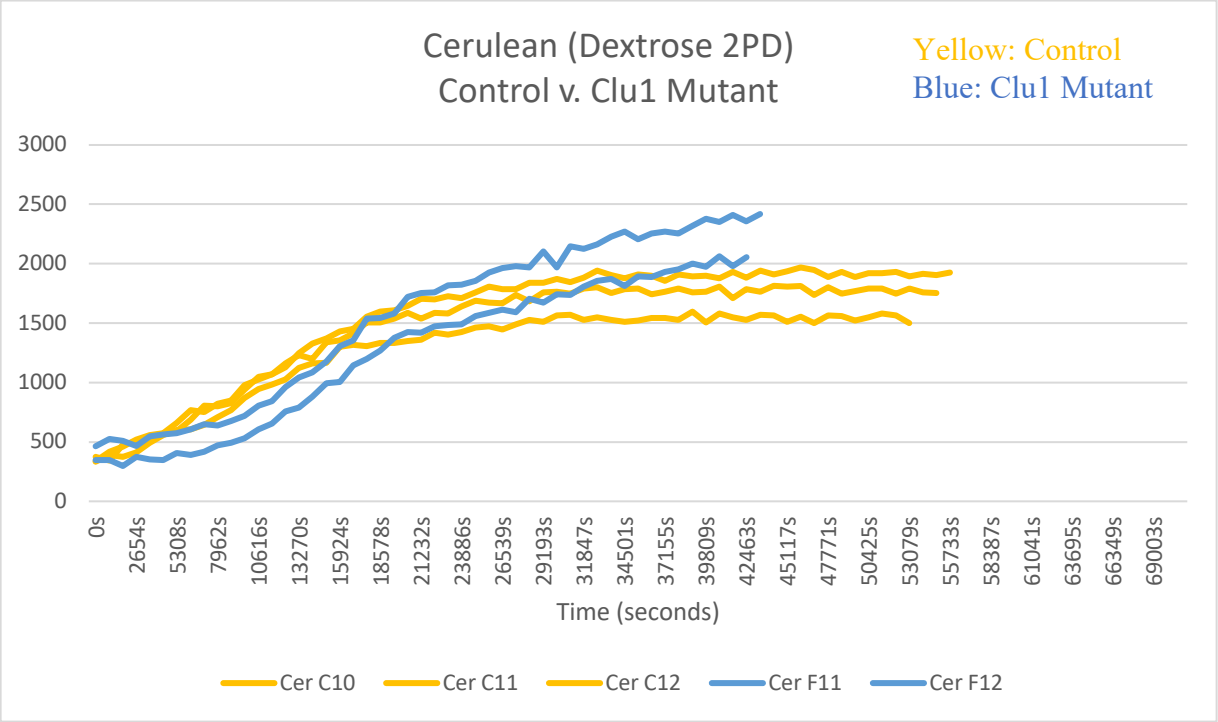


Figure 4: Cerulean and Ypet Production in Clu1 Mutant



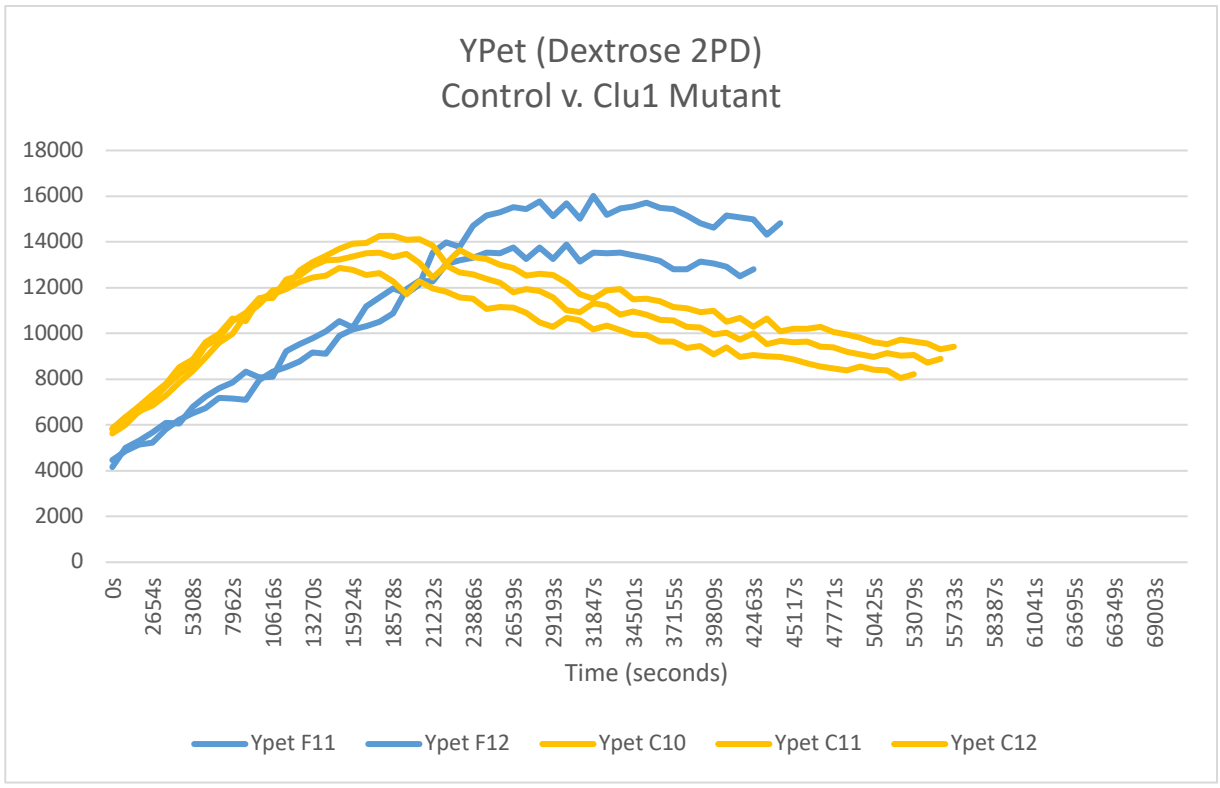
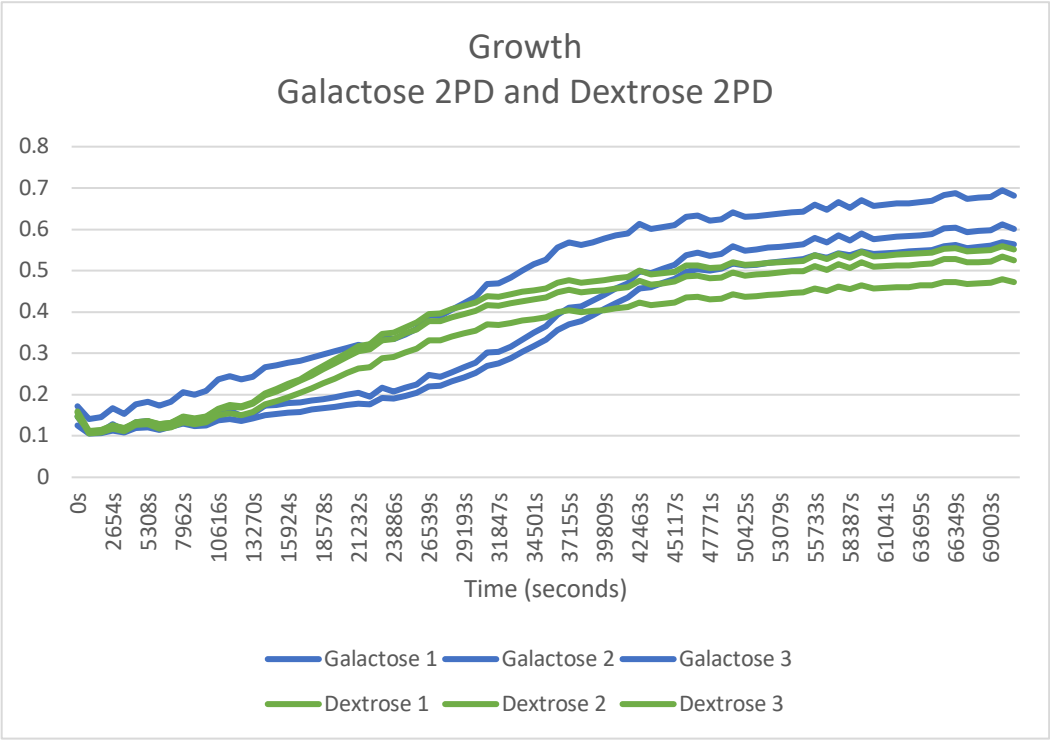
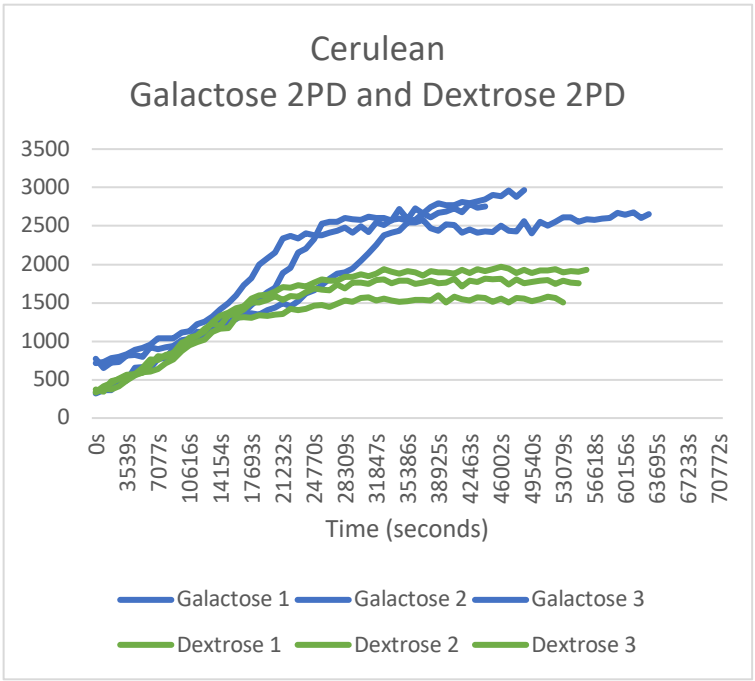
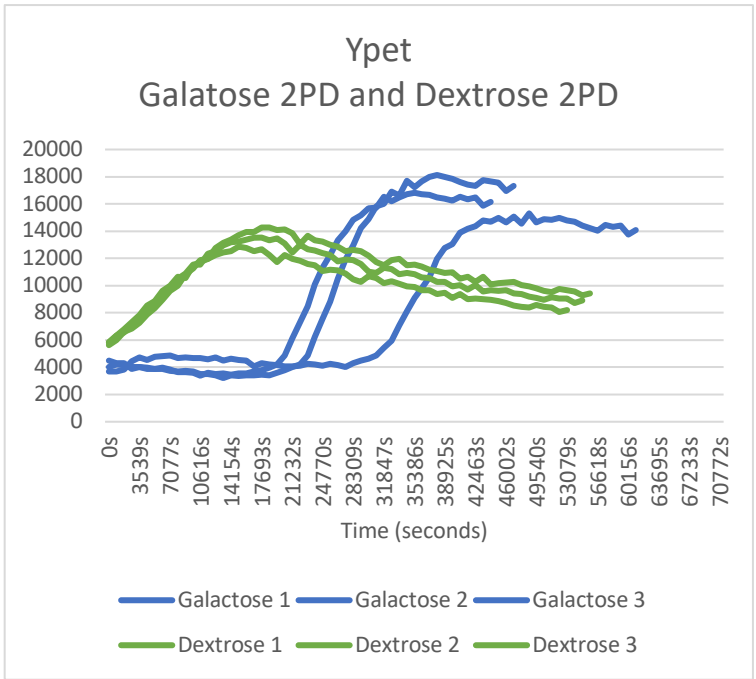


Figure 5: Growth, Cerulean Production, and Ypet Production in Galactose v. Dextrose







***Saccharomyces cerevisiae* Cells**

Fluorescence from the four proteins (mCerulean3, Ypet, mOrange2, and mCardinal) in the transformed yeast cells was measured across varying densities in the Tecan plate reader. The four proteins were not all produced in the same amounts at the same times. Shown in Figure 1 are the curves for blue and yellow protein in yeast with the processivity construct. As the yeast reach higher densities, they continue producing blue protein while they no longer produce yellow protein. Visually, this is illustrated in points further to the right on the horizontal axis, where the presence of yellow protein starts to decrease while the presence of blue protein continues increasing. When yeast reach high densities in a liquid medium, they enter a diauxic shift due to a lack of nutrients. The difference in the production of blue and yellow proteins suggests that at the diauxic shift processivity decreases.

In order to confirm that the differences in fluorescent signals from the blue and yellow proteins were not due to fluctuating mRNA levels, we also completed mRNA quantification using reverse transcription qPCR. We quantified the amount of ribosomal RNA, rRNA, and messenger RNA, mRNA. The ribosomal RNA was used as a baseline for comparison. Relative to the amount of rRNA, the amount of mRNA did not change as the density of yeast cells changed.

Fluorescence for Tor1, Rps6A, and Clu1 mutant yeast: Tor1, Rps6A, and Clu1 mutant yeast were transformed so that they produced fluorescent proteins of the processivity construct. Fluorescence was measured with the Tecan plate reader while the yeast grew to high densities. As shown in Figures 2, 3, and 4 the Rps6A mutant (red), Tor1 mutant (purple), and Clu1 mutant (blue) yeast produce lower amounts of all four proteins than the wild type yeast. This suggests that the Tor1 kinase, Rps6A component of the small subunit of the ribosome, and the Clu1

mutant of eIF3 are all important in translation. However, there does not appear to be a difference in processivity between the mutants and the wild type yeast, as the mutant produces lower amounts of all four proteins rather than producing only lower amounts of the proteins translated from the 3' end of the mRNA: yellow, orange, and red. Future data analysis needs to be conducted to create graphics for the results of the orange and red proteins. Moreover, more data collection needs to occur with the Clu1 mutant and Cerulean production, as the graph shows only small differences between the control and mutant strains.

Galactose and dextrose growth conditions: Wild type and Tor1 mutant yeast were transformed so that they produced fluorescent proteins of the processivity construct. The yeast were grown in one of two sugars: galactose or dextrose. Fluorescence was measured with the Tecan plate reader while the yeast grew to high densities. Although no statistical tests have been performed on the dataset to determine the significance of any differences, Figure 4 shows how the sugar in which the yeast is grown appears affect the rate at which it translates protein. Further research will be conducted with varying growth conditions to determine if the sugar significantly impacts processivity or translation. Our wild type yeast grown to the diauxic shift were all grown in dextrose. This means that our mRNA quantification needs to be retested again with wildtype yeast grown in galactose.

Western Blot

The wells containing protein from the TIF 4632 and TIF 4631 mutants appear to show two distinct bands in the image. The band further down the gel indicates shorter length proteins, which we believe can be attributed to truncated protein production and non-processive ribosomes. The bands are difficult to distinguish and there is a significant amount of background

that interferes with the clarity of the image. We plan on running the procedure again to produce a higher quality image, but the initial results we found are promising.

NIH3T3 Cells

The lab grew and attempted to image transfected cells throughout the summer, but it was difficult to obtain useful images. Therefore, the lab continues to grow and transfect the cells, as we are still working to take better images. Additionally, future research may include a stable cell line, as one of the main issues encountered was the low transfection efficiency. Previous research conducted in this laboratory has demonstrated processivity changes in NIH3T3 cells treated with puromycin, so future research will continue to look for changes in processivity.

References

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