Spring 5-1-2009

Imaging of RNase Mitochondrial RNA Processing Localization Using Fluorescent Microscopy

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1. INTRODUCTION

The widely studied *Saccharomyces cerevisiae* is a unicellular fungus. Fungi are eukaryotes, which is defined as any cell or organism that possesses a clearly defined nucleus.[1] This particular species of yeast has been used for centuries in baking and brewing, and is also known as baker's, brewer's, and budding yeast. *S. cerevisiae* has many characteristics that make the organism a useful model system.

Model systems are chosen for their favorable size, cost, and ability to be manipulated genetically. Yeast cells are approximately five microns in diameter. Their small size allows for easy viewing under a light microscope and the ability to maintain multiple strains cheaply with little maintenance. The *S. cerevisiae* genome has been completely sequenced allowing for an understanding of how the yeast genome is organized, and therefore can be manipulated more easily. In addition, baker's yeast can be transformed, or induced to take up foreign genetic material. Conversely, to cause a deletion the yeast can go through the process of homologous recombination, where a DNA segment replaces another DNA segment of similar sequence.[2] Lastly, the yeast is able to reproduce quickly under optimal nutritional conditions in approximately ninety minutes at 30°C, and can be quickly cultured on plates or in liquid media. Storage is also available for long periods when frozen in a

[1] Encyclopedia Britannica, 2009 Encyclopedia Britannica, Inc.
[2] http://www.hhmi.org

50% glycerol solution at -80°C. These beneficial characteristics are why yeast has remained a popular model system in genomics.

The life cycle of yeast involves reproduction by a process known as budding. The budding cell is referred to as the mother, and the bud as the daughter cell. The bud forms as the mother cell begins mitosis and separates to form two cells after cytokinesis and telophase have been completed. In the life cycle of *S. cerevisiae*, the yeast is able to survive as both a haploid and a diploid. A diploid cell has two copies of each homologous chromosome where as a haploid cell would only have one copy of the homologous chromosome. The normal haploid chromosome number for *S. cerevisiae* is sixteen with a size range of 200 to 2,200kb representing approximately 6,200 ORFs and 5,800 protein-encoding genes.[3] Furthermore, diploid cells can undergo sporulation when starved of adequate nutrients where through the process of meiosis a tetrad of haploid spores are formed and encased in a sac. These haploid spores can be mated together to form a diploid when the proper nutrients are available. Haploid cells can exist as either mating type MATa or MATα that can conjugate to form the diploid MATa/MATα.[4] This ability of yeast to survive as a haploid has allowed for the knockout collection to be easily created using homologous recombination in the PCR-based gene deletion strategy that placed a start- to stop- codon deletion at each of the

[3] http://dbb.urmc.rochester.edu/labs/Sherman_f/yeast/5.html
[4] Amberg 2-4

ORFs in the yeast genome.[5] This knockout collection has become invaluable in determining the function of many of the unknown ORFs that has given us a better understanding of how the cell cycle is controlled in *S. cerevisiae*.

The cell cycle, or how a cell replicates, consists of four phases. The first phase is Gap 1, G_1, where the cell grows and prepares for synthesis. The next phase synthesis, S, is when the DNA is replicated. The cell then proceeds to Gap 2, G_2, for further growth and preparation for mitosis. Lastly, the cell enters mitosis where the cell divides and finishes replication. While not in mitosis the cell is said to be in interphase. As a review, the regulatory molecules, cyclin and cyclin-dependent kinase, CDK, regulate how a cell progresses through the cell cycle depending on their levels. Genes encoding cyclins and CDKs are called cdcs. This is short for "cell division cycle." A number follows cdc indicating a unique identification number. CDKs are involved in specific signal cascades that regulate each step of the cell cycle. Each CDK must bind to the correct cyclin in order to activate the signal cascade required for proper progression through the cell cycle. One important factor in the completion of mitosis is the correct degradation of these cellular signals. RNase mitochondrial RNA Processing is one of these important enzymes necessary for correct degradation.

RNase mitochondrial RNA Processing, MRP, is an essential ribonucleoprotein endoribonuclease, meaning it is essential to the viability of

[5] http://www-sequence.stanford.edu/group/yeast_deletion_project/

the cell, and without it the cell cycle, among other things, will not function properly. Ribonucleoprotein means that RNase MRP is composed of RNA and protein components. Endoribonuclease describes the function of RNase MRP, mainly as an enzyme that cleaves the phosphodiester bonds within a ribonucleotide chain. Also, RNase MRP catalyzes the hydrolysis of RNA into smaller components. All eleven protein components are essential, and all are shared with RNase P except Rmp1 and Snm1.[6] These two unique proteins only found in RNase MRP are RNA binding proteins.[7] RNase MRP is evolutionarily related to RNase P (See Figure 1). Ancient cells must have had a need for RNA cell processing. This might have involved cell cycle control through RNA degradation. Cyclin proteins, such as yeast B-type cyclin, regulate how a cell progresses through the cell cycle depending on their levels. Completion of mitosis is dependent on the correct degradation of these cyclin proteins and their mRNAs. Since degradation of these signals is required for the completion of mitosis ancient cells might have used RNase MRP for this purpose.

[6] Schmitt and Clayton, 1992; Salinas et al., 2005
[7] Schmitt and Clayton, 1994; Salinas et al., 2005

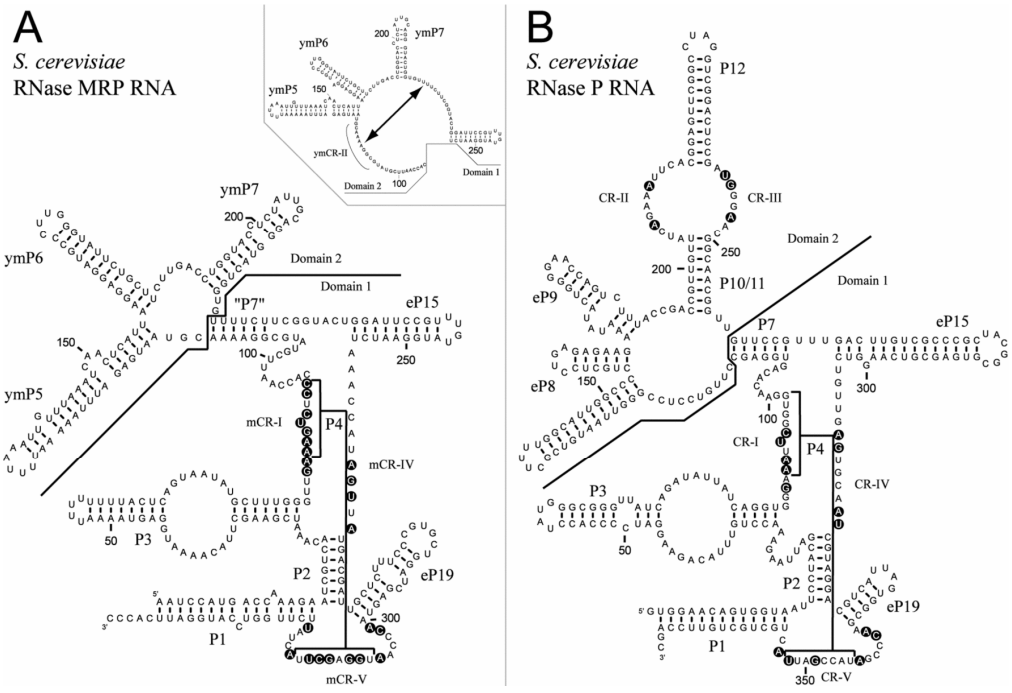

Figure 1: Side-by-side comparison of *S. cerevisiae* RNase MRP (A) and RNase P MRP RNA structure (B). (A) Similar domains and structures are labeled in each diagram, and conserved bases are highlighted in black. (B) Conserved regions in both RNase MRP RNA regions appear to occupy the same space (Walker et al., 2005).

Some of the functions of RNase MRP include mitochondrial primer formation, rRNA processing, and the degradation of specific mRNAs that control the cell cycle.[8] It is important to note that mitochondria have their own DNA, mtDNA, separate from the DNA located in the nucleus. The mtDNA, thought to be evolutionarily derived from bacteria, contains approximately 16,000 bp organized into a circular double-stranded helix. In addition, mtDNA is maternally inherited. RNase MRP cleaves RNA transcripts that form the RNA primer necessary for the initiation of mitochondrial DNA

[8] Gill et al., 2006

replication.[9] RNA MRP is also responsible for cleaving the pre-ribosomal rRNA to form the rRNA molecule 5.8S.[10] This process takes place in the nucleolus. The rRNA molecules 18S, 5.8S, 28S, and 5S come together to form the ribosome, which is the site of protein synthesis. Lastly, RNase MRP is essential in the degradation of specific mRNAs that control the cell cycle.[9]

RNase MRP was found to be essential to the degradation of specific mRNAs that control the cell cycle because several mutations of RNase MRP delay the cell cycle in late mitosis, and the result is a large budding yeast cell in the middle of telophase. Studies show that RNase MRP cleaves the mRNA for the yeast B-type cyclin, CLB2.[11] If a mutation in a RNase MRP component stops the function of RNase MRP, then CLB2 mRNA and the corresponding protein build up in the cell. Accumulation of CLB2 results in prolonged Clb2k/Cdk activity, and delays the completion of mitosis. In the normal cell division, RNase MRP would degrade CLB2 mRNA helping to contribute to the low levels of CLB2 protein signaling the completion of mitosis (See Figure 2).

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[9] Lee and Clayton, 1998
[10] Schmitt and Clayton, 1993; Lygerou et al., 1996
[11] Yeong et al., 2000

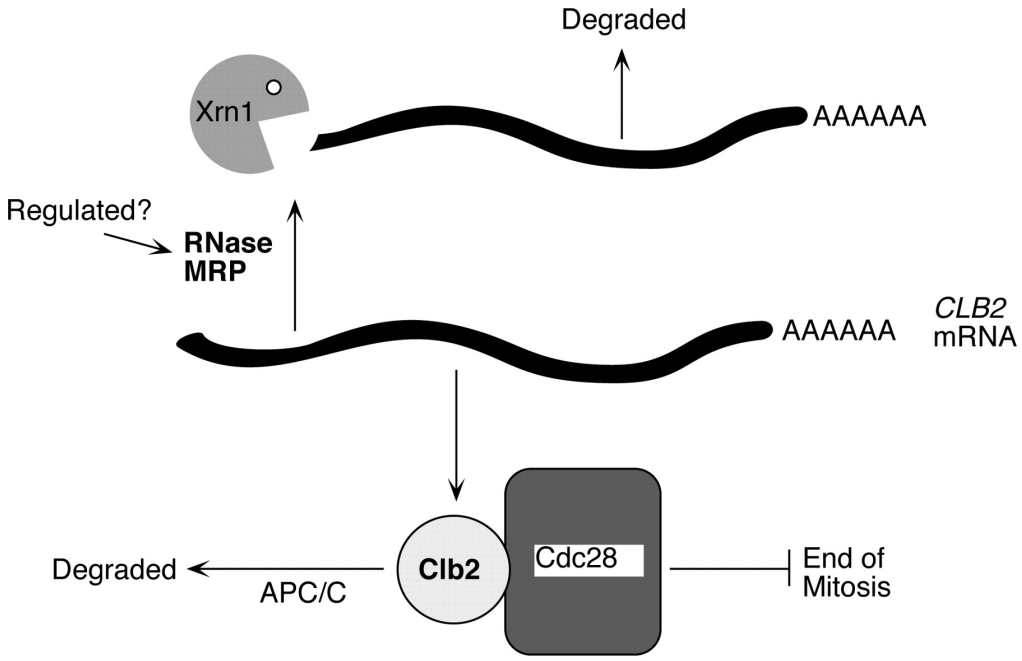

Figure 2: RNase MRP mechanism of action in cell cycle control. RNase MRP cleaves yeast B-type cyclin mRNA, also known as CLB2 mRNA. Degradation occurs by cleaving the CLB2 mRNA. RNase MRP mutations that stop the function of RNase MRP cause CLB2 mRNA to build up in the cell. Accumulation of CLB2 results in prolonged Clb2k/Cdk activity, and delays the completion of mitosis. Normal cell division involves RNase MRP degrading CLB2, and the low levels of CLB2 signal the completion of mitosis (Cai et al., 2002).

RNase MRP has been found to be localized in the nucleolus. This was observed by transforming two plasmids *pUN100[LEU2 CEN4 DsRED-NOP1]* and *pTD125[URA3 CEN GFP-POP1]* into yeast. The *pUN100* plasmid tags the nucleolar protein Nop1p with a DsRed fluorescent signal so that the nucleolus can be visualized using fluorescent microscopy. The *pTD125[URA3 CEN GFP-POP1]* plasmid tags the Pop1p protein subunit of RNase MRP with a GFP fluorescent signal so that RNase MRP can also be visualized using fluorescent microscopy. These two proteins signals co-localize indicating that RNase MRP localizes to the nucleolus during most of the cell cycle.[8] CLB2

has been assumed to be degraded in the cytoplasm. RNase MRP has been found to localize to a single spot in the daughter cell cytoplasm, and this location is where CLB2 mRNA is degraded.[8] In this model RNase MRP exits the nucleolus during mitosis, and localizes to a spot in the daughter cell that is a processing body like structure (See Figure 3). Processing bodies are sites in the cytoplasm where mRNAs are processed. Processing may involve de-capping of the mRNA to degrade it or other mechanisms of degradation to process the mRNAs. Certain mRNA processing and binding proteins are localized to these foci.[12] RNase MRP is thought to localize to such a special processing body specifically for the degradation of cell cycle specific signals that are daughter cell localized. These special processing bodies have been named TAM bodies, Temporal Asymmetric MRP bodies. We postulate that the TAM body is where CLB2 mRNA might be degraded.

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[12] Teixeira et al., 2005

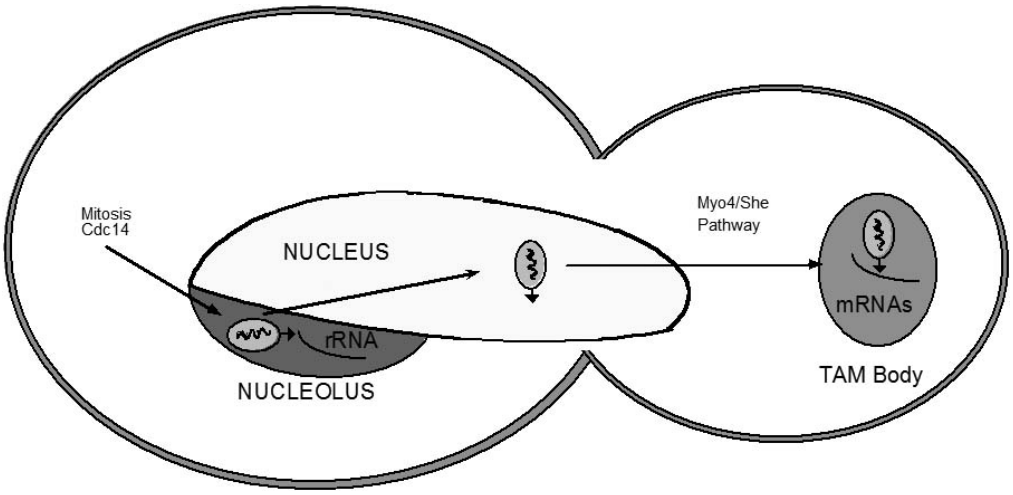

Figure 3: Localization of RNase MRP during mitosis. RNase MRP is localized to the nucleolus. During mitosis RNase MRP exits the nucleolus and moves into the cytoplasm. The RNase MRP moves to the TAM body site in the daughter cell. It is here at the TAM body where CLB2 mRNA might be degraded (Gill et al., 2006).

RNase MRP, as we have seen before, is required for cell growth. RNase MRP gene mutations lead to a decreased rate of cell growth because the mutated cell no longer has the ability to assemble rRNA and cyclin-dependent cell-cycle regulation no longer functions correctly. As a result of decreased cell growth and cell numbers, the mutation causes the disease cartilage-hair hypoplasia in humans. The disease is a rare defect that is recessively inherited. It is an autosomally recessive disorder, and not sex-linked. It is seen in mainly two populations the Amish and Finnish. In the Amish population, the frequency of the disease is 1 per 1340 in the population with a carrier rate of 1 per 19. In the Finnish population, the frequency of cartilage-hair hypoplasia is 1 per 23,000, with a carrier rate of 1 per 76.[13] Some of the more notable symptoms of the disease are short stature, defective

[13] Knutsen, 2007

immunity, and a predisposition to certain cancers such as leukemia and lymphoma (See Figure 4). Further research into the localization and function of RNase MRP may one day provide greater insight into new targets for cartilage-hair hypoplasia and cancer.

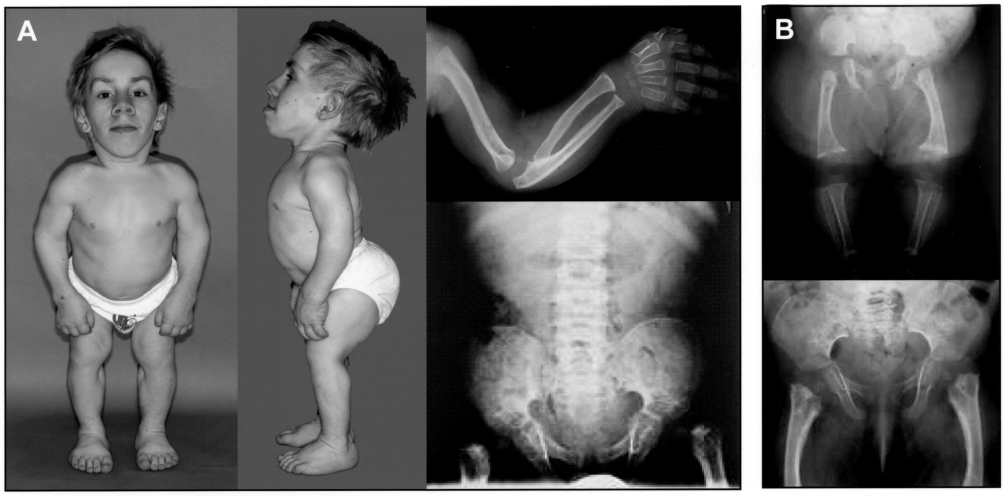

Figure 4: Clinical and x-ray characteristics of cartilage-hair hypoplasia. (A) Male patient aged 16.5 displays short stature due to advanced cartilage-hair hypoplasia. (B) X-rays show the extent of the curvature of the spine and severe bone deformities (Thiel et al., 2005).

To study the localization of RNase MRP fluorescent microscopy will be used. A fluorescent microscope is a type of microscope that uses fluorescent molecules to detect areas of interest on a specimen rather than just reflected white light that is used by a traditional light microscope. When an area of interest is tagged in a specimen with fluorescent molecules the area is then illuminated with a specific wavelength of light that is absorbed by the fluorescent molecules. The absorbed light is then given off in a longer wavelength of a different color, and can be captured and separated from the source light producing images of a single color in black and white. Multi-

color images of different fluorescent molecules can be produced by combining several of these single color black and white images.

The two fluorescent proteins that will be used in this project are green fluorescent protein, GFP, and red fluorescent protein, DsRed. GFP is composed of 238 amino acids, originally isolated from the Pacific jellyfish that fluoresces green when exposed to blue light.[14] The gene for GFP has been isolated and has become useful for linking the GFP protein to other proteins where it functions as a fluorescent protein tag. GFP tolerates N- and C-terminal fusion to a broad variety of proteins. DsRed fluoresces red under green light. The gene for DsRed has been isolated from coral reef organisms and has become useful for linking the DsRed protein to other proteins where it functions as a fluorescent protein tag.[15] DsRed tolerates N- and C-terminal fusion to a broad variety of proteins.

This project will examine the localization of RNase MRP as the yeast cells progress through mitosis. The TLG205 strain that will be used for this experiment has RNase MRP tagged with GFP, and the nucleolus tagged with DsRed. Under fluorescence various stages of the cell cycle will be examined for their localization pattern. My Capstone Project will study the localization and regulation of RNase MRP so that we can learn more about cartilage-hair hypoplasia, and also answer questions about how RNase MRP localizes, if the

[14] http://www.conncoll.edu/ccacad/zimmer/GFP-ww/GFP-1.htm
[15] http://www.microscopyu.com/articles/livecellimaging/fpintro.html

RNase MRP localization focus is the site of *CLB2* mRNA degradation, and if RNase MRP re-localizes back to the nucleolus after mitosis is completed.

In addition, minimal constructs of RNase MRP will be created to further reduce the RNA component of RNase MRP and potentially be able to examine the structure using X-ray crystallography. X-ray crystallography is a procedure for determining the three-dimensional structure of atoms arranged in a crystal. The crystal scatters X-rays in various directions producing unique patterns based on the structure of the atoms and their arrangement in the crystal. These patterns can then be analyzed to determine the three-dimensional structure of the atoms within the crystal. Using these methods we hope to learn more about the three-dimensional structure of RNase MRP, and may one day provide greater insight into new targets for cartilage-hair hypoplasia and cancer.

2. METHODS AND MATERIALS

2.1. Microscope Experiment Strains

The microscope experiment uses the strain TLG205 with the genotype of *MATα ade2-1 leu2-1,112 trp1-Δ1 his3-Δ200 ura3-52 pUN100[LEU2 CEN4 DsRED-NOP1] pTD125[URA3 CEN GFP-POP1]*. The strain was created using basic molecular biology techniques. The strain includes two plasmids *pUN100[LEU2 CEN4 DsRED-NOP1]* and *pTD125[URA3 CEN GFP-POP1]*. The *pUN100* plasmid tags the nucleolar protein Nop1p with a DsRed fluorescent signal so that the nucleolus can be visualized using fluorescent microscopy. The *pTD125[URA3 CEN GFP-POP1]* plasmid tags the Pop1p protein subunit of RNase MRP with a GFP fluorescent signal so that RNase MRP can be visualized using fluorescent microscopy. Now that these two proteins have been tagged with fluorescent signals localization and co-localization patterns can be imaged.

2.2. Strain Growth Maintenance

In order to minimize auto-fluorescence, the TLG 205 strain was grown in dropout synthetic minimal (SD) media that has the amino acids leucine and uracil omitted. The SD minimal media is prepared by combining 10 grams of agar, 10 grams of dextrose, 0.85 grams of yeast nitrogenous base w/o amino acids, 2.5 grams of ammonium sulfate, and then adding ddH_2O up to 500 ml. The mixture is autoclaved and then 5 ml of 1% histidine, 5 ml of 1% lysine, 5

ml of 1% methionine, 5 ml of 1% adenine, and 5 ml of 1% tryptophan were added.

The TLG 205 strain is maintained for 3-4 generations below 5 x 10^6 cells/ml in a 3-5 ml overnight culture placed in a motorized rotor at 30°C. The strain is maintained for 3-4 generations in order to improve the fluorescence of the DsRed signal. The media is also replaced before mounting and imaging in order to minimize autoflourescence, and obtain the proper cell concentration for imaging.

Before mounting, 1.25 ml of the TLG 205 strain in taken from the overnight culture and placed in a flat bottomed Erlenmeyer flask with 48.75 ml of SD minus leucine and uracil media . The flask is then placed in a 30°C shaker for four hours or until the cells have reached logarithmic growth. This process not only aerates the yeast, but also facilitates the growth of the cells to the proper density around 1 x 10^7 cells/ml that is required for this experiment. Avoid centrifuging the cells before mounting as the extreme forces may damage the cytoskeleton of the yeast and delay growth.

2.3. Slide Preparation

In previous attempts to mount TLG 205 and hold the cells in place for extended periods, up to 4-6 hours, we have found that simply wet mounting the cells only allows the cells to remain stationary for a few minutes. Instead an agarose pad is required. To prepare the agarose pad, 0.6 ml 2% agarose

and 0.4 ml SD minimal media minus leucine and uracil are combined to form a 1.2% final agarose composition. The 2% agarose is easiest to work with when aliquots of 0.6ml are made and placed in 1.5 ml microcentrifuge tubes. The SD media and agarose can be combined in the 1.5 ml microcentrifuge tube and warmed up in a heating block to approximately 60°C. After heating, the agarose and SD media mixture is transferred to a test tube and aerated by vortexing for 15-30 sec. If the agarose begins to solidify in the test tube, then it may be heated gently by a Bunsen burner.

Once the agarose and SD media mixture has been made approximately 200 µl is placed into the depression of a 60°C prewarmed slide with a single 18mm concavity depression. To smooth the top of the agarose another regular 1.5mm thick glass slide is quickly, before solidification of the agarose mixture, placed parallel to the single depression slide and clamped onto either end with a paper clamp. It is normal for excess agarose to squeeze out from the edges of the two slides. The clamped slides should then be flamed for a few seconds. This process ensures that no air bubbles will be trapped in the agarose interfering with the smooth surface. Also note that both of the slides and the coverslip should be free of any contaminates and should be cleaned with ethanol. To remove dust from the slides and coverslip a small dry brush may be used with the addition of pressurized air. After the agarose mixture has solidified, gently remove the top regular glass slide from the bottom depression slide, and remove excess agarose with a razor blade. With the cells

properly grown up to a density of approximately 1 x 10^7 cells/ml place 2 µl of cells onto the flat agarose surface. A regular glass coverslip measuring 22 x 22 mm, no. 1.5, is then lightly laid on top of the cells and agarose.

The slide is sealed with VALAP which is a 1:1:1 mixture of paraffin wax, petroleum jelly, and lanolin. A 1:1 mixture off capillary wax and petroleum jelly can also be substituted. Once the waxes are measured out they are heated and stirred in a beaker, and left to cool for later use. To seal the coverslip the VALAP is placed in the microwave until melted and spread evenly around the edges of the coverslip with a microspatula. The VALAP is used to keep the coverslip from moving, and to seal the internal environment of the slide off to prevent evaporation that would cause cell movement (See Figure 5).

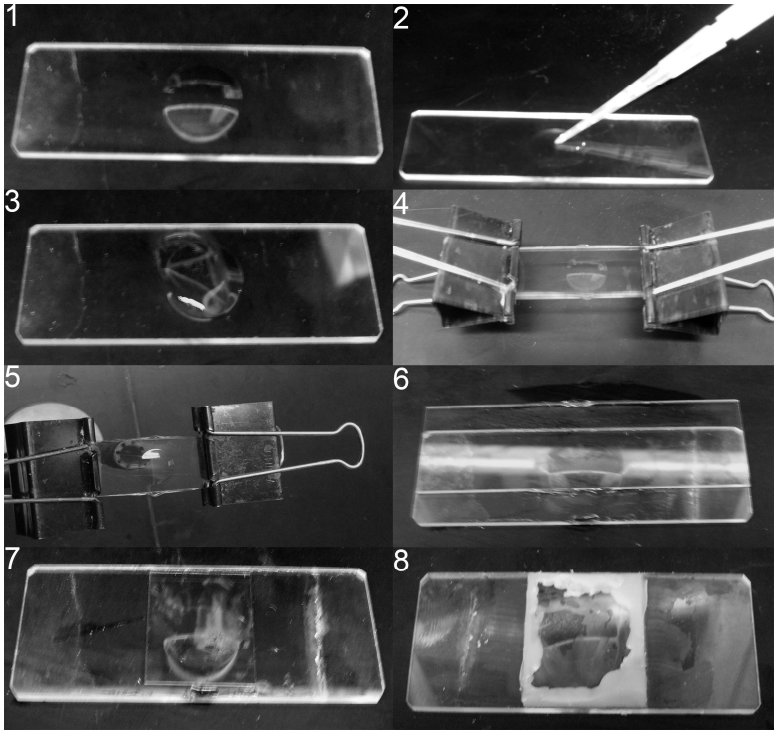

Figure 5: Preparing agarose slide and mounting *S. cerevisiae* cells. (1) Slide should be free of any contaminates and be cleaned with ethanol. (2) 200 µl of agarose and SD media is placed into the depression of a 60°C prewarmed slide. (3) Another regular glass slide is placed on top before solidification. (4) The two glass slides are paper clamped together. (5) The clamped slides are flamed to remove air bubbles. (6) After solidification, the top slide is slid off. (7) 2 µl of cells are placed on the agarose pad and a 22 x 22 mm, no. 1.5 coverslip is set on top. (8) VALAP is spread evenly around the edges of the coverslip with a microspatula.

2.4. Fluorescent Imaging

The microscope that was used is an upright fluorescent Carl Zeiss AxioImager Z1. The camera is a black and white 1.37 million pixel CCD Hamamatsu ORCA-ER with a progressive scan interline CCD chip. The objectives being used are the Carl Zeiss A-Plan 10x/ 0.25, EC Plan-neofluar 40x/ 0.75, and the α Plan-Apochromat 100x/ 1.46 oil DIC (UV) VIS-IR. The microscope camera is connected via IEEE 1394 to a PC running Windows XP SP3 and the AxioVision software version 4.6.3.0 SP1.

To set up an experiment, the slide is loaded onto the stage and brought into focus under the 100x objective. Using the experiment window in AxioVision illumination type, binning, exposure time, z-stack coordinates, time lapse settings, and gain settings can all be adjusted and set up for the experiment. Once setup, the microscope will then carry out the automated experiment. After imaging has finished the project can be viewed in 3D or in slices. AxioVision includes many image enhancing features such as deconvolution, z-stack correction, and time-lapse correction that can be used after imaging to enhance the areas of interest.

2.5. DNA Fragments and pMES145 Plasmid

The DNA fragments of RNase MRP with included site mutations were ordered from Epoch Biolabs who synthesized these fragments using basic molecular biology techniques. The fragments will be placed into the plasmid *pMES145* for later transformation into yeast.

2.6. Electroporation of E. coli

The electroporation of *E. coli* is preformed in two parts first the preparation of competent *E. coli* cells and then the electro-transformation. To prepare the competent cells, an overnight culture of the *E. coli* is placed in a test tube with 3-5 ml of LB media (10g Bacto-tryptone, 5g yeast extract, 10g NaCl, and 800 ml ddH20; adjust to pH of 7.5 with NaOH) and incubated at 37°C overnight. 200 ml of L-Broth (1% Bacto-tryptone, 0.5% Bacto yeast

extract, and 0.5% NaCl) and 2 ml of the overnight culture are then placed in a flat bottomed Erlenmeyer flask and shook vigorously at 37°C for two and a half hours or until the cells are in early to mid log phase at an approximate ABS_{600} of 0.5-0.7. The cells are harvested in a cold centrifuge bottle in a cold rotor at 4000 x g for fifteen minutes. The supernate is removed, and then resuspended in 200 ml of ice-cold 10% glycerol. The sample is repelleted as per the instructions before, and resuspended in 100 ml of ice-cold 10% glycerol. The sample is again repelleted as per the instructions before, and resuspended in 50 ml of ice-cold 10% glycerol. The sample is once again repelleted as per the instructions before, and resuspended in 3-4 ml of ice-cold 10% glycerol. Lastly, the aliquots are frozen on dry ice and stored at -70°C for up to six months.

The electro-transformation is performed by first thawing the competent *E. coli* cells at room temperature, and then placing them on ice. Also place the sterile cuvettes and cuvette slide on ice as well. In a cold 1.5 microcentrifuge tube, add 40 µl of the competent *E. coli* cells and 1-2 µl of DNA. Let the cold tube sit with the mixture on ice for one minute. Set the *E. coli* Pulser unit to 1.80 kV for the 0.1 cm cuvette, and 2.50 kV for the 0.2 cm cuvette. Place the cold cell mixture into the cuvette and tap to shake the suspension to the bottom. Place the cuvette into the cold slide and into the Pulser unit. Pulse once and immediately add 1 ml of SOC media (2% Bacto-tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM

$MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose). Using a Pasteur pipette resuspend the cells in the SOC thoroughly. Transfer the cells into a 1.5 ml microcentrifuge tube and incubate at 37°C for one hour. Lastly, plate onto selective media.

2.7. Plasmid Purification Miniprep

To begin, inoculate an overnight culture with *E.coli*, 3 ml of LB media plus ampicillin (10g Bacto-tryptone, 5g yeast extract, 10g NaCl, and 800 ml ddH20; adjust to pH of 7.5 with NaOH; add 100mg/ml of ampicillin). Place overnight culture into a motorized rotor at 37°C overnight. Place 1.5 ml of the overnight culture into a 1.5 ml microcentrifuge tube. Centrifuge the tube at 14,000 x g for thirty seconds. Pour off the supernate, and resuspend the pellet in left over media. Add 300 µl of TENS (5 ml 1M Tris-HCl pH 8.0, 12.5 ml 20% SDS, 5 ml 10N NaOH, and fill up to 500 ml with ddH20) to the microcentrifuge tube. Vortex the solution immediately for five seconds, and place on ice. Add 150 µl of 3M Na Acetate pH 5.3. Spin the tube in the microcentrifuge for five minutes at 14,000 x g. Pour off the supernate into a new tube, and precipitate the DNA by adding 1 ml of cold 100% ethanol. Afterwards, the sample is spun in the microcentrifuge at 14,000 x g for five minutes. The DNA pellet is then washed with cold 80% ethanol and dried in the speed vacuum. Next, the DNA is re-suspended in 200 µl of TE and 1 µl RNase A is added. The sample is incubated at 37ºC for 5 minutes, and lastly stored at -20º C.

2.8. Plasmid Digest

To digest a plasmid 10 µl of plasmid, 1.2 µl of buffer, and 0.8 µl of the restriction enzyme are added to a microcentrifuge tube and placed in a 37°C heating block for two hours or overnight.

2.9. Gel Electrophoresis

Gel electrophoresis was used in this experiment to separate plasmid DNA based on size and charge. The 0.8% agarose gel is made by adding 30 ml 1x TAE buffer (40 mM Tris; 20 mM acetic acid; 1 mM EDTA) and 0.25 grams of agarose to a flat bottom Erlenmeyer flask. The agarose is heated in the microwave until boiling stirring periodically, and then the flask is rinsed under cold water until it is just warm to the touch. The agarose is poured into the electrophoresis unit with the gel blocks installed. Once the agarose is solidified the gel blocks can be removed. The electrophoresis unit is then filled with TAE buffer. As a control, 5 µl of DNA marker is added to 1 µl 6 x DNA running dye and loaded into the first well. Each DNA sample is loading into its own separate well consisting of 10 µl of DNA sample and 3 µl of DNA running dye. The power supply is hooked up to the electrophoresis unit and turned to 80-100V for approximately one hour. After the loading dye can be seen about half way down the gel the power supply is turned off. The gel can now be removed and placed in a small container. The container is filled

with water and 100 µl of ethidium bromide is added. The container is placed on a laboratory shaker for ten minutes. Afterwards, the gel is rinsed with water and placed on the shaker again for ten minutes suspended in water. After staining and rinsing, the gel can now be visualized using a UV light.

2.10. Eppendorf Perfect Prep Gel Cleanup

The Eppendorf Perfect Prep Gel Cleanup is used to purify the DNA from a gel. After gel electrophoresis and staining the desired DNA band is excised from the agarose gel. The excised gel band is placed in a pre-weighed microcentrifuge tube. The tube is pre-weighed in order to determine the correct amount of buffer to add based on the weight of the gel. Three volumes of Binding Buffer are required for every one volume of agarose gel. Incubate the sample at 50°C for 5-10 minutes in a heat block, vortexing every 2-3 minutes. Make sure that all the agarose gel has been dissolved completely. Afterwards, one volume of isopropanol is added that is equal to the original gel volume. Mix the solution by inversion or by pipetting up and down. Insert a spin column into a 2 ml collection tube, and add 800 µl of the sample at a time to the spin column. Spin the tube assembly in a microcentrifuge at 10,000 x g for one minute. Discard the filtrate, and replace the spin column into the collection tube. For large samples, reload the spin tube and centrifuge again. Add 750 µl of diluted wash buffer to the spin column, and centrifuge at 10,000 x g for one minute. Discard the filtrate, and replace the spin column into the collection tube. Centrifuge for an additional minute at 10,000 x g to

remove any residual diluted wash buffer left behind. Place the spin column into a new 2 ml collection tube, and add 30 µl of elution buffer to the center of the spin column. Lastly, centrifuge for one minute at 10,000 x g. The spin column can now be thrown away and the collection tube stored at -20°C for future use.

2.11. Yeast Transformation

The yeast transformation is preformed in two parts first the preparation of competent yeast cells and then the LiAc transformation. To prepare the competent cells, an overnight culture of the desired yeast cells are placed in 1.5 ml of liquid YPD, and then the test tube is incubated in the 30°C motorized rotor. The overnight culture is grown to approximately 2 x 10^8 cells/ml and then 1.25 ml is diluted into 50 ml of YPD media. The flat bottom Erlenmeyer flask is placed in the 30°C shaker for four hours to grow to mid-log phase. The cells are transferred to a 50 ml corning tube and pelleted at 4,000 x g for five minutes at room temperature. The supernate is poured off, and the pellet is resuspended in 15 ml of 1x TE (10ml 1M Tris-HCl pH 8.0, 400µl 2.5M EDTA, and 990 ml ddH20). The sample is repelleted as per the instructions before, and is resuspended in 15 ml of TE/ 0.1 M LiAC (10 ml 1M LiAC, 1 ml 1M Tris-HCl pH 8.0, 0.2 ml 2.5M EDTA, and 88.8 ml ddH20). The sample is again repelleted as per the instructions before, and the supernate is poured off. Resuspend the pellet in 500 µl of TE/ 0.1 M LiAC, and incubate at 30°C for one to four hours with moderate shaking. The

competent cells can either be placed on ice to be used immediately or refrigerated at 4°C for up to 10 days.

The LiAc transformations are performed by first aliquoting 200 µl of competent yeast cells into 1.5 ml microcentrifuge tubes. 15 µl of herring sperm DNA (preheated at 95°C for five minutes) and 10 µl of plasmid DNA is added to the tube and vortexed lightly. The sample is then incubated at 30°C for thirty minutes while taped to a motorized rotor for light shaking. After incubation 750 µl of TE/ 0.1 M LiAC/ 40% PEG 4000 (8 vol 50% PEG 4000, 1 vol 10x TE pH 8.0 100mM Tris-HCl and 10mM EDTA, and 1 vol 10x LiAc) is added. The sample is then heat shocked in a water bath at 42°C for five minutes. After the heat shock, the samples are centrifuged for five seconds at 14,000 x g. The supernate is removed, and 100 µl of TE is added to resuspend the cells. Lastly, the cells are spread onto selective plates and grown at 30°C for three days.

2.12. Smash and Grab

The smash and grab procedure is performed by first growing an overnight culture of the desired yeast cells in 1.5 ml of liquid YPD, and then placing the test tube in the 30°C motorized rotor. The next day, 1.5 ml of the overnight culture is placed in a 1.5 ml eppendorf microcentrifuge tube, and centrifuged for five seconds at 14,000 x g. The YPD is decanted off and the pellet is resuspended in the remaining media. 200 µl Smash and Grab Buffer (1% SDS, 2% Triton X-100, 100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA,

pH 8.0), 200 µl phenol pH 8.0, 200 µl chloroform, and 0.3g of acid washed glass beads are then added to the resuspended cells. The acid washed beads are added in order to perforate the membrane and release the cellular contents into the solution. The microcentrifuge tube is then placed in the vortex for two minutes. After the vortex, the samples are pelleted in the centrifuge for five minutes at 14,000 x g. The aqueous layer is transferred to another 1.5 ml microcentrifuge tube, and the DNA is precipitated out with the addition of 200-500 µl cold 100% ethanol. Afterwards, the sample is spun in the microcentrifuge at 14,000 x g for five minutes. The DNA pellet is then washed with cold 80% ethanol and dried in the speed vacuum. Next, the DNA is re-suspended in 100 µl of TE and 1 µl RNase A is added. The sample is incubated at 37ºC for 5 minutes, and lastly stored at -20º C.

2.13. DNA Sequencing

DNA sequencing requires five main components: a single stranded DNA template, a DNA primer, a DNA polymerase, free nucleotides (dNTPs), and dideoxynucleotides (ddNTPs) to terminate DNA elongation. The ddNTPs terminate elongation to leave several shortened DNA fragments of varying lengths. The DNA sequencing is run in four different reactions for each of the four different ddNTPs. After the reaction the varying lengths of DNA fragments are separated by gel electrophoresis, and the sequence can then be read in order off of the gel.

SUNY Upstate Medical Center has its own DNA sequencing facility that performs the above DNA sequencing process using automated machines. After DNA sequencing is complete, the facility sends an electronic file containing the sequence. This electronic file can be read by many programs including DNA Strider. This technique was used to verify the correct insertion of an RNase MRP mutation into the *pMES145* plasmid in this experiment.

3. RESULTS

3.1. Determining a Method of Slide Preparation

In order to image the localization of RNase MRP as the yeast cells progress through mitosis, the cells must be held in place for extended periods, up to 4-6 hours. A wet mount slide was the first method of slide preparation that was tried. This procedure involved using a flat slide and coverslip with the cells mounted in liquid media, approximately 1µl. The problem with this type of mounting is that it is not suitable for long term imaging. The cells move around quickly as the media the cells are mounted in evaporates. The cells only remain in place for a few minutes at most. This procedure does not allow for time-lapse or z-stack imaging since the cells move rapidly in the media. At best, this method is suitable for taking individual images one at a time.

The next method that was tried was mounting the cells on a continuous flow slide. This procedure involved modifying a wet mount slide to include a small piece of Kimwipe on either side of the coverslip to act as a wick to minimize evaporation. The Kimwipe can be rewetted with media as evaporation occurs. Again, the problem with this type of mounting is that it is not suitable for long term imaging. The cells moved around just as quickly as

with the wet mount slide only allowing for a few minutes of imaging. As with wet mounting, this method is not suitable for time-lapse or z-stack imaging.

The third method that was tried was mounting the cells in a slide with wells. This procedure involved placing liquid media into the individual wells of a slide, and gently covering with a coverslip. Similarly, the problem with this type of mounting is that it is not suitable for long term imaging. The cells moved around just as quickly as with the wet mount and continuous flow slides only allowing for a few minutes of imaging.

The fourth method that was tried was mounting the cells on a thin agarose pad. Using two clamped together flat slides a thin agarose pad was created in between. The coverslip was then locked down with nail polish to prevent evaporation and movement. This method allows for imaging of cells for extended periods of time including time-lapse and z-stack imaging. Also, the agarose pad was mixed with media allowing the cells to continue growth during mounting. The main problem with this method was that creating the thin agarose pads was difficult, and often resulted in unusable torn pads. Even with experience this method was difficult to produce a flat agarose surface to mount cells on.

The last method that was tried was a modified version of the above procedure. Using a depression slide, the agarose pad now had a place to sit and a smooth agarose surface could be created easily every time. In addition, VALAP was used to seal the coverslip to prevent movement and evaporation.

This method was found to produce the best results for long term imaging, and was used for the remainder of the experiment. This method allowed for z-stack and time-lapse images to be taken in approximately thirty minute intervals. Every thirty minutes the microscope had to be refocused due to the stage drifting.

3.2. Determining a Procedure to Reduce Photobleaching

In order to image the localization of RNase MRP as the yeast cells progress through mitosis, images need to be taken in quick succession. The problem of photobleaching occurs when quicker and more frequent images are taken, and the fluorescent molecules begin to fade. Photobleaching is when a fluorescent molecule, fluorophore, suffers chemical damage caused by photons. This damage causes the fluorophore to lose its fluorescence permanently. By using digital and analog gain, binning, and small exposure times photobleaching can be minimized.

The purpose of analog gain is to reduce exposure time allowing for longer imaging periods without photobleaching. The downside to increasing analog gain is that using this setting increases background noise. Analog gain works to decrease the exposure time by amplifying and increasing the sensitivity of the analog signal before the signal is converted into a digital signal. On the other hand, digital gain works to decrease the expose time by amplifying and increasing the sensitivity of the digital signal after the analog to digital conversion has taken place. Using either gain settings will shift the

histogram, and produce an image that has an amplified signal, but will increase the amount of background noise. Through trial and error I have found that an analog gain setting of approximately 100 offers the best signal amplification without an excess of background noise. Any analog setting over 125 greatly increases background noise and will significantly decrease image quality. Digital gain, as stated above, increases the background noise more than analog gain, and should only be turned onto one of the lowest setting if at all.

Another method to reduce photobleaching is to use binning. Binning is available on most CCD cameras and offers the ability to combine pixels in both the horizontal and vertical directions into one larger pixel. Thus, a square of 2 x 2 pixels can be combined into one larger pixel. A binning of 2 x 2 would increase the light sensitivity of the camera by four times. By increasing the sensitivity to light a shorter exposure time can be used to minimize photobleaching and allow for a longer time lapse acquisition period. The down side to using binning is that the resolution of the camera in 2 x 2 binning is reduced by a quarter. Many times 2 x 2 binning can be used without any significant loss in quality affecting the acquired image because of the high 1 x 1 resolution of many newer CCD cameras. Through trial and error the best binning setting has been found to be 2 x2. Anything greater than 2 x 2 binning reduces the quality of the images too much.

Reducing the exposure time by using binning and gain settings allows for a greater reduction in the amount of photobleaching. This is because the shorter the time the fluorescent molecules are exposed to photons the less chemical damage they suffer allowing the fluorescent signal to remain for a longer period of time. Using the binning and gain settings the exposure time can be minimized to just 60 ms. With these settings it is possible to take z-stack images of 10 slices every six minutes for approximately 1-1.5 hours.

3.3. Imaging of RNase MRP Localization

Reaching the limits of the microscope, the slide mounting method allows for z-stack and time-lapse images to be taken in approximately thirty minute intervals. Every thirty minutes the microscope has to be refocused due to the stage drifting. An autofocus module was installed and has so far been unsuccessful in increasing the period of time that the cells need to be refocused. The microscope has also been preheated by turning on all the equipment and letting the microscope heat up for two to four hours without significant improvements to the period that the cells need to be refocused. The microscope must be preheated since the calibrations for stage drifting were made while the microscope is hot.

In addition, the limits of photobleaching have narrowed the time that successive z-stack images can be taken in. The most frequent that z-stack images of 10 slices can be taken in is every five minutes for approximately 1-1.5 hours. With several movies capturing dividing cells with z-stack images

spaced six minutes apart the localization of RNase MRP into the daughter cell during mitosis has been missed. In order to capture this localization process, the z-stack images will have to be taken in greater succession at around two minutes apart or even smaller. Confronted with problems of stage drift and photobleaching a movie of the localization of RNase MRP has yet to be captured. The exact timing of the localization of RNase MRP into the daughter cell is not currently known. From the many z-stack movies that were taken, it is believed that this process must occur at a shorter interval than six minutes.

3.4. Minimal Constructs

To create the mini1 MRP, the deletions that were made to the RNA of the RNase MRP were to remove the ymP5, ymP6, ymP7, and eP19 terminus. The ymP7 and eP19 termini were replaced by hairpins with the DNA sequence GGAAAC. This hairpin sequence acts to stabilize the missing terminus. The sequence was compiled in DNA strider, a DNA sequence editing program, and the DNA fragment was ordered from Epoch Biolabs (See Figure 6).

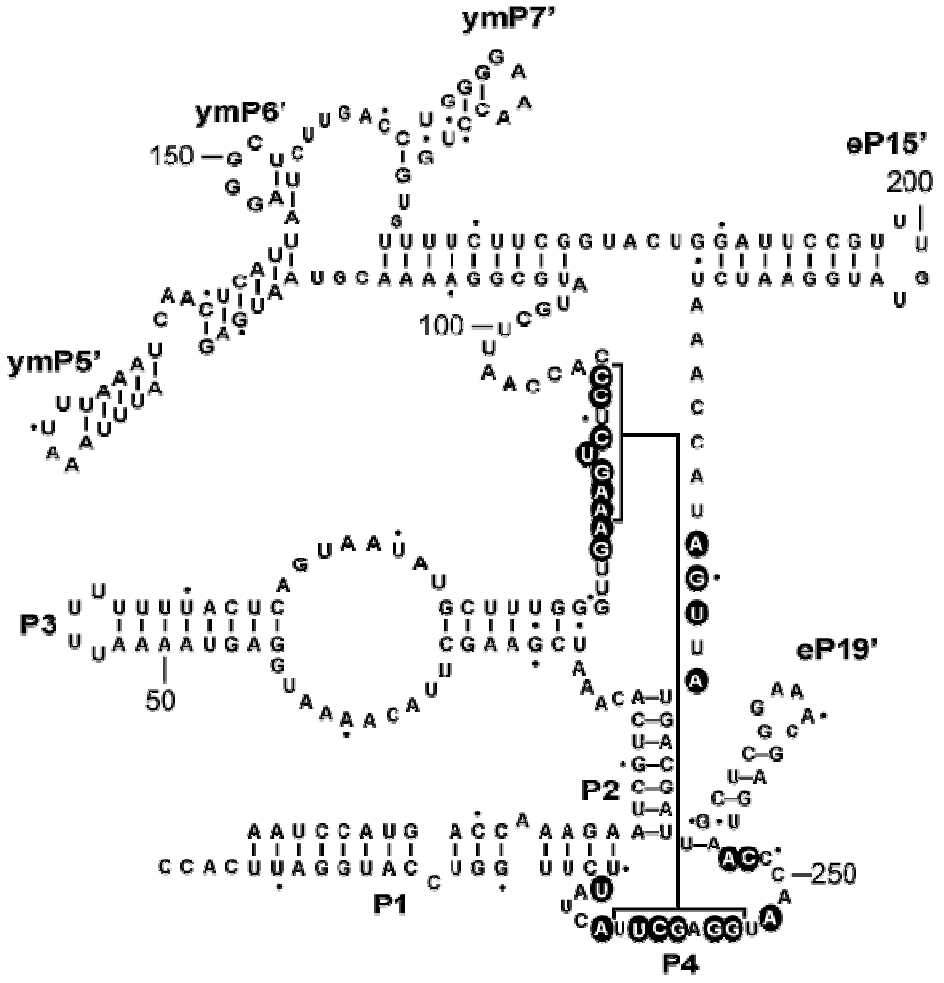

Figure 6: Mini1 RNase MRP secondary structure. The mini1 RNase MRP secondary structure includes the deletions of the ymP5, ymP6, ymP7, and eP19 terminus. The ymP7 and eP19 termini were stabilized with GGAAAC hairpins. The black circles outline bases that are conserved in all known MRP sequences.

The pMES145 plasmid was prepared by electroporation into *E. coli* cells, and then the cells were lysed to obtain the pMES145 plasmid using a plasmid purification miniprep. The pMES145 plasmid was digested overnight with the restriction enzymes *Hin*dIII and *Nco*I to cut out the MRP section of the pMES145 plasmid (See Figure 7). The digested plasmid was run on a

DNA mini gel to test the concentration of DNA. The concentration of DNA was found to be too high (See Figure 8). So the overnight double digest was redone with 20 µl of plasmid DNA diluted up to 100 µl with TE, 12 µl of buffer, and 4 µl of each restriction enzyme. Ten wells were run out on a DNA mini gel. The top band containing the pMES145 plasmid was excised using the Qiagen gel extraction kit and run on a DNA mini gel (See Figure 9). The purified plasmid was then transformed along with the mini1 MRP DNA into the MES116 strain (*MATα lys2-801 his3-Δ200 leu2-3,112 ura3-52 trpl-Δl nmel-Δ2:TRP1* pMES127 [pRS316::*NME1 URA3 CEN*]). The cells were then plated onto two round of leucine minus media and one round of 5FOA plates to select for the yeast cells containing the pMES145 plasmid with the mini1 MRP DNA insert. Using a smash and grab procedure the yeast cells were lysed, and the pMES145 plasmid with the mini1 MRP DNA insert was recovered. The pMES145 plasmid with the mini1 MRP DNA insert was then electroporated into *E. coli* cells. The plasmid was harvested from the *E. coli* with a plasmid miniprep. A double digest with *Nco*I and *Eco*RI was done overnight, and the digested plasmid was run on a DNA minigel to confirm the proper size of the mini gene (See Figure 10). Lastly, the plasmid was sequenced and compared to the original ordered DNA fragment to check for the correct mini1 MRP insertion into the pMES145 plasmid. The mini1 mutant was viable and exhibited good growth rates at 30°C

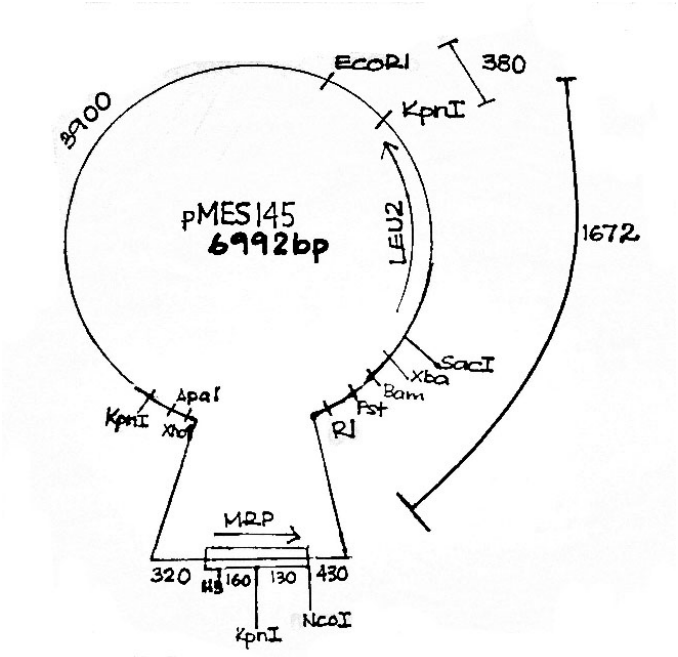

Figure 7: pMES145 plasmid restriction map. The restriction map lists all of the popular restriction sites for the pMES145 plasmid.

Well Number: 1 2 3 4 5 6 7 8 9 10

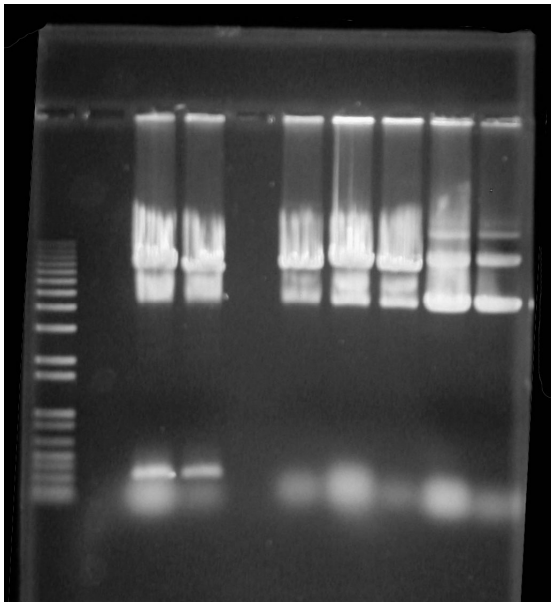

Figure 8: The pMES145 plasmid was digested overnight with the restriction enzymes Hind3 and Nco1 and run on a DNA mini gel to test the concentration of DNA. The concentration of DNA was found to be too high. (1) DNA ladder. (2) Empty. (3) Both *Nco*I and *Hind*III digest of pMES145. (4) Both *Nco*I and *Hind*III digest of pMES145. (5) Empty. (6) *Hind*III digest of pMES145. (7) *Nco*I digest of pMES145. (8) *Nco*I digest of pMES145. (9) pMES145 plasmid with no restriction enzyme digest. (10) pMES145 plasmid with no restriction enzyme digest.

Well Number: 1 2 3

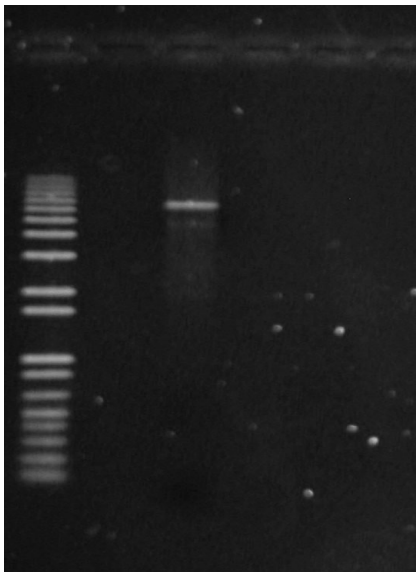

Figure 9: pMES145 plasmid was excised using the Qiagen gel extraction kit and run on a DNA mini gel. (1) DNA ladder. (2) Empty. (3) Purified pMES145 plasmid.

Well Number: 1 2 3 4 5

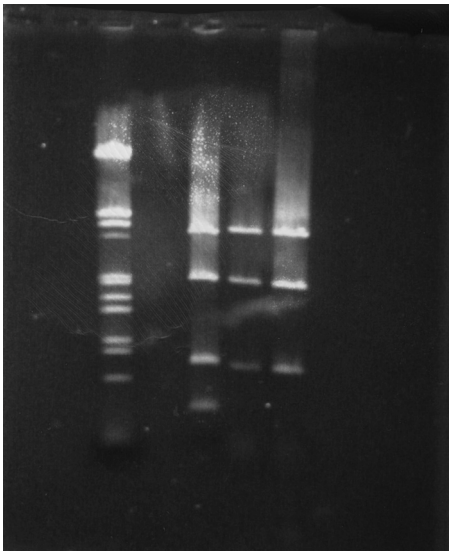

Figure 10: The pMES145 plasmid was digested overnight with the restriction enzymes *Nco*I and *Eco*RI and run on a DNA minigel to confirm the proper size of the mini gene. (1) DNA ladder. (2) Empty. (3) pMES145 plasmid. (4) pMES145 plasmid with mini1 MRP insertion. (5) pMES145 plasmid with mini1 MRP insertion.

To further reduce the RNA of the RNase MRP, four additional mutations were made to the mini1 MRP. To create the mini1-1 MRP, nucleotide pairs 5-13 and 260-268 in the P1 region were deleted to remove the base at positions 12 and 264, an adenine and cytosine respectively, since both do not have a base pair on the opposite RNA strand (See Figure 11). To create the mini1-2 MRP, the P3 terminus was removed and replaced by the DNA sequence GGAAAC (See Figure 12). The mini1-3 MRP was created by removing the eP15 terminus and replacing the DNA sequence with GAA (See Figure 13). The mini1-4 MRP was created by deleting the remaining base pairs between ymP6 and ymP7 (See Figure 14). The sequences were compiled in DNA strider, and the DNA fragments were ordered from Epoch Biolabs. The four mini1 DNA fragments will be cloned into the pMES145 plasmid and transformed into the MES116 strain. Afterwards, the viable strains will be mated together to produce an MES116 strain that contains all of the mini1 RNA mutations. Although the mini1 mutant was viable and exhibited good growth rates at 30°C, not all of the mini1-1, mini1-2, mini1-3, and mini1-4 mutants did. Future research will investigate which of these four mutant strains possess good growth rates at 30°C.

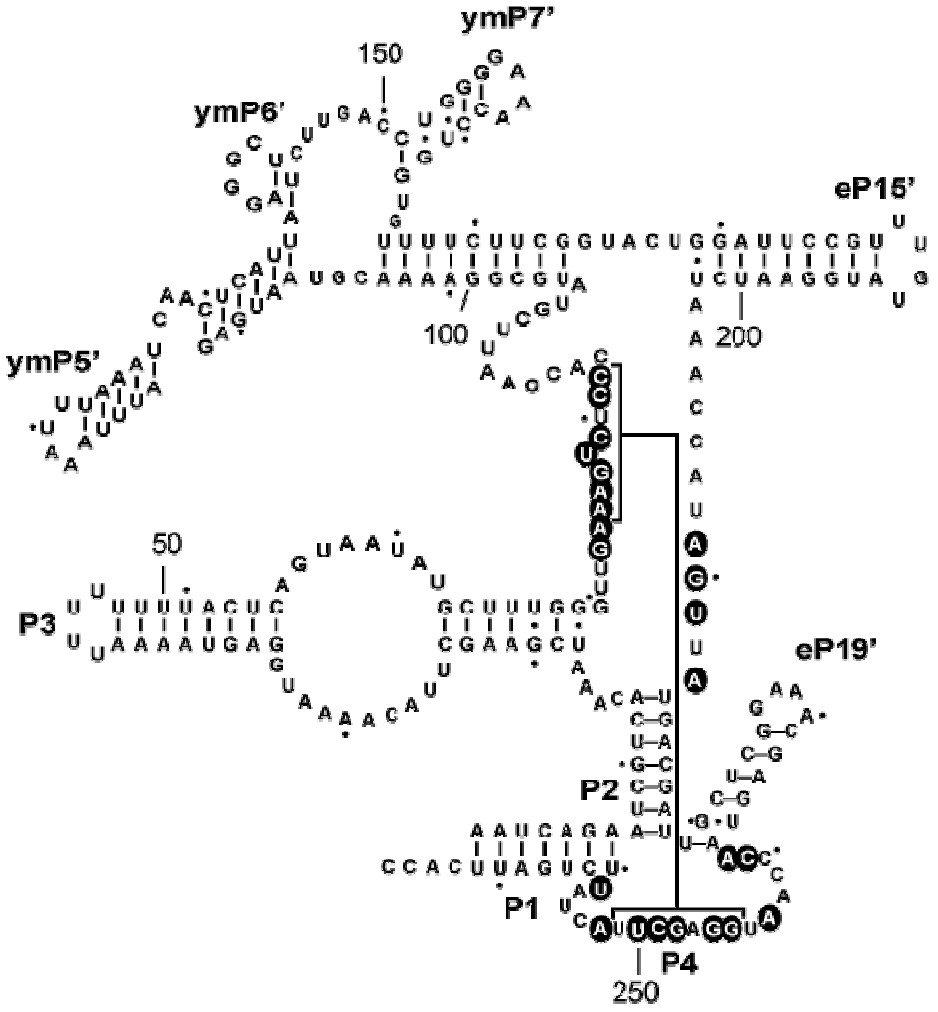

Figure 11: Mini1-1 RNase MRP secondary structure. The mini1-1 RNase MRP secondary structure includes the deletions of the ymP5, ymP6, ymP7, and eP19 terminus. The ymP7 and eP19 termini were stabilized with GGAAAC hairpins. In addition, base pairs 5-13 in the P1 region were deleted to remove the base at position twelve, an adenine, that does not have a base pair on the opposite RNA strand

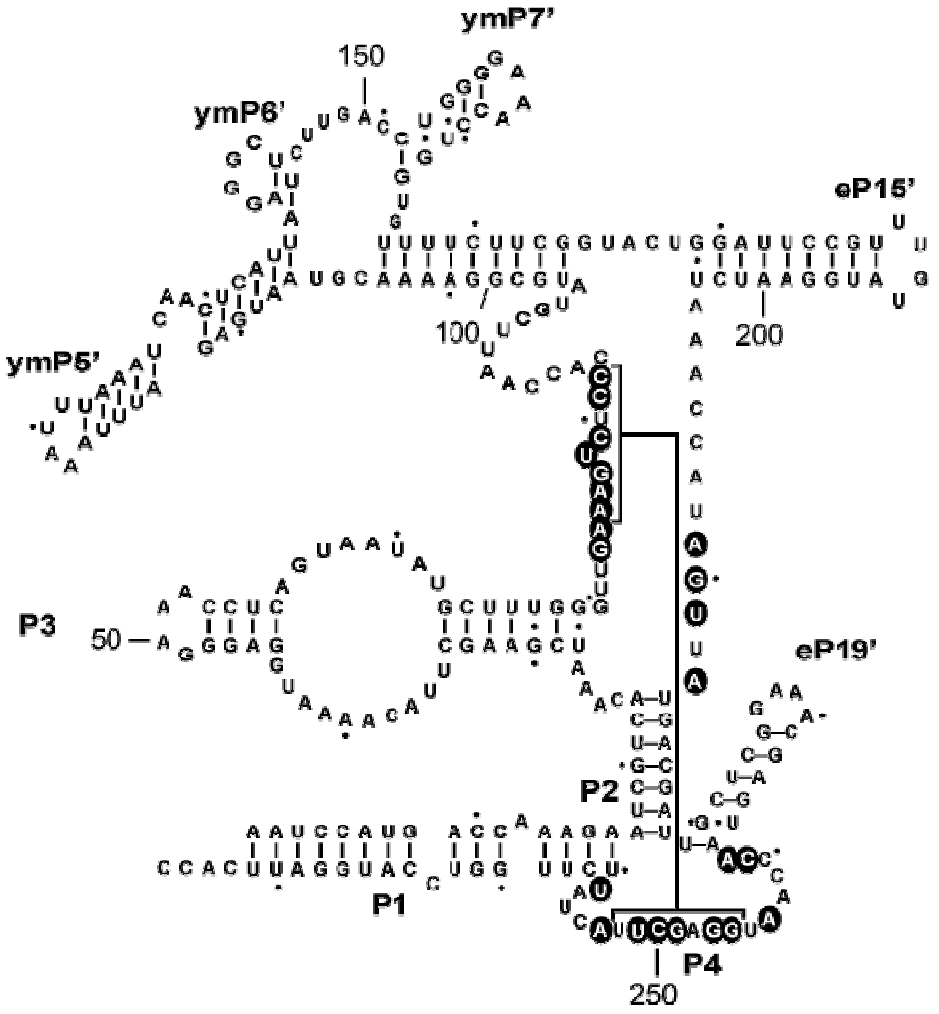

Figure 32: Mini1-2 RNase MRP secondary structure. The mini1-2 RNase MRP secondary structure includes the deletions of the P3, ymP5, ymP6, ymP7, and eP19 terminus. The P3, ymP7, and eP19 termini were stabilized with GGAAAC hairpins.

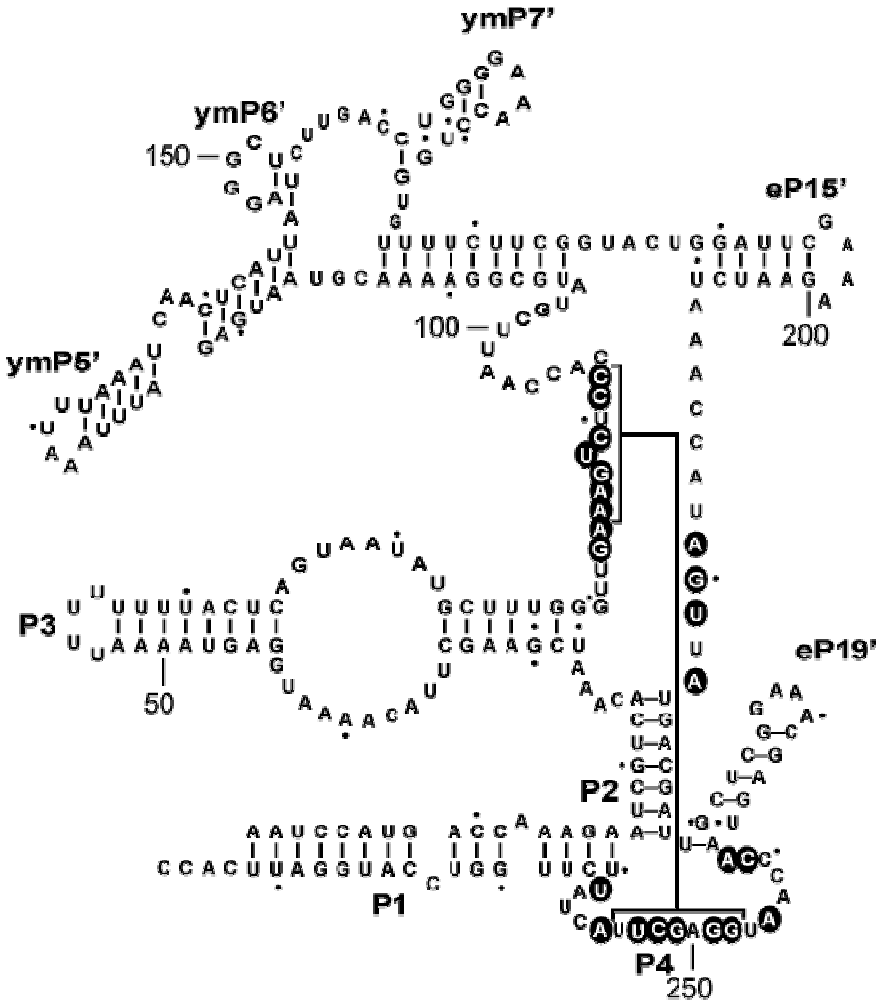

Figure 13: Mini1-3 RNase MRP secondary structure. The mini1-3 RNase MRP secondary structure includes the deletions of the eP15, ymP5, ymP6, ymP7, and eP19 terminus. The ymP7 and eP19 termini were stabilized with GGAAAC hairpins. The eP15 terminus was stabilized with a GAA hairpin.

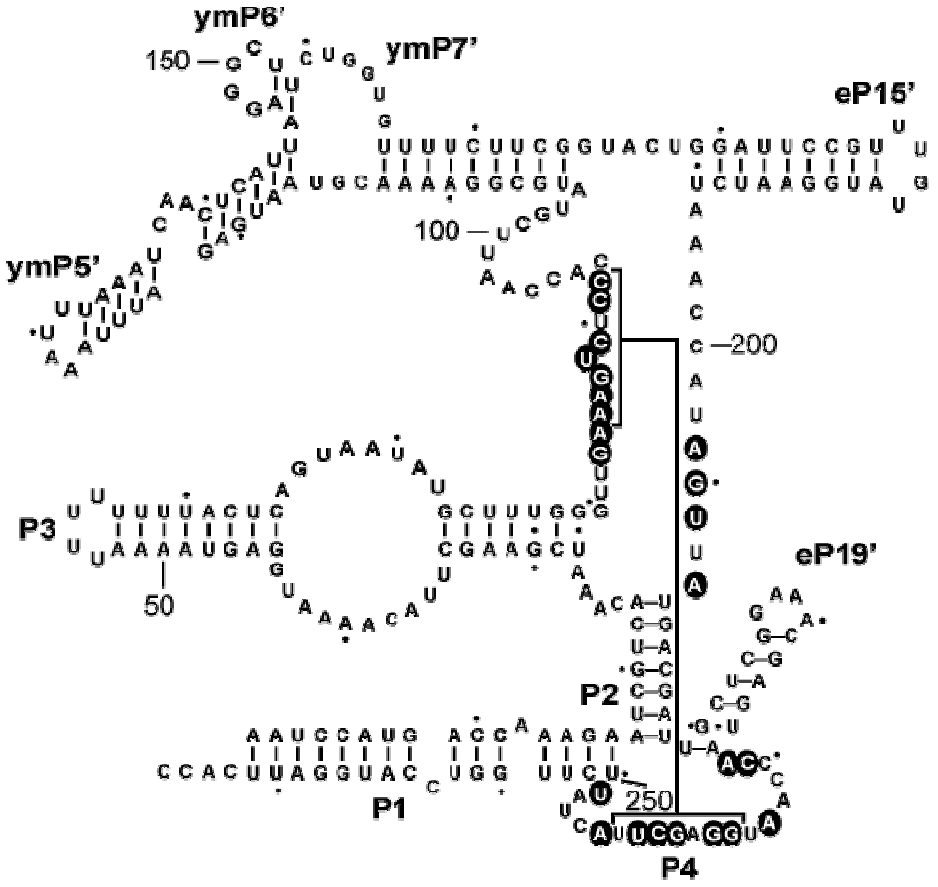

Figure 14: Mini1-4 RNase MRP secondary structure. The mini1-4 RNase MRP secondary structure includes the deletions of the ymP5, ymP6, ymP7, and eP19 terminus. In addition, the remaining bases between the ymP6 and ymP7 termini were deleted. The ymP7 and eP19 termini were stabilized with GGAAAC hairpins.

4. DISCUSSION

4.1 Imaging of RNase MRP Localization

In order to examine the localization of RNase MRP as the yeast cells progress through mitosis methods for mounting and reducing photobleaching had to be devised. Five different methods of slide preparation were compared including wet mount, continuous flow, wet mount on a well slide, an agaorse pad on a depression slide, and an agarose pad on a standard flat slide. The slide preparation technique that was found to hold the cells in place longest was the agarose pad on a depression slide. All other slide preparation techniques had limitations that would only allow for limited time-lapse periods. The agarose pad on the depression slide allowed for z-stack and time-lapse images to be taken in approximately thirty minute intervals. After thirty minutes the microscope had to be refocused due to the stage drifting. To minimize stage drifting the microscope was preheated for two to four hours. Using the binning and gain settings on the microscope the exposure time was reduced to just 60 ms. With these settings z-stack images of 10 slices were taken every six minutes for approximately 1-1.5 hours stopping every thirty minutes to refocus. Due to these limitations, a movie of the localization of RNase MRP has not yet been captured.

In order to capture a movie of the localization of RNase MRP another method for slide preparation, photobleaching reduction, and stage drift reduction will need to be worked out. An additional method of slide

preparation that could be used is a perfusion chamber. Live-cell imaging perfusion chambers consist of two, usually round, coverslips that are spaced apart by rubber O-rings held in place with two metal plates. These chambers isolate the cells from the outside environment while offering control of the inside environment by using heated pumps and other accessories. Using a heated slide at 30°C in this experiment may allow for better yeast cell growth. In addition, the perfusion chamber coverslips, which are made of high quality glass, can further reduce autoflourescence and background noise normally observed when using regular glass coverslips. The cells are grown on media in the optical cavity of the perfusion slide where they are held in place during imaging. Using a perfusion chamber slide may alleviate some of the problems with mounting and photobleaching. The microscope may also need to be recalibrated by a Carl Zeiss technician to compensate for the stage drift.

If a solution to the stage drift and photobleaching problems cannot be found the experiment may need to be modified. Instead of capturing z-stack time-lapse movies, individual z-stack images may need to be taken while refocusing in between. Since the process is not automated it would allow the user to begin capturing z-stack images when cell division first begins minimizing exposure time and photobleaching. The downside to this method is that it is more time consuming to take individual z-stack images every minute or two for an hour.

4.2 Minimal Constructs

Five minimal constructs of RNase MRP were created (mini1, mini1-1, mini1-2, mini1-3, and mini1-4) to further reduce the RNA component of RNase MRP. Interestingly in the mini1 mutant, the ymP5, ymP6, ymP7, and eP19 terminus could be deleted and still retain good growth rates at 30°C. These terminal regions are not essential to the enzymatic function of the protein and do not affect RNA folding to the extent of loss of function to the enzyme. Not all of the mini1-1, mini1-2, mini1-3, and mini1-4 mutants exhibited good growth rates at 30°C. Future research will examine which mutations can sustain good growth rates at 30°C. The viable strains of the four minimal constructs will be mated together to produce an MES116 strain that contains a combination of all the mini1 RNA mutations.

Previous studies have suggested that only a short portion of the bases forming the hairpin region are essential for function. The bases forming the hairpin region contribute to the proper folding of the RNase MRP RNA, but these portions may not have specific targets for the protein components of RNase MRP.[16] For the mini1-4 mutant, it will be interesting to see how in future research the deletion of the remaining ymP7 bases forming the hairpin region will affect viability, and to see if these bases play a role in the folding of the RNase MRP RNA. Similarly, for the mini1-1 mutant it will be interesting to see how in future research a deletion in the P1 region will affect

[16] Li et al., 2004

viability due to the protein footprint of RNase MRP extending into this P1 RNA region.

A large amount of the remaining RNA molecule is essential to the enzymatic function of RNase MRP. For the remaining RNA component of RNase MRP, all of the associated eleven protein components are arranged on this small RNA segment. A majority of the protein footprint of RNase MRP is located in this remaining RNA segment. Therefore, deletion of these areas will affect the enzymatic function of RNase MRP. Deleting areas of RNA-protein interaction will cause loss of function so future research will focus of finding the remaining RNA regions not associated with proteins and deleting them.

By further reducing the size of the RNase MRP RNA X-ray crystallography may become possible. This is because the current size of the RNase MRP minimal construct is not yet small enough to be crystallized properly. Using these methods to further examine the tertiary structure we hope to learn more about the three-dimensional structure of RNase MRP, and this may one day provide greater insight into new targets for cartilage-hair hypoplasia and cancer.

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Written Capstone Summary: Imaging of RNase Mitochondrial RNA Processing Localization Using Fluorescent Microscopy

For my Honors Capstone Project I worked with Professor Mark E. Schmitt at Upstate Medical University on the imaging of RNase Mitochondrial RNA Processing, MRP, localization using fluorescent microscopy and the creation of minimal RNase MRP constructs. RNase MRP is an essential ribonucleoprotein endoribonuclease, meaning it is essential to the viability of the cell, and without it the cell cycle, among other things, will not function properly. Ribonucleoprotein means that RNase MRP is composed of RNA and protein components. Endoribonuclease describes the function of RNase MRP, mainly as an enzyme that cleaves the phosphodiester bonds within a ribonucleotide chain catalyzing the hydrolysis of RNA into smaller components.

RNase MRP is evolutionarily related to RNase P, which means that ancient cells must have had a need for RNA cell processing. This might have involved cell cycle control through RNA degradation. Cyclin proteins, such as yeast B-type cyclin, regulate how a cell progresses through the cell cycle depending on their levels. Completion of mitosis is dependent on the correct degradation of these cyclin proteins and their mRNAs. Since degradation of these signals is required for the completion of mitosis ancient cells might have used RNase MRP for this purpose.

Some of the functions of RNase MRP include mitochondrial primer formation, rRNA processing, and the degradation of specific mRNAs that control the cell cycle. It is important to note that mitochondria have their own DNA, mtDNA, separate from the DNA located in the nucleus. RNase MRP cleaves RNA transcripts that form the RNA primer necessary for the initiation of mitochondrial DNA replication. RNA MRP is also responsible for cleaving the pre-ribosomal rRNA to form the rRNA molecule 5.8S. This process takes place in the nucleolus. The rRNA molecules 18S, 5.8S, 28S, and 5S come together to form the ribosome, which is the site of protein synthesis. Lastly, RNase MRP is essential in the degradation of specific mRNAs that control the cell cycle.

RNase MRP was found to be essential to the degradation of specific mRNAs that control the cell cycle because several mutations of RNase MRP delay the cell cycle in late mitosis, and the result is a large budding yeast cell in the middle of telophase. Studies show that RNase MRP cleaves the mRNA for the yeast B-type cyclin, CLB2. If a mutation in an RNase MRP component stops the function of RNase MRP, then CLB2 mRNA and the corresponding protein build up in the cell. Accumulation of CLB2 results in prolonged Clb2k/Cdk activity, and delays the completion of mitosis. In the normal cell division, RNase MRP would degrade CLB2 mRNA helping to contribute to the low levels of CLB2 protein signaling the completion of mitosis.

RNase MRP has been found to be localized in the nucleolus. This was observed by transforming two plasmids *pUN100[LEU2 CEN4 DsRED-NOP1]* and *pTD125[URA3 CEN GFP-POP1]* into yeast. The *pUN100* plasmid tags the nucleolar protein Nop1p with a DsRed fluorescent signal so that the nucleolus can be visualized using fluorescent microscopy. The *pTD125[URA3 CEN GFP-POP1]* plasmid tags the Pop1p protein subunit of RNase MRP with a GFP fluorescent signal so that RNase MRP can also be visualized using fluorescent microscopy. These two proteins signals co-localize indicating that RNase MRP localizes to the nucleolus during most of the cell cycle. CLB2 has been assumed to be degraded in the cytoplasm. RNase MRP has been found to localize to a single spot in the daughter cell cytoplasm, and this location is where CLB2 mRNA is degraded. In this model RNase MRP exits the nucleolus during mitosis, and localizes to a spot in the daughter cell that is a processing body like structure. Processing bodies are sites in the cytoplasm where mRNAs are processed. Processing may involve de-capping of the mRNA to degrade it or other mechanisms of degradation to process the mRNAs. Certain mRNA processing and binding proteins are localized to these foci. RNase MRP is thought to localize to such a special processing body specifically for the degradation of cell cycle specific signals that are daughter cell localized. These special processing bodies have been named TAM bodies, Temporal Asymmetric MRP bodies. We postulate that the TAM body is where CLB2 mRNA might be degraded.

RNase MRP is required for cell growth. RNase MRP gene mutations lead to a decreased rate of cell growth because the mutated cell no longer has the ability to assemble rRNA and cyclin-dependent cell-cycle regulation no longer functions correctly. As a result of decreased cell growth and cell numbers, the mutation causes the disease cartilage-hair hypoplasia in humans. The disease is a rare defect that is recessively inherited. It is an autosomally recessive disorder, and not sex-linked. It is seen in mainly two populations the Amish and Finnish. Some of the more notable symptoms of the disease are short stature, defective immunity, and a predisposition to certain cancers such as leukemia and lymphoma. Further research into the localization and function of RNase MRP may one day provide greater insight into new targets for cartilage-hair hypoplasia and cancer.

This project examined the localization of RNase MRP as the yeast cells progress through mitosis. The TLG205 strain used for this experiment has RNase MRP tagged with GFP, and the nucleolus tagged with DsRed. Under fluorescence various stages of the cell cycle were examined for their localization pattern. My Capstone Project studied the localization and regulation of RNase MRP so that we can learn more about cartilage-hair hypoplasia, and also answer questions about how RNase MRP localizes, if the RNase MRP localization focus is the site of *CLB2* mRNA degradation, and if RNase MRP re-localizes back to the nucleolus after mitosis is completed.

In addition, minimal constructs of RNase MRP were created using basic techniques in yeast genetics to further reduce the RNA component of RNase MRP and potentially be able to examine the structure using X-ray crystallography. X-ray crystallography is a procedure for determining the three-dimensional structure of atoms arranged in a crystal. The crystal scatters X-rays in various directions producing unique patterns based on the structure of the atoms and their arrangement in the crystal. These patterns can then be analyzed to determine the three-dimensional structure of the atoms within the crystal. Using these methods we hope to learn more about the three-dimensional structure of RNase MRP, and may one day provide greater insight into new targets for cartilage-hair hypoplasia and cancer.