IDENTIFYING GENETIC FACTORS OF NUCLEAR PORE COMPLEX ASSEMBLY IN SACCHAROMYCES CEREVISIAE

An Undergraduate Research Scholars Thesis

by

AVA PERRY

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Research activities involving the use of human subjects, vertebrate animals, and/or biohazards must be reviewed and approved by the appropriate Texas A&M University regulatory research committee (i.e., IRB, IACUC, IBC) before the activity can commence. This requirement applies to activities conducted at Texas A&M and to activities conducted at non-Texas A&M facilities or institutions. In both cases, students are responsible for working with the relevant Texas A&M research compliance program to ensure and document that all Texas A&M compliance obligations are met before the study begins.

I, Ava Perry, certify that all research compliance requirements related to this Undergraduate Research Scholars thesis have been addressed with my Faculty Research Advisor prior to the collection of any data used in this final thesis submission.

This project did not require approval from the Texas A&M University Research Compliance & Biosafety office.

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ABSTRACT

Identifying Genetic Factors of Nuclear Pore Complex Assembly in *Saccharomyces cerevisiae*

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The Nuclear Pore Complex (NPC), a 60-110 MDa protein complex embedded in the nuclear envelope, functions to transport all material across the nuclear membrane. While NPC structure is studied extensively, there is still much to learn about the assembly process. We are working to identify key factors to the assembly process by identifying the genetic mutation in the *Saccharomyces cerevisiae* NPC assembly mutant KRY141 through complementation testing. Fluorescence microscopy reveals the mislocalization of NPC proteins due to the failure to assemble NPCs. Since the single mutation confers both defects in NPC assembly (*npa*) and temperature sensitivity (*ts*) phenotypes, we use temperature sensitivity as a measure for complementation of the mutation. Previous work mapped the NPC assembly mutation near the *MAT*α locus on chromosome III. Since then, genes in the region have been isolated one at a time to attempt to rescue the mutant phenotype. In this way, we have ruled out 4 potential genes: *MAK32*, *PMP1*, *YCR024C-B,* and *YCR025C.* Identification of this gene required in the assembly

of the NPC would provide key information on an essential biological process and provide insight to NPC assembly related diseases associated with aging and cancers.

DEDICATION

To my friends, family and peers who have helped me through my undergraduate experience and this process.

ACKNOWLEDGEMENTS

Contributors

I would like to thank my faculty advisor, Dr. Kathryn Ryan, for her guidance and support throughout the course of this research.

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All other work conducted for the thesis was completed by the student independently.

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1. INTRODUCTION

1.1 Nuclear Pore Complex

1.1.1 Nuclear Pore Complex Function

The Nuclear Pore Complex (NPC) is embedded in the nuclear envelope (NE) and functions as the only transport for RNAs, proteins, and other metabolites across the nucleocytoplasmic divide. Nucleocytoplasmic transport can be characterized through either diffusion through the NPC for small molecules or through the Ran regualted system for larger macromolecules [1]. Nucleocytoplasmic transport of proteins in cells is elucidated with the help of NPC structural studies, Ran system studies, and studies on the nuclear localization signals along with their receptors [2]. Ran is one of the most abundant proteins in eukaryotic cells and functions to transport proteins and RNAs that have the proper localization sequence through the NPC [1]. First the cargos with the proper signaling sequence will be identified by karyopherin β which will then bind to docking sites on the NPC [1]. Some nuclear localization signals require the additional adaptor of karyopherin α before binding to karyopherin β [1]. Then, Ran Guanine Nucleotide Exchange Factor (RanGEF) will exchange the GDP on Ran to GTP, then RanGTP binds to karyopherin β to release the cargo in the nucleus [1]. After karyopherin β and RanGTP are recycled back to the cytoplasm through the NPC [1]. Once in the cytoplasm, RanGAP will bind RanGTP and activate the hydrolysis of RanGTP to RanGDP [1]. Nucleoporin Nup358 on the cytoplasmic filaments has homologous structures to the RanGAP, meaning there is some functional aspects of the NPC that directly impact the function of Ran during nucleocytoplasmic transport through the NPC [1]. There is also evidence that NPCs play a secondary function during cell division in cells which assemble the NPC at the end of mitosis [3].

1.1.2 Nuclear Pore Complex Structure

The Nuclear Pore Complex is a protein complex made of multiple copies of about 30 different proteins called nucleoporins (nups) in an 8-fold radially symmetric structure. NPCs are composed of nups organized into 3 main subcomplexes. There is a core subcomplex that is embedded in the nuclear envelope that interacts with the membrane, a nuclear basket that adds structure to the nuclear side of the complex, and filaments that function in the transport of proteins and RNAs across the nuclear envelope. While the nuclear pore can expand and contract slightly to allow for transport of larger biomolecules, the localization of the nuclear pores remain relatively consistent [4]. Their interaction with nuclear lamina – intermediate filaments on the inside of the nucleus that provide structure and aid in gene expression – on the nuclear side likely provide the stability of NPC localization.

Recently, the Nuclear Pore Complex has been studied using cryo-EM technology to visualize the structure. The Wu lab created multiangled cryo-EM visuals and predicted individual nucleoporin structures with AlphaFold technology [5]. The result was single particle cryo-electron microscopy of *Xenopus laevis* NPC within their native membrane and the generation of a reconstructed cytoplasmic ring map [5]. While cryo-EM is incredibly precise, AlphaFold was needed to predict the structures of obscured nucleoporins [5]. The combination of cryo-EM data and AlphaFold predictions, interactions among core-subcomplex nucleoporins, specifically Nups 214, 88, 62, 155, and 358, can be better understood [5]. While *Xenopus* NPCs are assembled at the end of mitosis rather than during interphase like *S. cerevisiae*, methods for better understanding the interaction between proteins and their associated membranes can only serve to build a base of knowledge for assembly studies.

Similarly, cryo-EM structures have been generated for NPCs in *S. cerevisiae*. These structures show the interaction between structural and flexible components of the Nuclear Pore Complex. The flexibility of some connectors between functional layers of the NPC allows for a dynamic shape during transport of different size molecules [6]. Some structural interaction motifs are homologous to transport protein interaction motifs pointing to a possible evolutionary connection between transport and structure. Overall, this study reveals nuclear basket and central transporter features and structural flexibility needed for transport [6]. Similar studies have been done in a large variation of organisms, showing structural conservation of NPCs and a variation of outer ring forms depending on the needs of the organism [6]. While most of the NPC can be visualized in vivo, the core subcomplex – sometimes called the central transporter – is harder to get clear images due to its interaction with the nuclear membrane. Even after removing the membrane with a detergent, this portion of the NPC is not imaged as clearly as the rest of the complex [6]. There is some variation of NPCs among yeast, typically seen as different organizations of outer membrane proteins. This type of variation is created through differing copy number of Y complexes and repurposing structural Nups for use in the outer ring [6]. The Y-complex, also referred to as the Nup107-160 complex forms key cytoplasmic and nucleoplasmic rings, with eight Y-complexes in each distinct ring [7].

Other proteins have been identified to work in the assembly process. Nic96, another nucleoporin associated with the nuclear pore basket as a linker between inner and outer rings of the nuclear pore complex, is thought to have a potentially integral role in the assembly process. Reversible phosphorylation of N-terminal domains of Nic96 seem to be targets for mitotic disassembly and reassembly [6]. Structural similarities to COPI and COPII families of vesicle coating proteins points to an evolutionary link between secretion transport and the assembly of

the Nuclear Pore Complex [8]. Similarities have also been pointed out between NPC nucleoporin structure and karyopherin- proteins associated with nuclear export. While other nuclear pore complex assembly mutants were being identified, some had some affiliation with secretion and membrane interaction genes [9]. This solidifies the idea that a combination of transport and the regular assembly of the NE are essential to a properly formed NPC.

1.1.3 Nuclear Pore Complex Assembly

There are two modes of assembly: one where the NPC assembles at the end of the mitotic phase and one where the NPC assembles during interphase. In *S. cerevisiae*, NPCs assemble continually throughout interphase to double the 70 NPCs to around 140 before cell division. Since humans assemble their NPC during interphase like *S. cerevisiae*, using yeast as a model organism to study the pathway will directly impact human health and medicine. To understand what is going on in the interphase assembly, it is important to understand NPC assembly as a whole by comparing the two modes.

During mitotic mode assembly, the nuclear envelope breaks down during the normal process of the cell cycle to separate the duplicated chromosomes. This also allows the NPC to embed into the NE as the membrane is being reassembled. However, during the interphasic assembly mode the NPC is assembled into an intact NE, which requires the NE to be deformed. The deformed nuclear envelope is infiltrated by nucleoporins which will bind to the nuclear membrane using amphipathic transmembrane domains and recruit subsequent nucleoporins to assemble the mature NPC. Regardless of the mode of assembly, nucleoporin MEL28 is generally associated with the initiation of NPC assembly [7]. Interphase assembly of the NPC is described as slow and sporadic in comparison to mitotic assembly modes. During interphase assembly modes, the inner and outer membrane of the nuclear envelope must bend and fuse, usually

though the binding and deforming properties of specific proteins, for example Nup53, Nup133, and Nup153 [7]. While these proteins have been seen to localize relatively early in interphase modes of assembly, the exact initiation of NPC assembly is still speculatory. For example, how the membrane is deformed has not been entirely confirmed to be the effect of membrane binding nucleoporins, as there is also data to suggest that these nucleoporins merely have an affinity to bind to previously deformed membranes and that the beginning of assembly is initiated by another mechanism [7].

During interphase, the nuclear pore seems to form from both sides of the membrane rather than just one side and creates a thinner portion of the nuclear envelope that early recruited nucleoporins have an affinity to bind. New nuclear pore complexes seem to be assembled de novo rather than through a splitting of a previously matured NPC [10,11].

During interphase assembly, Nup153 is the first nucleoporin to be recruited to the nuclear envelope and is a protein of study during assembly research. What has been found is that the Nup153 protein has a transmembrane domain that allows it to be embedded partially within the membrane. There also seems to be some interaction with transport proteins to assist localization of the nuclear pore [11,12]. Once Nup153 binds, other nucleoporins can be recruited to the nuclear envelope in what seems like previously assembled subcomplexes [13]. Nup153 preferentially binds to the nuclear membrane with the assistance of RanGTP and recruits the Y complex and Nup107-160 subcomplex and assists the formation of NPCs [13].

1.2 Nuclear Pore Complex Assembly Mutant

1.2.1 Nuclear Pore Assembly Mutant Phenotype

The NPC assembles in the NE in healthy cells. To visualize what this looks like in vivo, nups can be tagged with Green Fluorescent Protein (GFP) and imaged with fluorescent

microscopy. When the NPC is assembled correctly, nups will localize to the NE and create a characteristic punctate ring in the images taken (*Figure 1*). However, when the NPC is not assembled correctly, for example in *npa* mutant KRY141, nups will mislocalize and be dispersed in other parts of the cell, seen as a more sporadic distribution of the GFP tagged proteins (*Figure 1*).

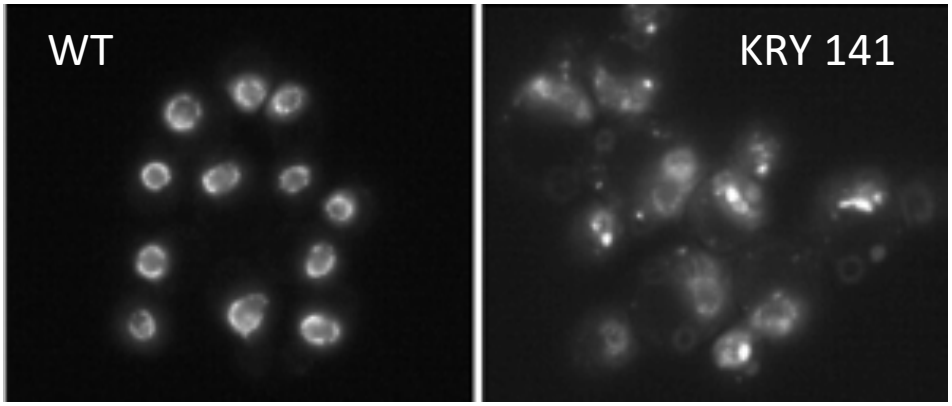


Figure 1: Fluorescent microscopy images taken with Nic96 and Nup170 tagged with Green Fluorescent Protein. Comparing localization of GFP-Nups in wild-type (WT) and the npa mutant KRY141 at non-permissive temperature (34˚C).

Since the NPC is essential to the cell, mutants studied must be conditional. In this case, temperature sensitivity was the most obvious choice due to the ease of control. At permissive temperatures, the NPC will assemble correctly, and cells will be able to grow and divide. However, at non-permissive temperatures, the NPC will fail to assemble properly, meaning the cells won't be able to perform normal nucleocytoplasmic transport, impacting gene regulation, killing the cell (*Figure 2*).

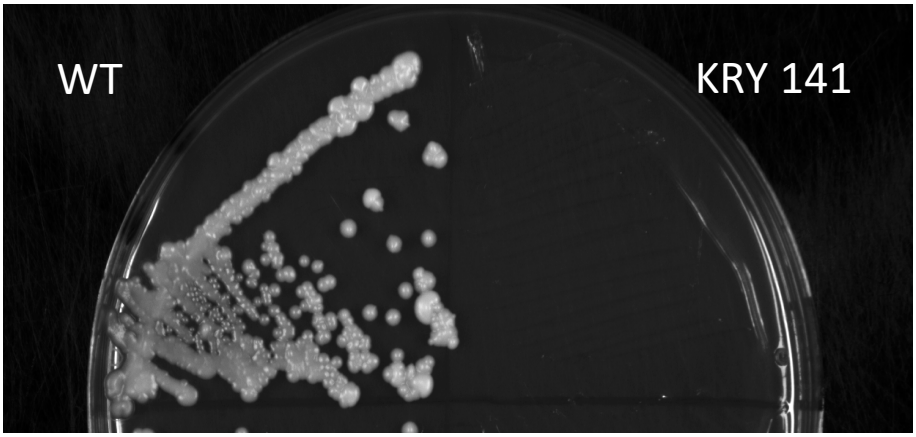


Figure 2: Plate growth of wild-type and npa mutant KRY14 at non-permissive temperature (34˚C). Showing npa mutant temperature sensitivity.

1.2.2 Previously Identified and Characterized

Previously in the Ryan lab, 121 NPC assembly mutants (*npa* mutants) were isolated in a screen of randomly generated temperature sensitive mutants. Due to the essential nature of the NPC, to study mutants, their mutations must be conditional, meaning they have wild-type (WT) phenotype most of the time, but under controlled conditions have phenotypes of the mutant. In this case, temperature sensitive mutants were selected for. Using this methodology, of those 121, 16 unique genes have been identified; 11 of which are published, and five unpublished [9,11,14,15]. Mutated genes were identified by screening for complementation with a genomic library [9,11,14,15]. This assay (*Figure 3*) involves taking a genomic library- a collection of plasmids each containing different random sections of the wild-type genome- and transforming it into cells of an *npa* mutant strain. Each cell will only take in one plasmid. If one of the genes provided on the plasmid are the same gene that is mutated, the wild-type version of the gene will act as a wild-type protein and confer proper assembly of the nuclear pore complex. Thus, the growth will be rescued the mutant at the nonpermissive temperature (34˚ C).

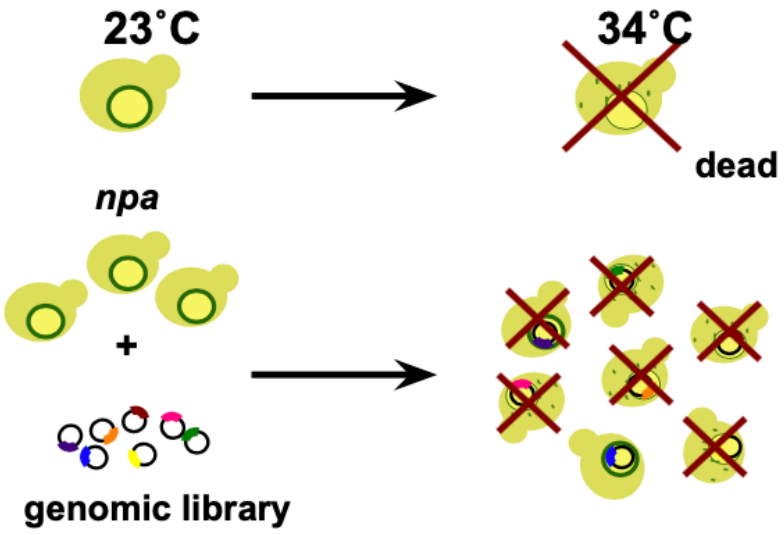


Figure 3. Genomic Library Screen used to identify genes mutated in 16 npa mutants.

After screening *npa* mutant, KRY141 with this genomic library complementation assay, none of the cells survived at 34˚ C. This mutant is the only npa mutant so far that has failed to be identified using this genomic screen. Next, an ordered, tiled library with minimum gaps and overlap was used in the same assay [16]. Despite having over 80% coverage of the *S. cerevisiae* genome, none of the plasmids complemented the mutated gene. This result meant the lab had to take a different approach.

1.2.3 Mapped to MATα locus on Chromosome III

While other mutants have been characterized through the complementation with a genomic library, *npa* mutant KRY141 failed this assay. After testing with a random genomic library, an ordered tiled library- which has 80% coverage of the *S. cerevisiae* genome- was assayed. Still, the complementation assay failed to show any possible sections of the genomes that corresponded to this mutant, so a different genetic approach had to be taken.

Thus, the mutated gene in KRY141 was mapped to the right arm of Chromosome III on the centromeric side of the MATα locus (*Figure 4*). Genes within this region were surveyed and candidates were chosen [17]. First, the genes already covered in the tiled genomic library along with non-protein coding genes unlikely to be related to the mutation had been eliminated from consideration. What was left was a collection of about 10 genes to be individually studied by cloning individual genes into an expression plasmid.

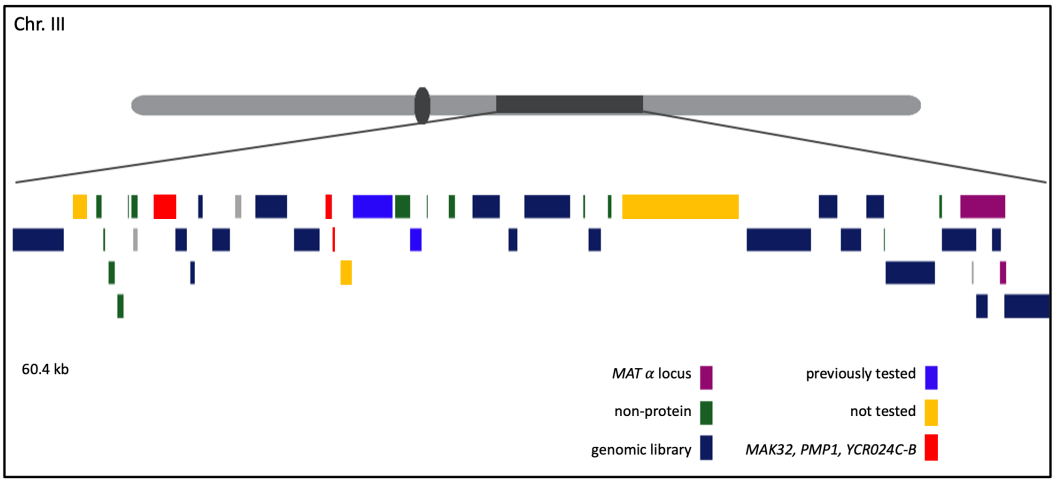


Figure 4. Genetic Map of Chromosome III where each gene is represented by a rectangle. Mat(alpha) is seen as fuchsia, non-protein genes are in dark green, genes covered in the genetic library screen are in navy blue, genes tested by other students in the Ryan lab are in royal blue, MAK32, PMP1 and YCR024C-B are in red, and every other gene are in yellow.

1.2.4 Complementation Testing Thus Far

Within the candidate genes for the KRY141 mutated gene, three genes have been studied by other students in the lab, four have been studied in this project, and three remain. To assay each individual gene, a more directed approach was taken. A complementation assay was still used, but here, instead of using the large pool of the genomic library, each individual candidate gene was amplified and cloned into a yeast expression plasmid and transformed into both WT and KRY141 strains. Due to the temperature sensitivity nature of the mutant, temperature

sensitivity was used as a read out of complementation of the mutation. Thus, once transformation was complete, cells were placed at nonpermissive temperature, and room temperature for control, and visualized for cell growth. If the plasmid transformed into the cell contained the wild-type copy of the gene mutated in KRY141, it will act as the wild-type within the cell and rescue the cell from its nuclear pore complex assembly phenotype and thus will restore the cell to normal function, seen as growth at 34˚ C. However, if the gene is not correct, then the cell will still act as a mutant and won't grow at 34˚ C.

In this way the following genes have been studied: *MAK32*, *PMP1*, *YCR024C-B*, and *YCR025C*. This paper will show results from these four genes, but it should be noted that the genes studied by other students within the lab failed this exact genetic assay and thus have been removed from the list of candidates.

1.3 Going forward

1.3.1 Understanding Nuclear Pore Complex Assembly

Despite the Nuclear Pore Complex being essential to normal cellular function, there is still more to investigate in the assembly process. Continued research on the assembly process lays the stepping-stones from what we know to what we don't. While we could speculate, we do not know the full capacity of the impact of our research. The unknown makes this research exciting and daunting, however, the more that we discover, the clearer the impact will be. For example, there was a time when little was known about nuclear lamina, a complex network of intermediate filaments surrounding the genetic material but just underneath the surface of the nuclear envelope [18]. Decades went by with only handful of researchers contributing to the field of nuclear lamina. Since then, laminopathies have been tied to rare genetic diseases, such as progeroid or premature aging syndromes, and some cancers [18,19]. The therapies developed for

these diseases would have been entirely deficient without the groundwork of early nuclear lamina researchers. The NPC assembly is associated with pathologies we don't yet understand, and so the early NPC assembly research is vital to the future of cellular biology and medicine.

Disfunction of nuclear import and export plays a major role in various diseases, one of which is related to some cancer types. Uncontrolled division seen in cancers is directly linked to upregulation of certain genes along with the pronounced multiplicity of cellular structures, including Nuclear Pore Complexes. Additionally, some viruses rely heavily on the NPC to transport viral proteins into the nucleus to turn off anti-viral responses. Some examples include: HIV-1, influenza A, dengue, and rabies [2]. Therefore, NPC transport are a potential target for future therapies.

2. METHODS

2.1 Building Complementation Plasmids

2.1.1 Polymerase Chain Reaction

For each candidate gene, adjacent oligonucleotide primers were designed that included the endogenous promoter and termination signals. Each oligo also included a restriction site for cloning into the expression plasmid. Each gene was amplified using wild-type genomic DNA through polymerase chain reaction. The PCR was set up using the primers (*Table 1*), wild type genomic DNA (YGS52), Taq polymerase, Taq 10x Buffer, and 2.5 mM dNTPs. Genes *PMP1* and *YCR024C-B* were tested together due to their proximity and size.

Table 1. Table of different primer oligosaccharides used in PCR for each gene studied.

Gene(s)	5’ Primer	3’ Primer
MAK32	TATGGATCCCTGTCAAAAG CACATCGTAT	TATCTCGAGACAACGCCTA ATCTCAAGTA
PMP1+ YCR024C-B	TATGGATCCGGAAAGCCG GGAATAGGTCG	TATCTCGAGCTCGACGGCC TAATCTCTGT
YCR025C	AATGGATCCACCCCGAAG AGACAGAACAA	AATCTCGAGTCAAAGTGTG TAGCGTCTTCC

2.1.2 Gel Electrophoresis

Once amplified, gel electrophoresis was used to confirm identity of the selected gene based on band size. Each gene was run on a 1% agarose gel against a 1 Kb DNA ladder. Gels were run at 140 Volts for about an hour. Gels were photographed.

2.1.3 Gel Extraction and DNA Purification

Confirmed bands were excised using a razor on a UV light table then the DNA extracted using a Qiagen purification kit. Standard manufacturer protocol was followed.

2.1.4 Restriction Digestion and Ligation

Each purified gene(s) was then digested using BamHI and XhoI restriction enzymes at 37° C. The expression vector pRS315 (*Figure 5*) was also digested using the same restriction enzymes and the gene was cloned into the vector using a T4 DNA Ligase [20].

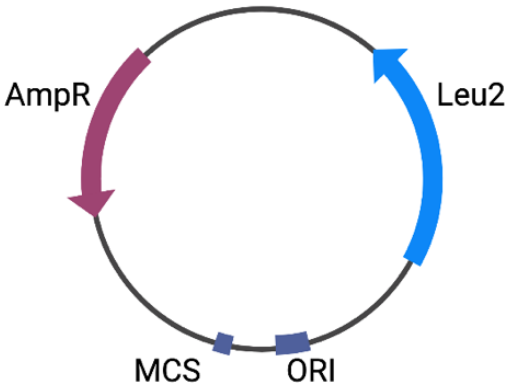


Figure 5. Plasmid map of pRS315 Yeast Expression plasmid. Including Multiple Cloning Site (MCS), Origin (ORI), Leu2 selection gene, and Ampicillin Resisitance Gene (AmpR). Figure created with BioRender.com.

2.2 *E. coli* Transformation and Mini Prep

2.2.1 Electroporation Transformation

DH5α *E. coli* cells were used for plasmid DNA replication. Electrocompetent cells and ligated plasmid products were loaded into a 2 mm electroporation cuvette. Cells were electroporated and then recovered in a 1 mL SOB solution at rotating while 37° C for 1 hour before streaking on LB+ ampicillin plates. Since the plasmid contains an ampicillin resistance gene, only cells with transformed plasmids will survive.

2.2.2 E. coli *Mini Prep*

Transformed colonies from the incubated plates were harvested for mini prep to isolate the plasmid. Colonies were inoculated into 5 mL of SOB media and incubated at 37° C overnight. Used a standard miniprep procedure.

To confirm the identity of the isolated plasmids, a restriction digest was used. A 1% agarose gel electrophoresis at 140 Volts for about 1 hour to confirm band sizes. Band sizes were compared to an empty pRS315 control and a standard 1 Kb ladder. Gels were photographed.

2.3 *Saccharomyces cerevisiae* Transformation and Complementation

2.3.1 S. cerevisiae *Transformation*

Cells of both YGS52 (WT) and KRY 141 (*npa*) strains were grown to early log phase at room temperature. Then, a lithium acetate yeast transformation protocol was followed to transform the confirmed plasmids into both strain types. An empty pRS315 plasmid was used as a control. Transformed cells were selected on -leu plates.

2.3.2 Temperature Rescue Assay

Colonies from the Transformed YGS52 and KRY141 were simultaneously struck on a single YPD plate. Multiple replicates of this setup were created, and one set of plates were places

at 23° C while the other set were placed at 34° C (*Figure 6*). Since the mutant is temperature sensitive, at 34 ° C we should see growth on both transformed and non-transformed YGS52 colonies, no growth of non-transformed KRY141 colonies, and if the gene complements the mutation, growth of the transformed KRY141 colonies.

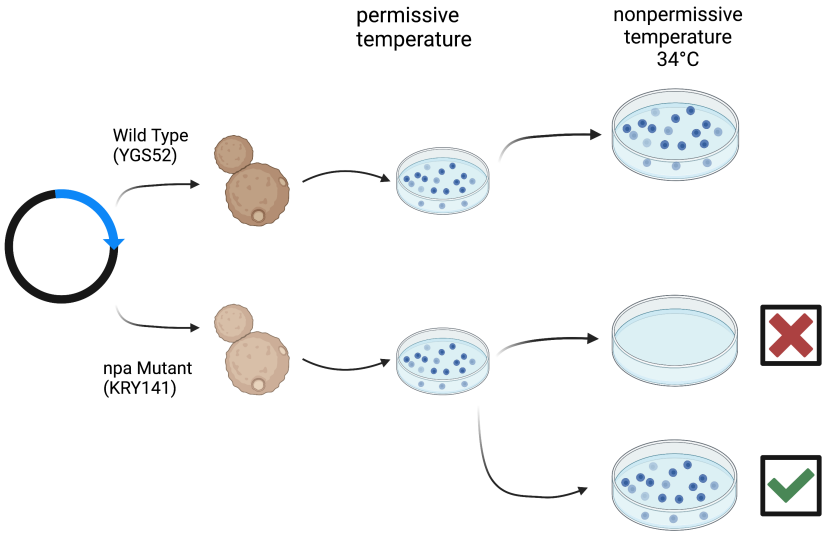


Figure 6. Single-gene complementation assay. Cloned single gene into yeast expression plasmid, transformed into both wild-type and npa mutant KRY141. Placed streaked plates at non permissive temperature (34˚C) to test if transformed gene of interest complemented the mutant gene and rescued the mutant phenotype- seen as growth at the non-permissive temperature. Figure created with BioRender.com.

3. RESULTS

3.1 *MAK32* Fails to Complement the Mutation in KRY141

To understand NPC assembly, we sought to find additional factors that are required for NPC assembly in yeast. To identify the gene mutated in the NPC assembly (*npa*) mutant KRY141, candidate genes linked to the mapped genetic locus were individually cloned into a yeast expression plasmid and transformed into both WT (YGS52) and mutant strains (KRY141). This mutation is temperature sensitive, so at non-permissive temperatures (34˚C), KRY141 NPCs will fail to assemble properly, and the cells will not grow. Alternatively, WT cells will have no issue assembling the NPC and growing at this elevated temperature (*Figure 2*). If the candidate gene is the gene mutated in KRY141, transforming the wild-type version of the gene into the npa mutant should provide the mutant what it needs to properly assemble the NPC and should also rescue growth at the non-permissive temperature (*Figure 5*).

The first candidate gene tested, *MAK32* was isolated and cloned into a yeast expression vector then transformed into both YGS52 and KRY141 strains. Empty yeast expression plasmid (pEmpty) was used as a control. Transformed cells were struck on YPD and incubated at both room temperature and non-permissive temperatures. At room temperature, both WT and KRY141 cells will grow with or without the transformed gene (*Figure 7*). At the non-permissive temperature, WT will grow with no issues, KRY141 with pEmpty will fail to grow due to the failed NPC assembly. However, even after supplying *MAK32* on the expression plasmid, growth of KRY141 was not rescued. This means that the wild-type version of *MAK32* failed to complement the *npa* mutated gene, the NPC failed to assemble, and the cells failed to grow.

MAK32 is not likely to be the gene mutated in KRY141. It should be noted there is a difference between KRY141 and WT colony sizes indicating the difference in growth rates between the two strains even at the permissive temperature.

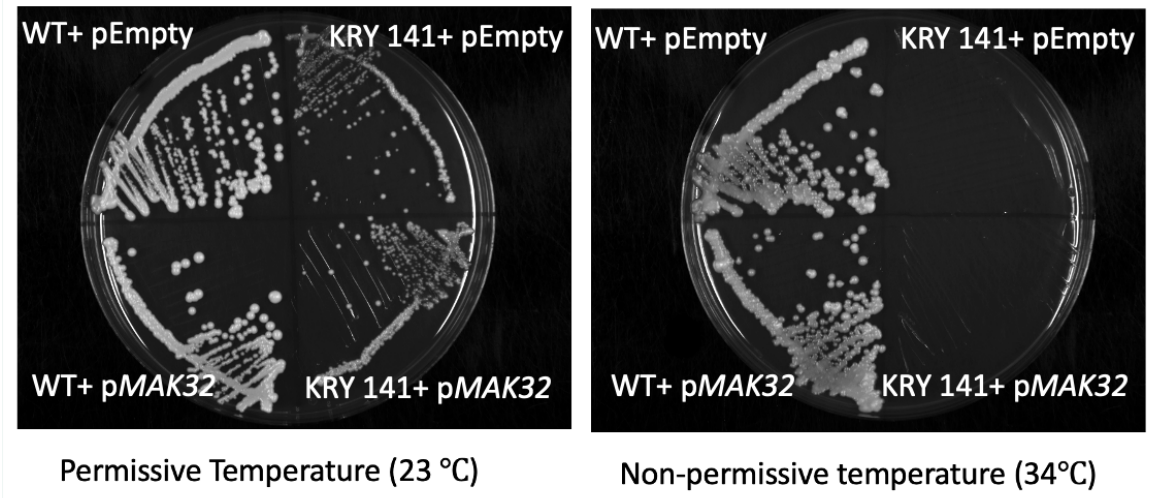
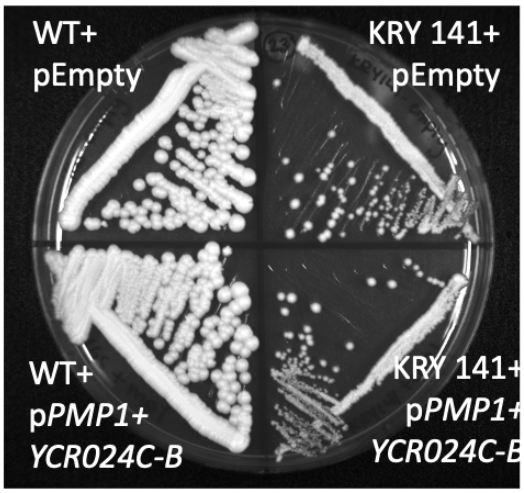


Figure 7. Temperature sensitivity assay of pMAK32. Plate on left: incubated at the permissive temperature (23˚C), where both wild-type and npa mutant (KRY141) grows. Plate on right, incubated at the non-permissive temperature (34˚C). Wild Type (WT) is not affected, KRY141+ pEmpty is negative control and shouldn't grow at 34˚C. When pMAK32 is introduced, the mutant phenotype isn't rescued.

3.2 Neither *PMP1* nor *YCR024C-B* Complements the Mutation in KRY141

The same approach was taken to test the next two genes, *PMP1* and *YCR024C-B*. Due to their proximity and size, the two genes *PMP1 + YCR024C-B* were tested together. Both *PMP1* and *YCR024C-B* were cloned into a yeast expression plasmid and transformed into both WT (YGS52) and *npa* mutant (KRY141) strains. Comparably to the results for *MAK32*, while all cell strains grow at permissive temperatures, only WT cell types grow at the non-permissive temperature even after providing the KRY141 mutant with the wild-type version of *PMP1* and *YCR024C-B* (*Figure 8*). Since both *PMP1* and *YCR024C-B* failed to complement the mutant phenotype, neither are likely to be the gene mutated in KRY141.



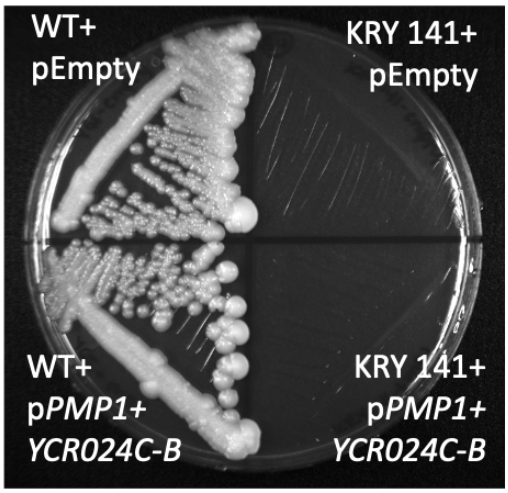


Figure 8. Temperature sensitivity assay of pPMP1+YCR024C-B. Plate on left, grown at the permissive temperature (23˚C), both WT and KRY141 cells will grow. Plate on right, placed at non-permissive temperature (34˚C), WT is not affected, KRY141 is negative control and shouldn't grow at 34˚C. When pPMP1+YCR024C-B is introduced, the mutant phenotype isn't rescued.

3.3 *YCR025C* Also Fails to Complement the Mutation in KRY141

Finally, *YCR025C* was tested with the same assay. Similarly, *YCR025C* was cloned into a yeast expression plasmid and transformed into both WT (YGS52) and *npa* mutant (KRY141) cells and tested for temperature sensitivity. Once again, if *YCR025C* was the gene mutated in KRY141, then the complementation with the wild-type version of the gene would rescue the npa mutant phenotypes, seen as growth at the non-permissive temperature (34˚C). However, after placing the plates at their respective temperatures, KRY141 growth was not rescued with the addition of p*YCR025C* (*Figure 9*). This means that the gene mutated is likely one of the three remaining candidate genes.

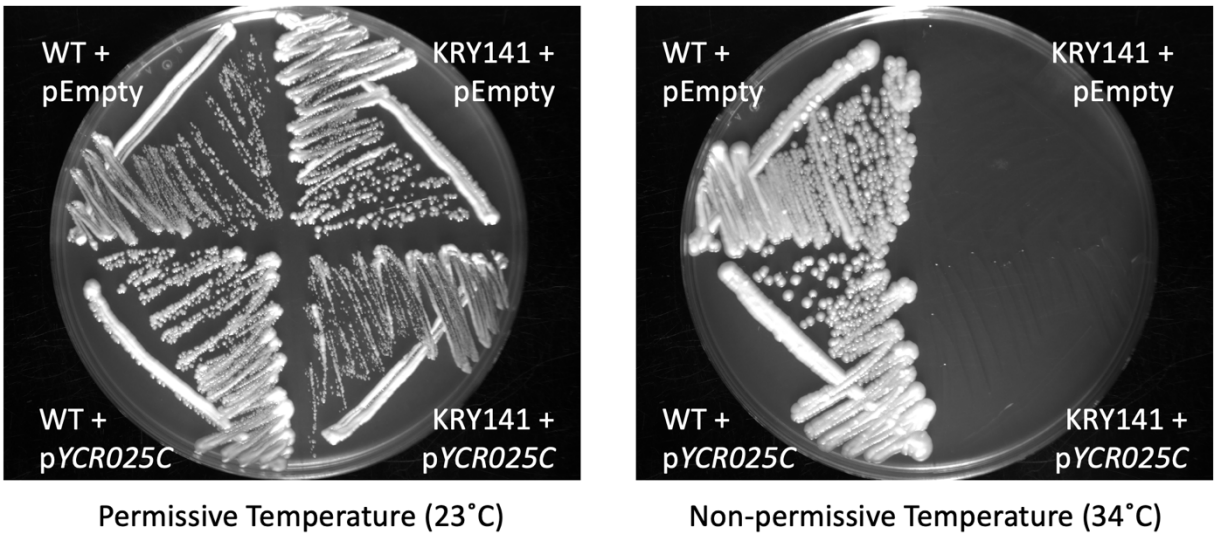


Figure 9. Temperature sensitivity assay of pYCR025C. Plate on left: Placed at permissive temperature (23˚C), both WT and KRY141 cells will grow. Plate on right: incubated at non-permissive temperature (34˚C). WT positive control grows, KRY141+pEmpty negative control does not. KRY141 still does not grow even after transforming pYCR025C.

4. CONCLUSION

Nuclear Pore Complexes (NPCs) are the only structures involved in nucleocytoplasmic transport and are essential to eukaryotic life. Without NPCs, cells would not be able to perform gene expression or regulation. While NPC structure has been studied extensively, the assembly process is still under investigation. In *Saccharomyces cerevisiae*, the NPC is assembled at a constant rate during interphase [21]. General interactions between NPC assembly and proteins like MEL28, Nup153, Nic96, COPI/II and Y-complex have been studied [6-8]. To assemble the NPC into the Nuclear Envelope (NE), the inner and outer membranes must deform and fuse to allow the large protein complexes to associate. More genetic interaction studies need to be completed to create a complete picture of the assembly process. The Ryan lab aims to elucidate key features in the NPC assembly process through the identification of factors required for NPC assembly by characterization of genes mutated in NPC assembly (*npa*) mutants. Through genomic library screening, the lab has been able to successfully identify 16 genes related to NPC assembly process by complementing the *npa* phenotype [9,11,14,15]. While this approach has been observed to work in other *npa* mutants, when working on *npa* mutant, KRY141, this approach failed to see a positive result. A direct approach was then needed to move forward with KRY141.

To identify possible candidates for the gene mutated in KRY141, the mutation was mapped to the right arm of Chromosome III. Genes included in the tiled genomic library assay were retested and removed from consideration, and 10 genes remained. These final genes have been studied individually through a temperature sensitivity complementation assay. Since the NPC is essential to growth, the *npa* mutant collection was made through a temperature sensitivity screen, where mutant cells will assemble the NPC normally at room temperature but

fail to assemble the NPC at non-permissive temperatures. Each candidate genes was amplified individually and cloned into a yeast expression plasmid. These plasmids were transformed into both wild-type (WT) and KRY141 strains. Once transformation was confirmed, cells were placed at both room temperature and non-permissive temperatures to see if the newly transformed plasmid with the candidate gene rescued the NPC assembly at the non-permissive temperature. If the gene failed to complement the phenotype, it would be removed from the list of candidates.

Using this assay, this project tested 4 candidate genes (*MAK32*, *PMP1*, *YCR024C-B*, and *YCR025C*). All four genes failed to complement the mutant phenotype. Three other genes were tested in the same way with the same results by other students in the lab. Three genes remain on the list of candidates: *SRD1*, *BPH1*, and *SLM5*. *SRD1*, a gene involved in rRNA processing, has been previously attempted by the lab [22]. Due to high AT percentage in the sequence, PCR amplification has not been successful so far. The gene product for *BPH1* is involved in endocytosis and autophagy [23]. *BPH1* is homologous to Chediak-Higashi syndrome and beige proteins, both of which are involved in both human and mouse disease syndromes, making *BPH1* an exciting candidate [24]. *SLM5*, however seems to be the most likely candidate out of those which remain. *SLM5* codes for a mitochondrial asparaginyl-tRNA synthetase but is also observed to have negative genetic interactions with Nup159 and Nup60, meaning the double mutant of SLM5 and either Nup159 or Nup60 is worse than the single mutant [25]. This points to a possible correlation to the NPC assembly. Going forward, the lab will be testing *SLM5* using a knockout complementation approach. An *SLM5* knockout mutant will be crossed with KRY141 to see if there is a complementation of the mutant phenotype. Once the gene mutated in KRY141 is found, the next step will be to sequence the gene and do structural studies on the protein gene

product. Likely the sequence will be studied in the context of protein structure through Alpha Fold protein structure database technology. The amino acid sequence will elucidate possible binding sites and sites of chemical interactions with Nups and other proteins within the Nuclear Pore Complex Assembly Pathway. Ultimately, biochemical methods, like affinity chromatography or western blots, will be completed to elucidate interactions of the gene with others in the NPC assembly pathway.

The mutation in KRY141 might have an impact on other structures within the cell. In studying the genetic mutation, it will be important to do a comprehensive study on the impact of all aspect of the cell. This might include colocalization visualization of organelles and the mutated gene product using fluorescence proteins and microscopy. If other perturbations are observed this might point to a connection between the Nuclear Pore Complex assembly pathway and other biochemical processes in the cell, which may highlight an overall organization to cellular processes. For example, if it is determined there are effects on mitochondrial function in the *npa* mutant cell, another study could be done to confirm the connection between Nuclear Pore Complex function and metabolic processes. Which could make sense because some of the proteins needed in the mitochondria were made from genes housed in the nucleus; and possibly metabolic products from the mitochondria have functions within the nucleus.

While this research has a direct impact on the scientific community through the knowledge of the essential Nuclear Pore Complex assembly pathway, there is also some potential to impact human diseased states. As we have seen, complete loss of the nuclear pore complex causes cell death. No transcripts can leave the nuclear pore complex and thus the cell won't be able to perform normal functions. If there are diseased states associated with the nuclear pore complex assembly, they would most likely be only partial mutants or full loss of

function in only certain cell types. Additionally, there might be an upregulation of NPC assembly in cancer cells, as they must produce more transcripts than any other type of cell as they divide uncontrollably [26]. Targeting cells with only upregulated NPCs might be a viable option for some cancer therapies going forward [26]. Currently, there are some ties to brain development and cognition, such as Huntington's Disease, in the function of the nuclear pore complex in neural cells [27]. Perhaps understanding the assembly process of the NPC will highlight possible therapy targets for affected individuals. Since nuclear lamina are observed to function in rare genetic aging diseases and are important not only in the aging process but gene regulation, it can be postulated that the NPC might have similar functions [28]. The NPC works tightly with exported mRNAs from the nuclear envelope and their interaction with RNA polymerases within the nucleus might have some key impacts on gene expression. As we understand more of our genetic code at an increasing pace, understanding the method the transcripts leave the nucleus and interact with the forming NPCs will only increase in importance.

The overall knowledge of molecular biology will only be increased by the knowledge of such an essential complex within the cell. Understanding how transcription factors enter the nucleus, how the genes interact and react accordingly to outside stimulus with the NPC will be beneficial in our understanding of the innerworkings of the cell. As we understand the assembly, we only stand to know more about the interactions within the cell.

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