Phosphorylation at MELT Domains and its Importance for Stu1 Function

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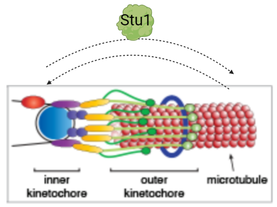
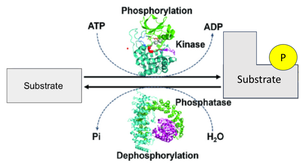
Introduction and Aims

Chromosomal Segregation

Chromosomal segregation is an essential function in ensuring that cells accurately inherit chromosomes during cell division. The segregation of chromosomes is led by kinetochore-microtubule attachments in which the chromosomes are properly aligned by the kinetochores and, once aligned, pulled apart by the microtubules.





Objective

In order to further understand the function and behavior of proteins in kinetochores, budding yeast serves as the organism of study due to its simplicity in kinetochore structure and conservation of its functions and sub complexes in eukaryotic organisms. Stu1p, a component of the budding yeast mitotic spindle, is essential for growth and is known to be well conserved in the MELT domain, an area of protein that has been demonstrated to be phosphorylated. Is phosphorylation at these domains essential to Stu1p function? In order to test its importance, CRISPR was used to create a mutation at the codon that codes for Threonine to a Valine at the 1034 position, which would prevent phosphorylation.

Experimental Approach

We began by designing a guide RNA and repair template which would be cloned and used in the CRISPR vector. We then did a Gibson assembly to insert our guide RNA into the CRISPR vector and confirmed our success through DNA sequencing. Finally, we introduced the CRISPR vector into budding yeast and performed initial testing on phenotype. Although we have yet to confirm whether or not the CRISPR system was successful in making a mutation in our budding yeast, we can confirm colony growth of various sizes. Sequencing to confirm transformation followed by further phenotype analysis are the next steps in further development of this research.

References

Biggins, Sue. 2013. The Composition, Functions, and Regulation of the Budding Yeast Kinetochore. Yeastbook Genome Organization and Integrity. 10.1534/genetics.112.145276

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Acknowledgments

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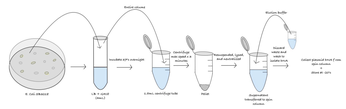
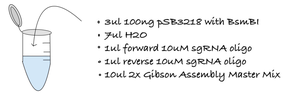
Special thanks to Dr. Jack Vincent for his patience and guidance throughout this project.

Methods

Purification of the pSB3218 CRISPR Plasmid.



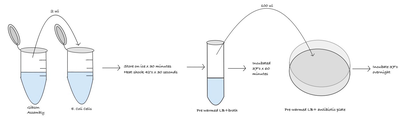
Gibson Assembly



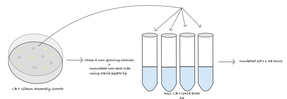
- 3ul 100ng pSB3218 with BsmBI
- 7ul H2O
- 1ul forward 10uM sgRNA oligo
- 1ul reverse 10uM sgRNA oligo
- 10ul 2X Gibson Assembly Master Mix

Sample was then incubated in thermocycler for 60 minutes at 50 degrees C then stored on ice during preparation of transformation into competent *E. coli* cells.

Transformation into *E. coli*



Inoculation of Four Chosen *E. coli* Transformed Colonies



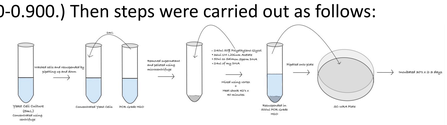
Mini Prep

Each of the tubes containing the inoculation of its designated colony was purified using the same protocol listed above under "Purification of the pSB3218 CRISPR Plasmid." All 4 purifications were then sent off for sequencing to be further analyzed.

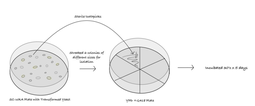
Resuspension of HDR Oligos and Preparation of Yeast Transformation

Yeast Transformation with sgRNA Vector Combined with Repair Template

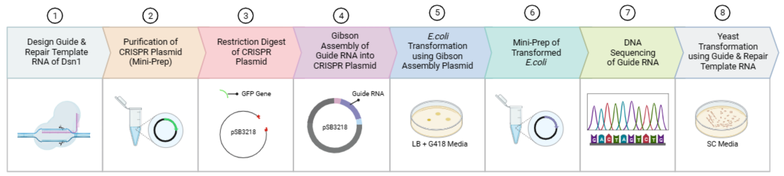
It was first confirmed that the yeast cell culture was in log-phase by measuring its absorbance (target absorbance between 0.200-0.900.) Then steps were carried out as follows:



Streaking of Transformed Yeast Cells onto YPD plate



Experimental Overview



Results

Design of Stu1 Mutation

sgRNA forward oligo:
5'-ggctgggcaacaccttcgggtggcgaatgg**AAATGATGTTAAGAAATGCA**-3'
sgRNA reverse oligo:
5'-ggctgggcaacaccttcgggtggcgaatgg**TGCATTTCTTAACATCATTT**-3'

Wild Type STU1: PAM · Silent Mutation · Threonine

5'-GTA GAA GAC ATC ATT TCT AGA GAA AGT TCT GTA AGC TTC ACT CCC ATC GAC AAT AAA AAA TCT GAA
GGG GAT GAG GAA TCC GAC GAT GCT GTA GAC GAA AAT GAT GTT AAG AAA TGC ATG GAA ATG ACA ATG ATT
AAT CCC TTC AAA AAC TTG GAA ACT GAT AAA ACA CTA GAG TTG AAG AAT AAC GTT GGA AAA AGA ACA

HDR Template: Pam · Silent Mutation · Valine

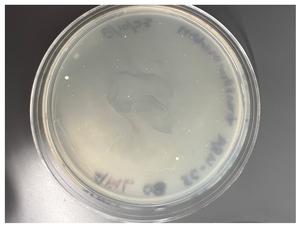
5'-GTA GAA GAC ATC ATT TCT AGA GAA AGT TCT GTA AGC TTC ACT CCC ATC GAC AAT AAA AAA TCT GAA
GGG GAT GAG GAA TCC GAC GAT GCT GTA GAC GAA AAT GAT GTA AAG AAA TCU ATG GAA ATG GUA ATG ATT
AAT CCC TTC AAA AAC TTG GAA ACT GAT AAA ACA CTA GAG TTG AAG AAT AAC GTT GGA AAA AGA ACA

Gibson Assembly and *E. coli* Transformation

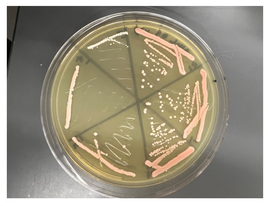
This image represents the results of the Gibson assembly after incubation overnight. There are many colonies present, several of them non-glowing. The 4 colonies that were chosen for further analysis are numbered and circled.

Yeast Transformation on SC-URA Plate

This image represents growth after 2-3 day incubation of the transformed yeast cells on an SC-URA plate. As seen to the right, there are multiple white colonies that vary in size.



Yeast Transformation YPD Plate



This image represents the results of streaking transformed yeast cells for isolation after 5 days of incubation. All 6 colonies were successfully grown.

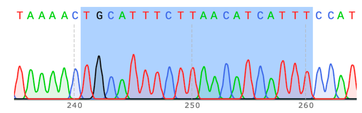
Data and Conclusions

Successful isolation of pSB3218 plasmid confirmed by end concentration of 332.6 ng/ul

Gibson assembly with BsmBI successful with several non-glowing colonies grown on LB plate. These colonies no longer contained the GFP gene

Successful isolation of all four chosen non-glowing colonies

Insertion of pSB3218 plasmid confirmed by sequencing results of four chosen colonies



Growth of colonies varying in size on SC-URA plate support successful transformation of plasmid containing sgRNA and Cas9 enzyme into budding yeast cells.

YPD results showed growth of all six chosen colonies isolated from SC-URA plate. The growth on this plate will be used for future analysis of the various colonies isolated.

Future Implications

Sequencing analysis to further confirm transformation

Phenotype analysis after variable applications of stress

Since Stu1 is essential for growth, analysis and comparison of variability in colony sizes should be performed