Using CRISPR to mutagenize phosphorylation sites of the *STU1* and *DSN1* genes in *Saccharomyces cerevisiae*

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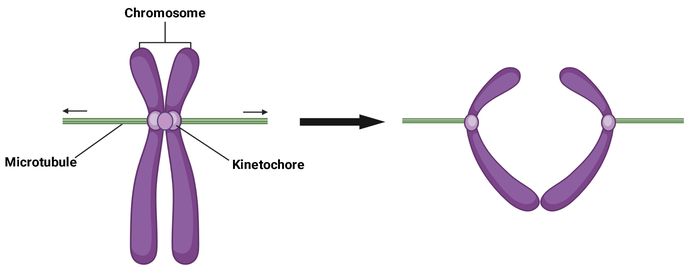
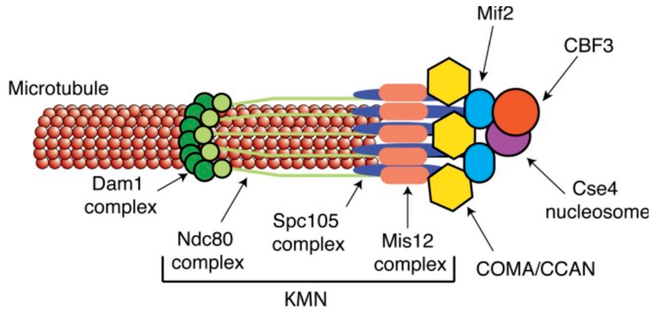
Introduction

Kinetochore & Chromosome Segregation

The separation of chromosomes during cell division relies in part on successful interactions between the kinetochore and microtubules.



Errors in kinetochore-microtubule (KT-MT) attachment can result in cells with abnormal numbers of chromosomes and can lead to cell death. Unsuccessful KT-MT attachment can result in the initiation of the spindle assembly checkpoint (SAC) to prevent cell division from proceeding further. The SAC is communicated by phosphorylation events. The kinetochore is a protein complex that is involved in monitoring KT-MT attachment and in regulating SAC activation to ensure that daughter cells obtain accurate amounts of genetic material.



Dsn1

- Dsn1 is a part of the Mis 12 complex
- Ipl1 phosphorylation of Dsn1 is vital for kinetochore function
- Through mass spectroscopy we know Dsn1 has phosphorylation at serines 546, 547, and 554 (Biggins Lab)

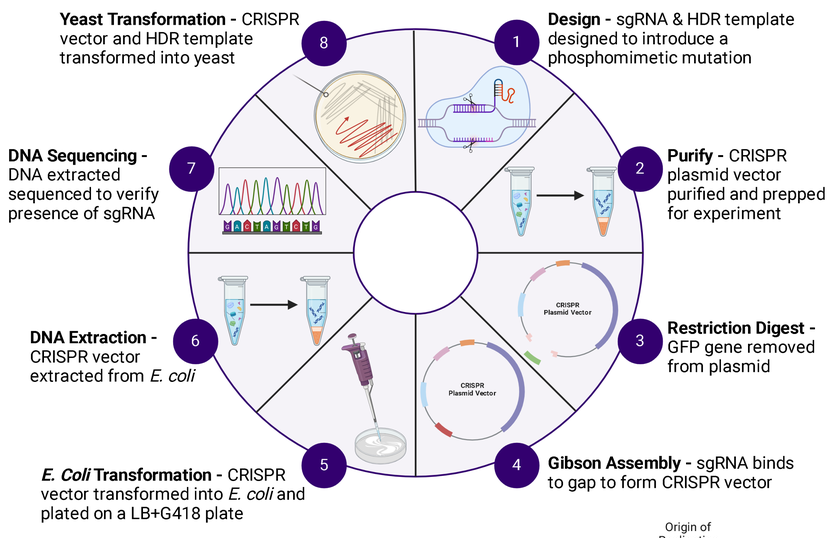
Stu1

- Stu1 is an outer kinetochore protein that has a role in recapturing detached kinetochores
- Previous research has shown that phosphorylation of the outer kinetochore protein Spc105 at "MELT" sites initiates the SAC (Kolenda et al. 2018)
- Research has shown that the Stu1 sequence contains 2 highly conserved MELT-like sequences: IDLT 716 and MEMT 1031 (Juchau 2020)

Objective

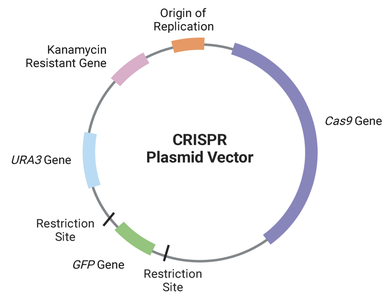
Our main research objective is to mutate the kinetochore proteins Stu1 and Dsn1 at phosphorylation sites to observe how these changes may affect protein function.

Methods



Key parts of CRISPR plasmid vector:

- *Cas9* gene: Codes for Cas9 enzyme
- *GFP* gene: Glows green; replaced with sgRNA
- *URA3* gene: Yeast normally can't make *URA3*, allows us to select for yeast that took up our plasmid
- Kanamycin resistant gene: *E. coli* with this gene can grow in presence of G418 antibiotic



Construction of CRISPR Vector Targeting *STU1*

sgRNA Forward Oligo: 5'-gctgggcaacaccttcgggtggcgaatgg**GATTAATCCCTTCAAAAACT**-3'
sgRNA Reverse Oligo: 5'-attttaacttgctatttctagctctaaaac**AGTTTTTGAAGGGATTAATC**-3'

Wild type *STU1*:
5'-A 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 TGG GAA ACT GAT AAA ACA CTA GAG TTG AAG AAT AAC GTT GGA AAA AGA ACA TCA AGC ACA GAC AGC GTA GTT A-3'

HDR Template:
5'-A 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 **GAC GAT** ATG ATT AAT CCC **TTT** AAA AAC TGG GAA ACT GAT AAA ACA CTA GAG TTG AAG AAT AAC GTT GGA AAA AGA ACA TCA AGC ACA GAC AGC GTA GTT A-3'

Fig. 1. Designed sgRNA and HDR template for *STU1*. Pink codons code for amino acid threonine in the wild type and aspartic acid in the HDR template. Blue highlighted region represents portion of *STU1* gene used to designed sgRNA.

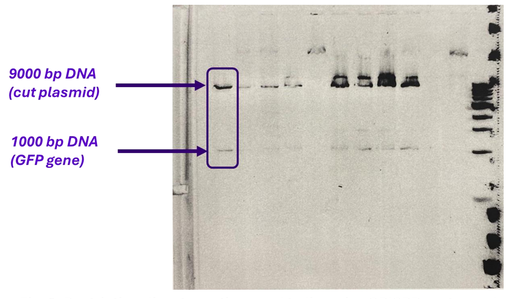


Fig. 2. Restriction digest reaction used to linearize CRISPR vector and remove *GFP* gene.

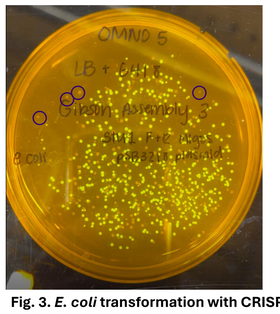


Fig. 3. *E. coli* transformation with CRISPR vector containing designed sgRNA oligos

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Query  121  TGGCGAATGG----------------------GTTTTAGAGCTAGAAATAGCAAGTTAAAAT  160
            ||||||||||                      ||||||||||||||||||||||||||||||
Sbjct  268  TGGCGAATGGAATCCCTTCAACAGCTGTTGGTTTTAGAGCTAGAAATAGCAAGTTAAAAT  209
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Fig. 4. Comparison between CRISPR vector sequence without cloned sgRNA (query) to CRISPR vector sequence with cloned sgRNA (subject). Gap in query sequence highlighted with a purple box indicates success in constructing CRISPR vector.

Construction of CRISPR Vector Targeting *DSN1*

sgRNA forward oligo: 5' -gctgggcaacaccttcgggtggcgaatgg**GAGAAAGATCTCAAGCGAAA** 3'
sgRNA reverse oligo: 5'-attttaacttgctatttctagctctaaaac**TTTCGCTTGAGATCTTTCTC** 3'

Wildtype Dsn1:
5' CAG TCG CAT ATA TTA AAC TCG CAC TCA CTA GCC TTA AAC GAA ATA ACA AAT TCA AAA GTG AAT AAA TTG AAC ATA GAA ACA ATG AGA AAG ATC TCA AGC GAA ACG GAC GAT GAC CAC TCA CAA GTG ATT AAT CCT CAA CAG CTG TTG AAG GGA TTA AGT TTA TCT TTC AGT AAA AAA CTG GAT TTA TGA AAT AAA AGA AAT CA

HDR template:
5' CAG TCG CAT ATA TTA AAC TCG CAC TCA CTA GCC TTA AAC GAA ATA ACA AAT TCA AAA GTG AAT AAA TTG AAC ATA GAA ACA ATG AGA AAG ATC GAT GAC GAA ACG GAC GAT GAC CAC GAT CAA GTG ATT AAT CCT CAA CAG CTG TTG AAG GGA TTA AGT TTA TCT TTC AGT AAA AAA CTG GAT TTA TGA AAT AAA AGA AAT CA

Fig. 5. Designed sgRNA and HDR template sequences for *DSN1*. Pink codons code for amino acid serine in the wild type and aspartic acid in the HDR template. Blue highlighted region represents portion of *DSN1* gene used to design sgRNA.

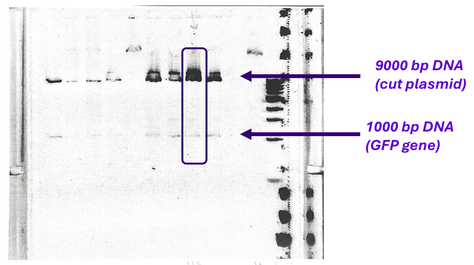

Fig. 6. Restriction digest reaction used to linearize CRISPR vector and remove *GFP* gene.

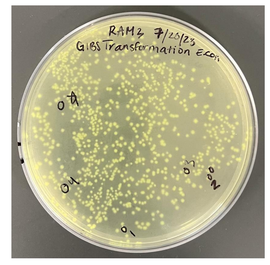

Fig. 7. *E. coli* transformation with CRISPR vector containing designed sgRNA oligos

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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  152  CGCTGGCGCCGGCTGGGCAACACCTTCGGGTGGCGAATGGGAGAAAGATCTCAAGCGAAA  93

Query  121  GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  92   GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGT  33
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Fig. 8. Comparison between expected CRISPR vector sequence with cloned sgRNA (query) to CRISPR vector sequence without cloned sgRNA (subject). Identical match between the query and subject sequences highlighted by the purple box indicates success in constructing CRISPR vector.

Conclusion

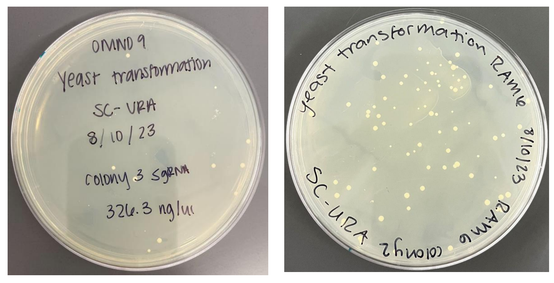

Fig. 9. Yeast transformation with CRISPR vector and HDR template.

- **We were successful in constructing the CRISPR vectors containing sgRNA encoding sequence designed to mutate specific phosphorylation sites in *STU1* and *DSN1***
- The CRISPR vectors were transformed into *S. cerevisiae* and colonies were formed
- We are awaiting sequence confirmation that the *STU1* and *DSN1* genes contain our intended mutations
- In the future, phenotypic analysis can be performed to test the effects of the mutations

References

Acknowledgments

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