Designing a CRISPR-Cas9 system for site specific mutagenesis of the Dsn1 protein in *S. cerevisiae*

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TBIOMD 495

Background

During normal cell division, chromosome segregation is a critical step in ensuring daughter cells receive the correct number of chromosomes. A step in the chromosome segregation process involves kinetochores, large protein complexes located on centromeres where spindle microtubules attach. Before a cell can proceed through metaphase and into anaphase, there must be correct attachment between both the kinetochore and microtubules, as well as adequate tension, through a process known as the spindle assembly checkpoint (SAC).

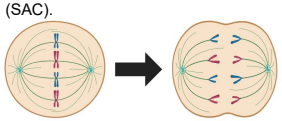

Figure 1. A cell undergoing metaphase into anaphase. This process requires accurate assembly of the spindle microtubules. Created with biorender.com.

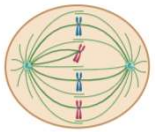

Figure 2. Incorrect attachment of spindle microtubules to sister chromatids. This condition leads to aneuploidy. Created with biorender.com.

Dsn1, a protein located within the outer kinetochore, plays an important function in the attachment of spindle microtubules and the assembly of kinetochore subcomplexes involved in the SAC. Mps1 kinase, a component of the SAC, has been shown to phosphorylate specific sites on Dsn1, which are known to impact chromosome segregation. Mps1 kinase is prone to phosphorylating specific sites where amino acid threonine is flanked by two acidic amino acids.

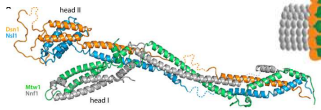

Figure 3. Proteins that comprise the outer kinetochore on the model organism *Saccharomyces cerevisiae*. Our study investigated the outer protein Dsn1, as highlighted in orange. (Dimitrova et al. 2016)..

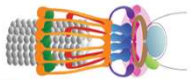

Figure 4. Structure of the kinetochore. Highlighted in blue is the complex to at which the Dsn1 protein can be located. Image credit to the Sue Biggins lab at Fred Hutch Cancer Center.

We have identified site 491 of the *Saccharomyces cerevisiae* Dsn1 protein as a potential Mps1 kinase target sequence due to this context and known conservation of this amino acid sequence throughout closely related species. To measure whether this phosphorylation site is important to the function of Dsn1, we are designing a CRISPR-Cas9 system to mutate codon 491 and test for the function of that mutation. We designed a small guide RNA (sgRNA) encoding sequence and cloned it into a CRISPR vector. We also created a homology directed repair DNA template (DR) that was designed to target site 491 and cause a phospho-null missense mutation. We combined these designed templates into a transformation involving *S. cerevisiae* cells. Future continuation of this project will determine if the mutation at site 491 was correctly inserted into *S. cerevisiae*.

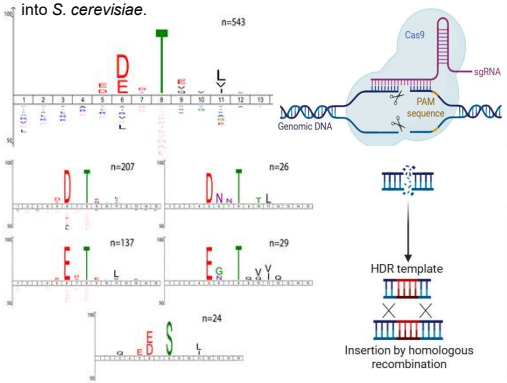

Figure 5. Phosphorylation by Mps1 kinase occurs in an acidic context. These are the results from a study that examined mass spectrometry analysis of peptide sequences exposed to Mps1 kinase. Their research found that when threonine, flanked by two acidic residues, experienced higher levels of phosphorylation events (Hennrich et al. 2013).

Figure 6. CRISPR-Cas9 induced mutagenesis. A designed plasmid with sgRNA template is cloned into *S. cerevisiae* cells, along with an HDR designed template with two mutations: one silent mutation at the PAM sequence, and a phospho-null mutation at site 491. Created with biorender.com.

Method/Results

1. sgRNA and Homology-Directed repair design
2. Restriction Digest of CRISPR-Cas9 vector
3. Cloning of sgRNA template DNA into CRISPR vector
4. Transformation of *E. coli* with Gibson Assembly reaction
5. Sanger sequencing to confirm insertion of sgRNA template
6. Transformation of yeast with CRISPR vector and HDR template

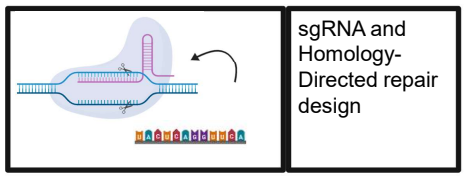

Figure 7. Homology-Directed Repair DNA template. The bottom DNA strand is a section of the wildtype *DSN1* gene. Highlighted in pink is the sgRNA template that will guide the CRISPR-Cas 9 system The top strand is a portion of the HDR template with changes to both the PAM sequence (silent mutation) G→A, and Threonine at the 491st codon (ACA) → Valine (GTA).

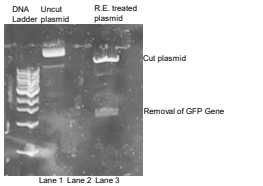

Figure 8. Gel electrophoresis results after restriction enzyme digest was completed on the CRISPR vector. Uncut plasmid untreated with restriction enzyme digest in lane one, and lane three is treated with a restriction enzyme digest and the *GFP* Gene. Lane two served as a blank. Cut plasmid was isolated and used for future experiments.

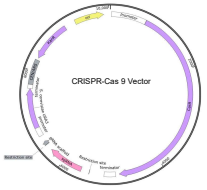

Figure 9. A map of our CRISPR vector containing the gene encoding Cas9, a section encoding our sgRNA, along with selectable markers for *S. cerevisiae* (URA3) and Kanamycin resistance in *E. coli* as well as replication origins for both *S. cerevisiae* and *E. coli*.

Figure 10. Transformation of our CRISPR vector into *E. coli*. The non-glowing colonies represent *E. coli* with sgRNA cloned into the CRISPR vector.

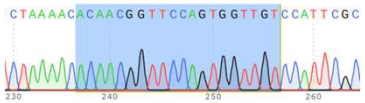

Figure 11. Sanger sequencing results of the *E. coli* transformation indicating correct insertion of the sgRNA encoding region into the CRISPR vector. This sequence is an identical match to the sgRNA template from Figure 7.

Figure 12. Transformation of *S. cerevisiae* cells with CRISPR vector and HDR template.

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Discussion/Conclusion

- Gel electrophoresis ran after the restriction digest of the CRISPR vector shows successful removal of the *GFP* marker gene.
- We used Gibson Assembly to create our CRISPR-Cas9 vector with our designed sgRNA template.
- The CRISPR vector was successfully transformed into *E. coli* cells.
- Sanger sequencing of CRISPR vectors purified from an *E. coli* colony confirmed the successful integration of sgRNA template sequence.
- Our CRISPR-Cas9 system was successfully transformed into *S. cerevisiae* cells.

Future Works

- Isolation of separate colonies and Sanger sequencing to verify mutagenesis.
- Phenotypic testing (benomyl and heat stress) on colonies to observe for cell division discrepancies.
- Conduct mutagenesis on other noted potential phosphorylation sites within the Dsn1 protein.

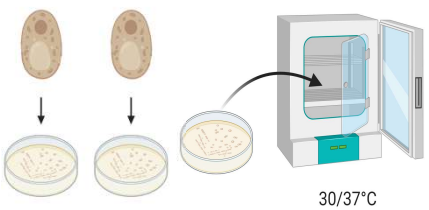

Figure 13. Future testing of *S. cerevisiae* colonies using both 15 and 30 µg/mL benomyl agar plates. Created with biorender.com.

Figure 14. Future testing of *S. cerevisiae* colonies under 30ºC and 37ºC conditions. Created with biorender.com.

Acknowledgements

Thank you to my fellow TBIOMD 495 research team-you were instrumental in assisting me through the whole term. Thank you to the Sue Biggins lab at the Fred Hutch Cancer Center for your collaboration on this project, and providing feedback on current research. Lastly, thank you Hannah Neir for the template for the HDR figure.

Literature Cited