CRISPR Mutagenesis of Phosphorylation Sites T380/T386 in Dsn1 Protein in *Saccharomyces cerevisiae*

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TBIOMD 495

Background

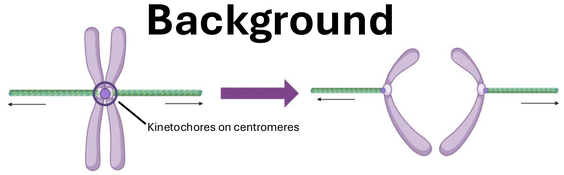

Figure 1. Diagram of kinetochore-microtubule interactions allowing for chromosome segregation during mitosis. Kinetochores form on DNA sequences called centromeres. Microtubules then bind to kinetochores and pull sister chromatids into two forming daughter cells during anaphase of mitosis. (Made with BioRender).

- The kinetochore, a multi-protein complex, assembles on centromeres and connects to microtubules during chromosome segregation.
- Chromosome segregation is an essential process mediated by the kinetochore.

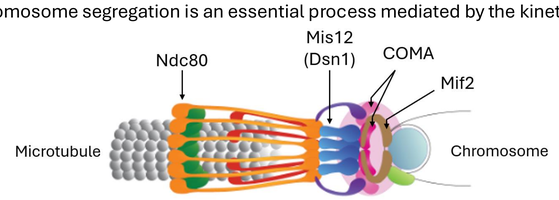

Figure 2. Components of the *Saccharomyces cerevisiae,* or budding yeast kinetochore. (Adapted from the Biggins lab).

- The Mis12 subunit of the kinetochore connects inner components, COMA/Mif2, that contact centromeric nucleosomes, to the outer component Ndc80, which contacts microtubules.
- The process of phosphorylation recruits the Mis12 subunit to the assembling kinetochore and activates the spindle assembly checkpoint.

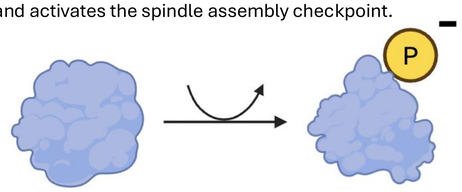

Figure 3. Diagram demonstrating charge and shape change associated with phosphorylation events. (Made with BioRender).

- Kinases are enzymes that act as molecular switches during the cell cycle, adding phosphate groups to proteins to change their conformation and function.
- Phosphorylation of Mis12 by Ipl1 kinase allows for proper assembly of the kinetochore and promotes timely microtubule detachment.

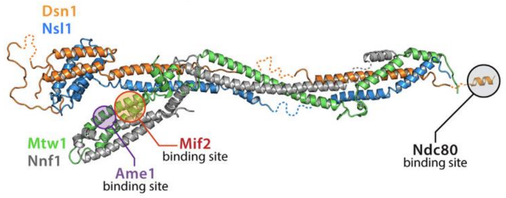

Figure 4. Predicted structure of the heterotetrametric Mis12 subunit of the kinetochore. Dsn1p shown in orange contains a binding site for kinetochore complex Ndc80 on its carboxyl end. (Dimitrova et al. 2016)

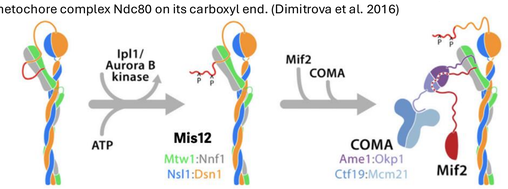

Figure 5. Phosphorylation of Dsn1p by Ipl1 kinase promotes the association between the Mis12 subunit and inner kinetochore components COMA and Mif2. (Adapted from Dimitrova et al. 2016).

- Phosphorylation of Dsn1p within Mis12 regulates kinetochore assembly.
- Dsn1p spans across the Mis12 subunit, with its N-terminus regulating binding to COMA/Mif2, and its C-terminus contacting Ndc80.
- Mass spectrometry has shown amino acid residues threonine 380 and 386 to be phosphorylated in Dsn1p.
- **Due to its unique placement within the kinetochore and within the Mis12 subunit itself, we explored how phosphorylation regulates Dsn1p's interactions within the kinetochore. More specifically, we examined the function of phosphorylation at *DSN1* T380/T386, located near the C-terminal end of the protein.**

Methods

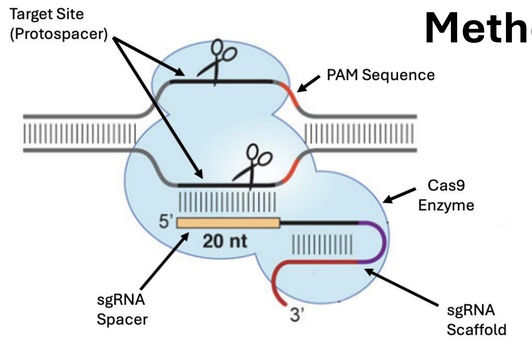

Figure 6. Cas9 enzyme and single guide RNA (sgRNA) interactions with target gene during CRISPR-Cas9 based mutagenesis. The Cas9 endonuclease shown in blue induces a double stranded break (DSB) in DNA. The 20-nucleotide spacer portion of the sgRNA shown in orange binds to the target DNA, aligning Cas9 into the correct position to cut the target site. The sgRNA scaffold shown in red and purple allows the sgRNA structure to bind to the Cas9 enzyme, forming the Cas9-guide RNA complex. The protospacer adjacent motif (PAM) is a short DNA sequence that is required for the Cas9-guide RNA complex to recognize, bind, and initiate endonuclease activity on the target DNA. (Adapted from Jinek et al. 2012)

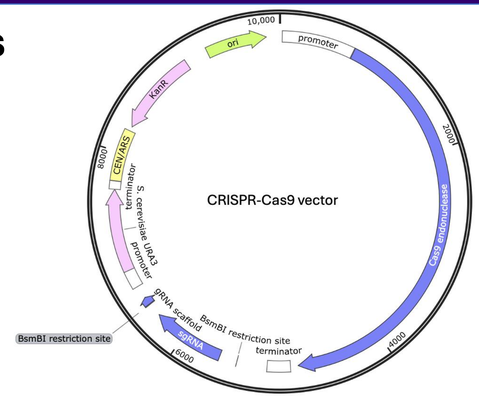

Figure 7. The CRISPR-Cas9 vector contains sequences encoding the components of the Cas9-guide RNA complex (Cas9 endonuclease, sgRNA, gRNA scaffold). S. cerevisiae *URA3* is essential for uracil synthesis and therefore viability. This acts as a selectable marker for yeast that have taken up the vector during transformation as the parent strain used cannot synthesize their own uracil. BsmBI restriction sites are recognized by the BsmBI restriction digestion enzyme and were used to construct this plasmid. (Made with SnapGene).

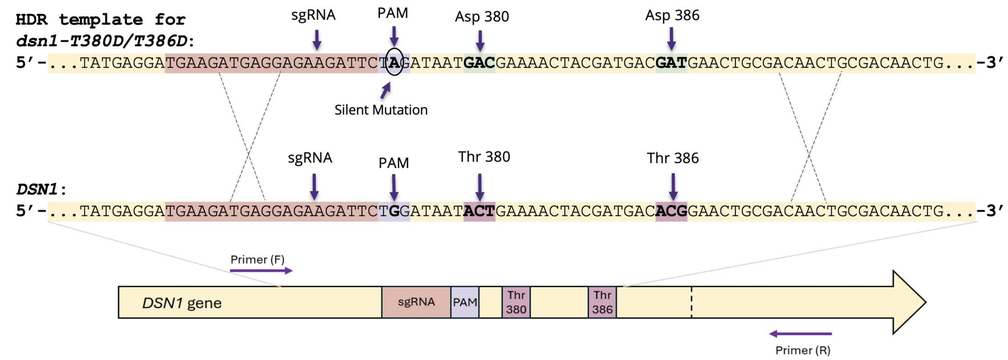

Figure 8. Homology directed repair (HDR) design to create phosphomimic mutations in *DSN1* gene after CRISPR-Cas9 induced DSBs. The wild type (WT) target *DSN1* sequence is compared with the designed HDR template. Codons 380 and 386 of the WT sequence encode the neutral amino acid threonine (T). The HDR template encodes aspartic acid (D) at these loci, giving a negative charge to mimic constant phosphorylation at these residues. A silent point mutation was also designed in the PAM sequence to prevent the Cas9-guide RNA complex from continuing to create breaks in DNA after the HDR template has been transformed into the yeast. The red sgRNA portion of the sequence is where the sgRNA spacer will recognize and bind to allow Cas9 to cut at the target locations. Forward and reverse primers were designed to amplify a 500 base pair (bp) portion of the *DSN1* gene containing the target sequence using PCR.

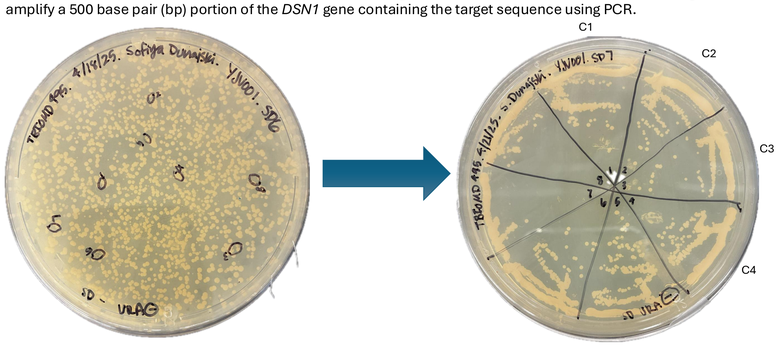

Figure 9. Selection of yeast after transforming with CRISPR-Cas9 vector and HDR template. Our yeast transformation reaction was plated on media without uracil to select for yeast that have taken up the CRISPR vector. Eight colonies (C) were pure cultured, and four continued to undergo genomic prep for subsequent PCR and sequencing.

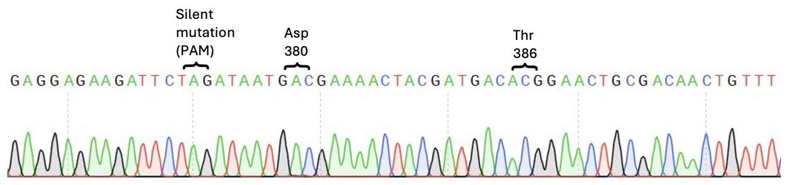

Figure 11. Sanger sequencing results for the PCR reaction of colony 4. Both the PAM and T380D mutations were implemented, but the T386D mutation was not made. Colonies 1-3 only contained the PAM mutation.

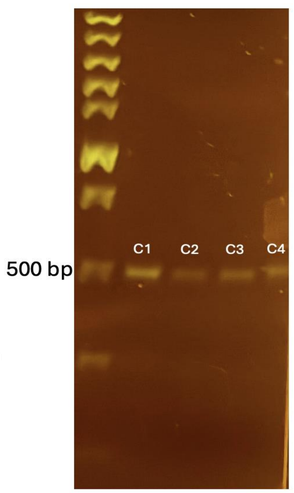

Figure 10. PCR results of the middle portion of the *DSN1* gene containing T380/T386. All reactions showed band sizes around 500 bp, indicating successful amplification of the target portion of *DSN1*.

Results

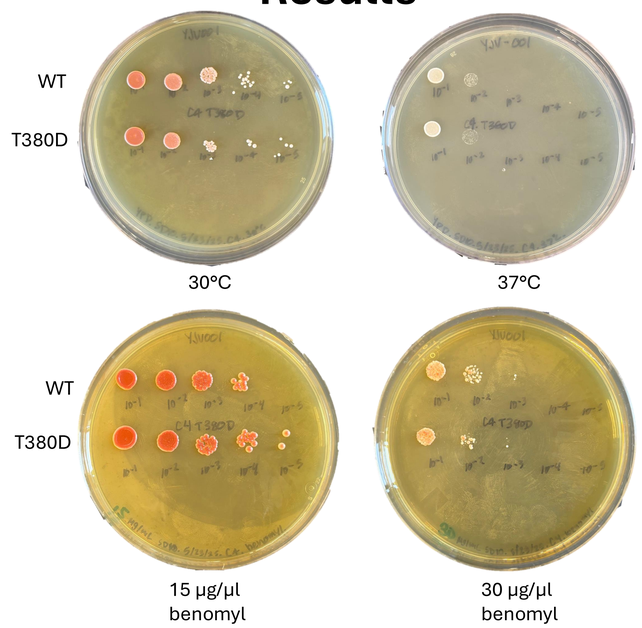

Figure 12. Dilution spot assay comparing growth between WT and T380D mutant strain. One assay included growing the strains on standard growth media at the optimal 30°C, and at 37°C for a temperature stress condition. The other assay was conducted on media containing the chemical stressor benomyl, which inhibits microtubule polymerization.

Conclusions + Future Directions

- **We successfully transformed *Saccharomyces cerevisiae* with the CRISPR vector.**
- **We mutated the 380th codon in the *DSN1* gene from threonine to aspartic acid using the HDR template.**
- **No changes in growth were observed between the mutant and wild type strains under the temperature and benomyl stressors.**
- Next steps include retransforming yeast with the CRISPR vector and HDR template to obtain the aspartic acid mutations at both the T380 and T386 codons, and conduct phenotypic testing to observe any changes in growth.
- A new HDR template should be designed that inserts two aspartic acid codons at each loci to create a larger local negative charge mimicking phosphorylation as done in other studies.

Acknowledgements

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References