CRISPR-Cas9 Mutagenesis of Phosphorylation Sites 380 & 386 in the Kinetochore Protein Dsn1

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Background

- The interaction between the kinetochore and dynamic ends of the spindle microtubule serves as a checkpoint during the transition from metaphase to anaphase in the cell cycle.[1]
- Bipolar microtubule attachment and tension sensing is required for successful segregation of sister chromatids and progression through the cell cycle.[1]
- Incorrect attachment leads to cells containing excess genetic material, or not enough; both of which will compromise the cell's survival.[1]
- Yeast is used as a model organism for our study, since the kinetochore on each sister chromatid interacts with a single microtubule.[2]

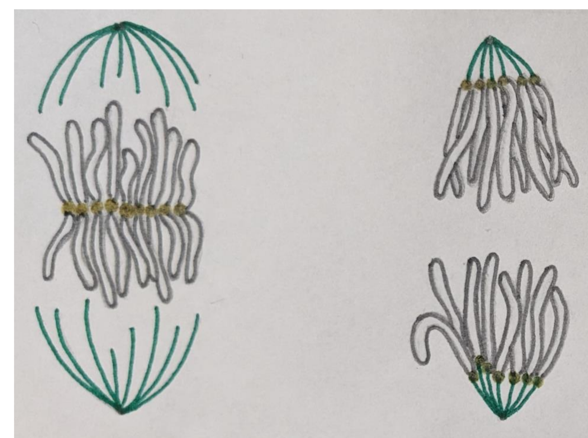

Figure 1: Sketch representing the transition from metaphase to anaphase in the cell cycle. Kinetochores are represented by gold circles. Spindle microtubules are shown in green.

- Proteins that make up the kinetochore are still being investigated for their role in chromosome segregation.
- Dsn1 is a protein located in the MIS/MIND complex, a component of the outer kinetochore, and bridges kinetochore subcomplexes involved in microtubule attachment and tension sensing.[3]

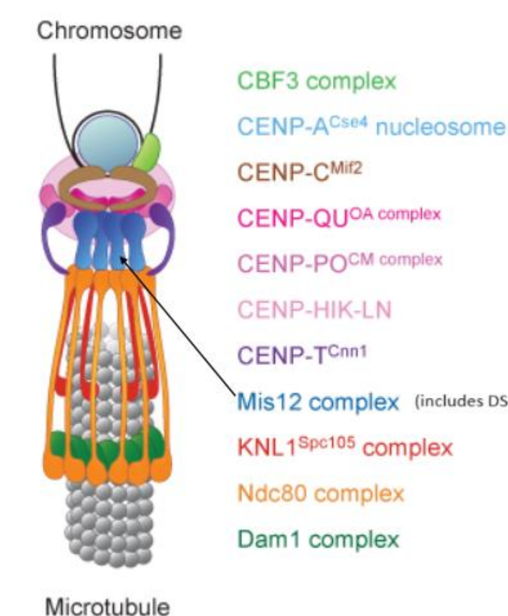

Figure 2: Model representing the kinetochore complex. Dsn1 is a protein within the Mis12 complex, indicated with an arrow. Dsn1 is associated with Ndc80, where it contributes to microtubule attachment and tension sensing.[3] *Credit: Biggins Lab* [1]

- Phosphorylation alters the structure and function of proteins.[4,5,6] We were interested in whether these phosphorylation events impacted the structure and function of Dsn1 within the kinetochore.
- We aimed to mutate the *DSN1* gene at codons that code for amino acids known to be phosphorylated.[7,8,9,10]

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  1 MSLEPTQTVS GTPPMLHQRT HKQVYPLRME TIPILESDSK ATLQSNEPTQ KDEEETEYFE
 61 NKQSVSNLSP DLKFKRHKNK HIQGFPTLGE RLDNLQDIKK AKRVENFNSS APIADDNHSG
121 DATANATANA TANATANVNA SAMPAPYMPY YYYYHPMNAP TPAMIPYPGS PMHSIMPNSS
181 LQPFYSQPTA AGGPDMTTPQ NISSSQQLLP APQLFPYGSF HQQQLQQPHY IQRTRERKKS
241 IGSQRGRRLS MLASQANGGS TIISPHKDIP EEDFYTVVGN ASFGKNLQIR QLFNWCLMRS
301 LHKLELKAKN QEEEGELEHL TKKSKLESTK AETDYVDPKR LAMVIIKEFV DDLKKDHIAI
361 DWEDEEKYED EDEEKILDNT ENYDDTELRQ LFQENDDDDD DDDEVDYSEI QRSRRKFSER
421 RKALPKEPKK LLPNSKNVEN TKNLSILTSK VNAIKNEVKE WAVTLDTSRP DLEWQELTSF
481 SSQPLEPLSD TEEPDLAIAD VETKLETKVD ELRYQSHILN SHSLALNEIT NSKVNKLNIE
541 TMRKISSETD DDHSQVINPQ QLLKGLSLSF SKKLDL*
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Figure 3: Dsn1 amino acid sequence. Amino acids highlighted in blue have been identified as modification sites through mass spec analysis. Our research investigates threonine, located at sites 380 and 386.

Methods & Results

- A Homology Directed Repair (HDR) template containing the intended phospho-null mutations was introduced to yeast cells with the use of the CRISPR-Cas9 system.

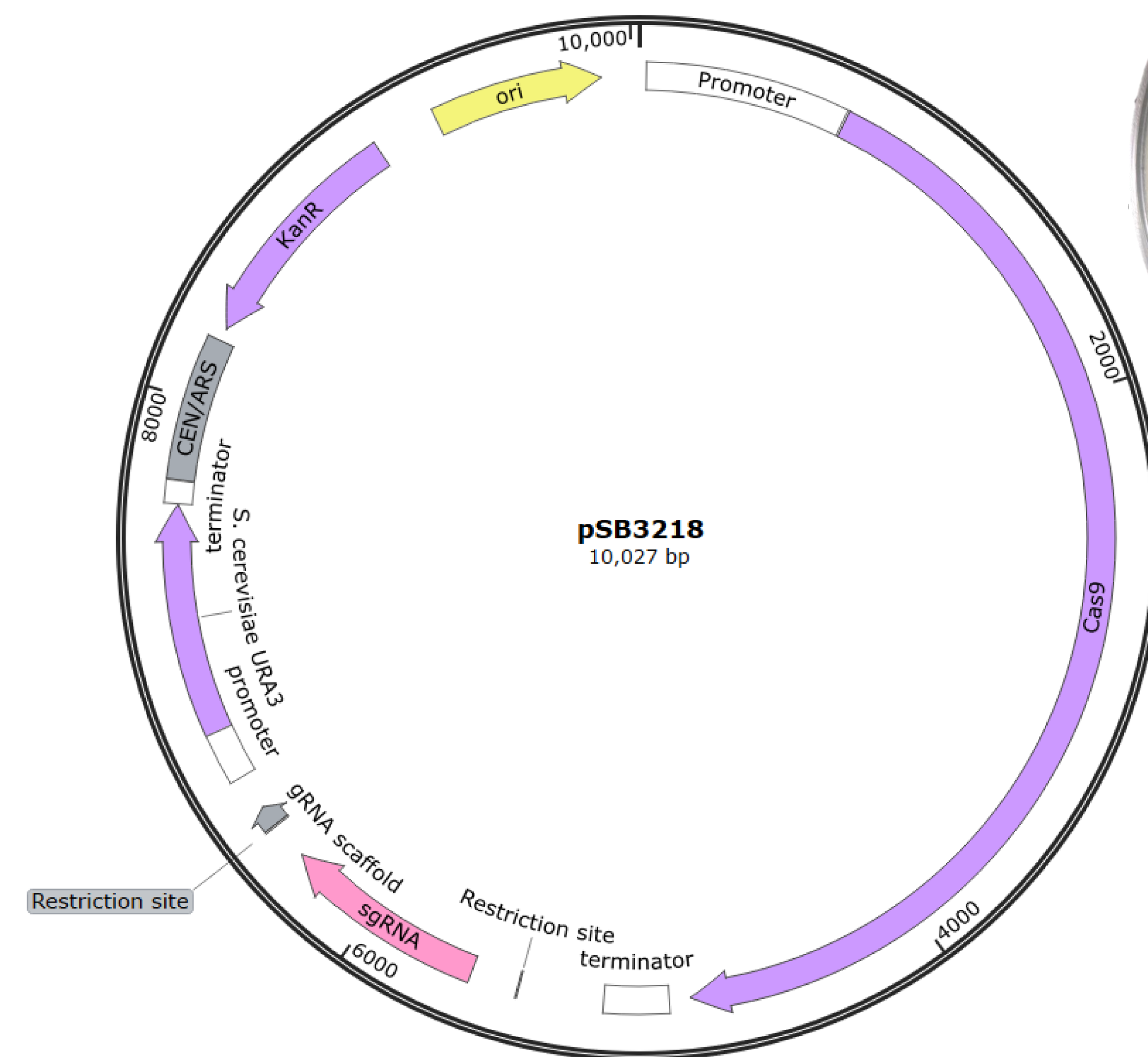

Figure 4: CRISPR-Cas9 vector. Alexandrea Pascua constructed the CRISPR vector containing a DNA sequence that codes for the guide RNA necessary to direct Cas9 to cut proximal to Dsn1 sites 380 & 386 used in our experimentation.[11]

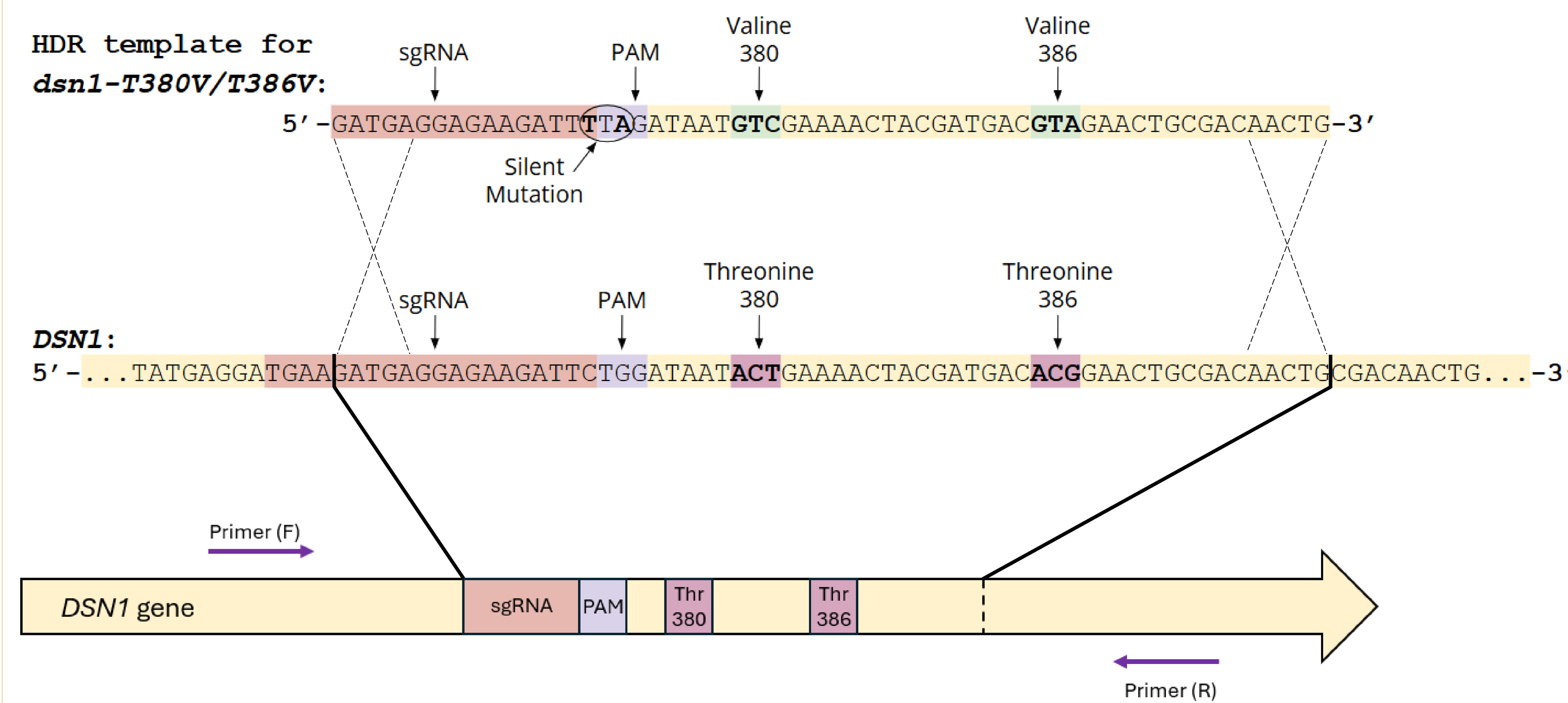

Figure 5: *dsn1-T380V/T386V* HDR template design. *DSN1* gene sequence annotated with the CRISPR recognition site, intended mutations, and primers. Primers were designed to amplify a 489bp DNA fragment containing sites 380 & 386 during PCR. We aimed to insert the 60bp HDR template (containing the intended mutations) within the magnified region.

- After transformation, a region of the *DSN1* gene containing sites 380 and 386 was amplified through a PCR reaction.
- Yeast DNA was purified and sent out for Sanger sequencing to confirm the presence of our intended mutations.

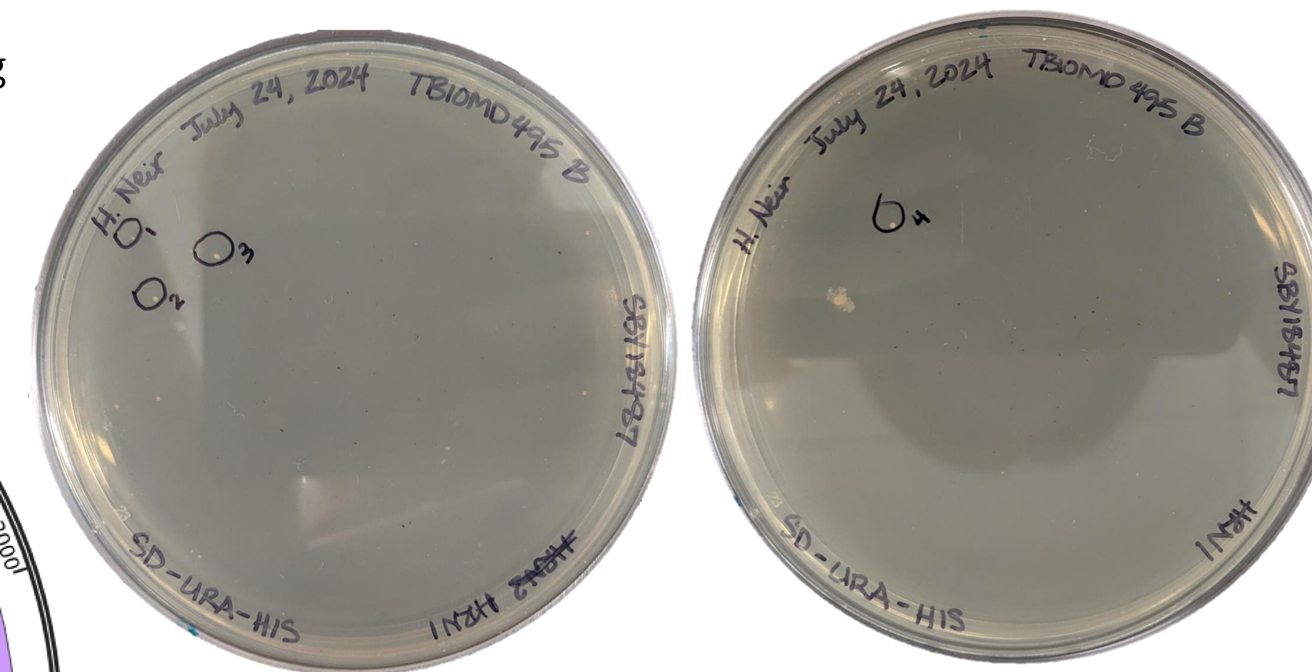

Figure 6: Transformation of SBY18487 with CRISPR vector and HDR template for construction of *dsn1*-T380V/T386V plated on selective media (SD-URA-HIS). Successful transformants are indicated with a black circle, labeled Colony 1-4.

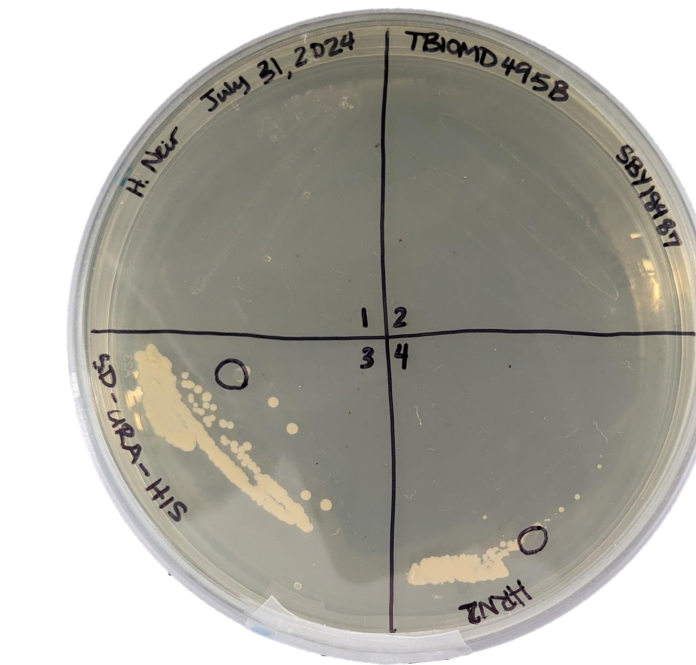

Figure 7: Transformed yeast pure culture plated on selective media (SD-URA-HIS), showing successful transformants that retained the CRISPR vector. Black circles represent yeast colonies that were used for PCR (originating from colony 3 and colony 4 from transformation plates).

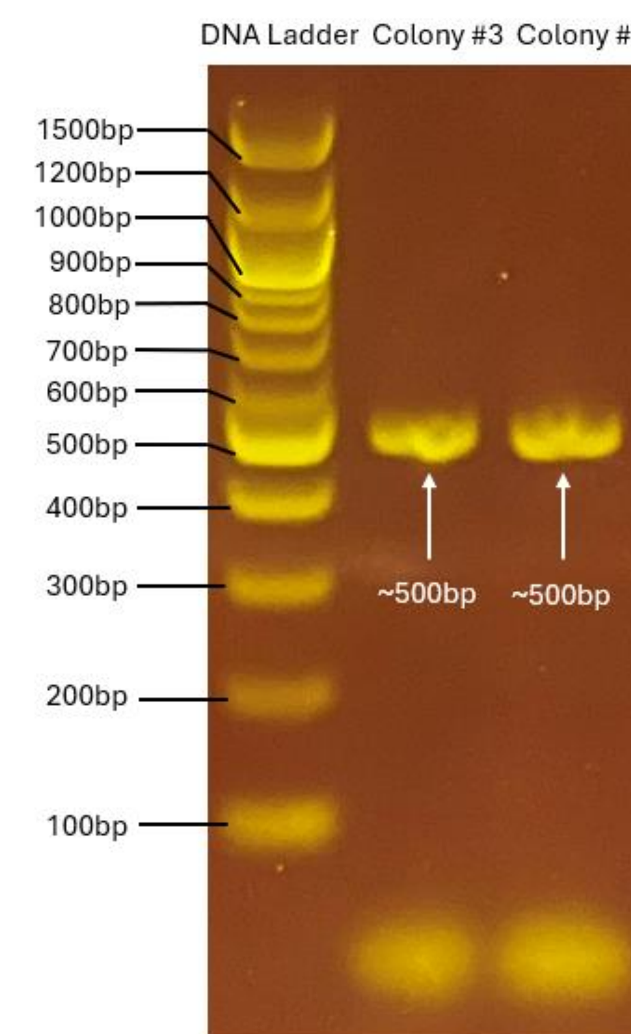

Figure 8: Gel electrophoresis of PCR amplified *DSN1* target sequence, including sites 380 & 386, between two successful transformations. (Samples from left to right: DNA Ladder, Colony 3, Colony 4).

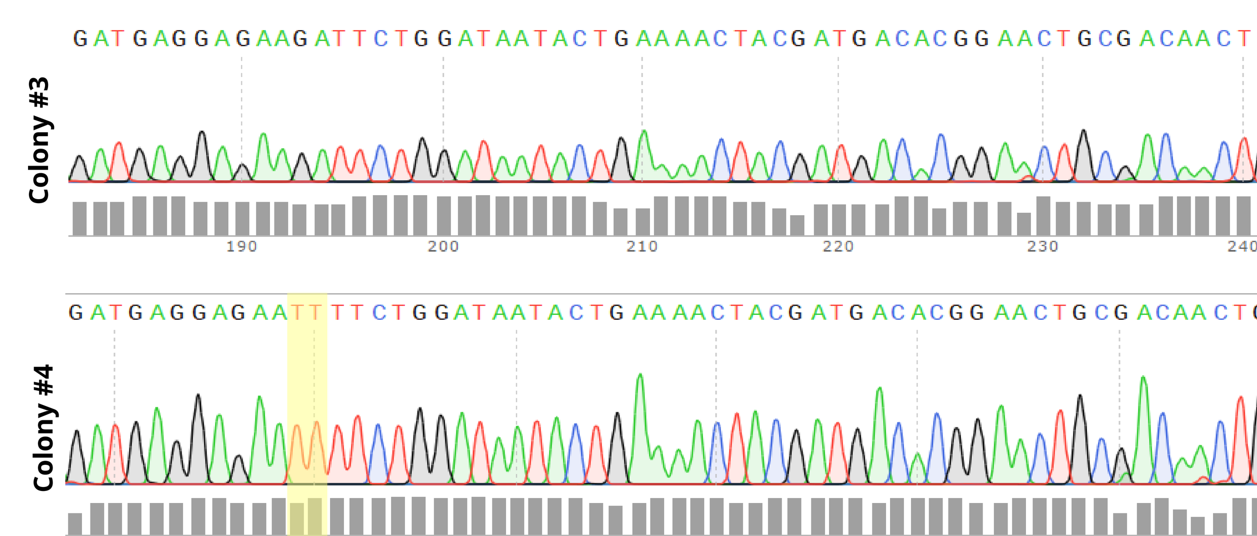

Figure 9: Sanger sequencing data of PCR amplified DNA, including sites 380 & 386, between colony #3 (top) and colony #4 (bottom). The sequences indicated above represent the targeted HDR template area in *DSN1*. The highlighted bases in Colony #4 indicate offsite mutations (within sites 375 & 376).

Conclusion

- Initial transformation of yeast with the CRISPR vector was successful. This was represented by colony growth present on media that selected for the URA3 gene on the CRISPR vector.
- PCR amplification of sites 380 and 386 in the *DSN1* gene was shown as successful through gel electrophoresis, which produced the expected band size (489bp).
- Sanger sequencing data showed that the integration of the T380V/T386V mutations was not successful.
- Sanger sequencing data supported the function of the CRISPR vector, since an offsite mutation was present.
- The HDR template introduced was 60 base pairs. Higher site directed mutagenesis success rates were associated with HDR templates 200bp in length.

Future Directions

- We are in the process of repeating the mutagenesis with a longer HDR template for the construction of *dsn1-T380V/T386V*
- This research will aid in the investigation of the kinetochore and the role of its associated proteins to successfully produce two equal cells during the metaphase-anaphase transition.
- Collaborative phenotypic characterization of yeast containing mutations in kinetochore protein subcomplexes will provide information relevant to mutations that result in aneuploidy and the associated diseases observed in humans.

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Scan Here for References