Mutagenesis of the *Saccharomyces cerevisiae STU1* Gene to Mimic Constant Phosphorylation of a MELT Motif

Ambre Fontana & Jack Vincent Division of Science and Mathematics University of Washington - Tacoma

Background

- The kinetochore plays a key role during the cell division process, mediating the process and acting as an adaptor between the mitotic spindles and the centromere (Biggins et al. 2013).
- Without proper connection, there can be non-disjunction and an unequal division of chromosomes during anaphase, called aneuploidy, which can result in cancer or cell death.
- The *STU1* gene codes for a microtubule plus-end-tracking non-motor protein (Stu1) and facilitates the connection between the kinetochores and mitotic spindles during cell division. It also participates in a checkpoint that ensures proper connections between the spindles and kinetochores before the cell continues into anaphase (Pasqualone et al.1994).
- Stu1 has two well-conserved MELT motifs. MELT motifs are highly conserved sequences of amino acids that are phosphorylated and targeted by the Mps1 kinase, which has been found to play a major role in the regulation of segregation and spindle checkpoints (London et al. 2012).

The overall objective of this study is to use CRISPR-Cas9 to mutate the *STU1* gene to see the effect of changing the MELT motif of IDLT at position 719 from threonine (T) codon to glutamic acid (E) codon. This change is to test whether turning a phosphorylation site into a constitutively negatively charged amino acid, or phospho-mimetic site will impact Stu1's role in chromosome segregation.

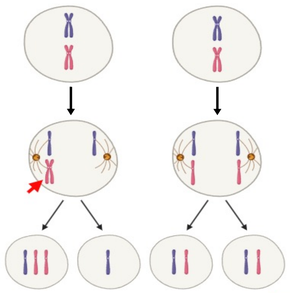

Figure 1: (Left) Diagram of a cell going through cell division, but there is an improper connection of the mitotic spindles, as is shown at the red arrow, so the daughter cells result in aneuploidy. (Right) Diagram of a cell going through normal cell division.

Wild type *STU1*:

5′-GCC CCT CCT TCT TCT ACT GCC GCC ACA AAA GTA TCT GAA AAT TAC ACA AAT TTT GAT GAC TTT CCG TCA AAC CAA ATC GAC TTG ACT GAT GAG TTA TCA AAT AGT TAC TCT AAC CCG TTG ATA AAG AAA TAT ATG GAT AAA AAT GAT GTT TCG ATG TCA TCT TCT CCA ATC TCA TTA AAA GGC AGT AAT AAA CTT GGT

HDR template STU1hdrT719V:

5′-GCC CCT CCT TCT TCT ACT GCC GCC ACA AAA GTA TCT GAA AAT TAC ACA AAT TTT GAT GAC TTT CCG TCA AAC CAA ATC GAC TTG GAA GAT GAG TTA TCA AAT AGT TAC TCT AAC CCG TTG ATA AAG AAA TAT ATG GAT AAA AAT GAT GTT TCG ATG TCA TCT TCT CCA ATC TCA TTA AAA GGC AGT AAT AAA CTT GGT

Figure 2: HDR Template STU1hdrT719V designed by Anh-Thu Truong and Son Nguyen in Summer 2023. The original sequence was edited via SnapGene Viewer to change the *STU1* original code from Threonine (ACT) to Glutamic Acid (GAA) at codon position 719. The red sections are the original, unchanged codons of the MELT motif IDLT, the highlighted section is the sequence bound by the sgRNA, and the purple section is the changed codons to switch the Threonine to Glutamic Acid (Truong & Nguyen 2023).

Methods

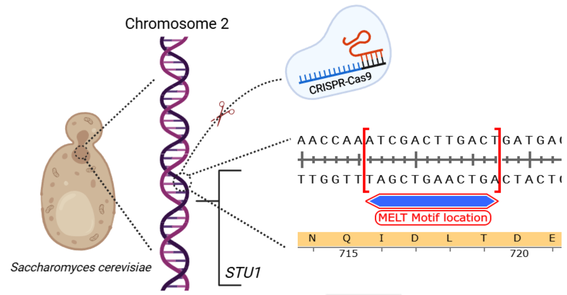

Figure 3: CRISPR-Cas9 methodology for targeting mutation to *STU1* in *Saccharomyces cerevisiae* cells.

- Pre-constructed samples made by students were used: Christian Gombio's *STU1*-targeting CRISPR vector and Anh-Thu Truong and Son Nguyen's HDR template, STU1hdrT719V.
- Transformed the vector and template into a *Saccharomyces cerevisiae* (budding yeast) cell that lacks a functioning *URA3* gene (Figure 3 & 4).
- Once yeast colonies have successfully appeared on a plate that lacked Uracil, they can be collected, and the DNA can be isolated.
- Then the purified gDNA can be used as a template during PCR to copy the middle section of *STU1*. Once the PCR is successfully done, the re-isolated DNA can be sent off for sequencing.

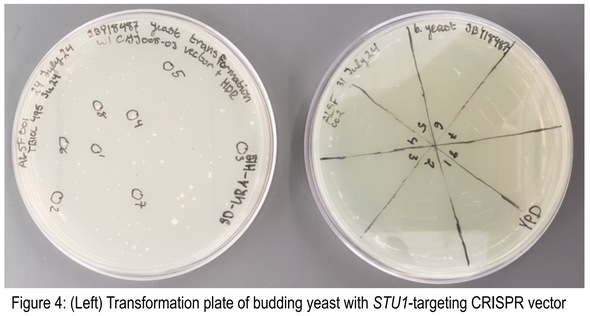

Figure 4: (Left) Transformation plate of budding yeast with *STU1*-targeting CRISPR vector and STU1hdrT719V HDR vector on an SD-URA-HIS plate. (Right) Single colony streak plate containing eight colonies from SD-URA-HIS plate onto a YPD plate. Circled colonies on left plate were plated onto the right plate.

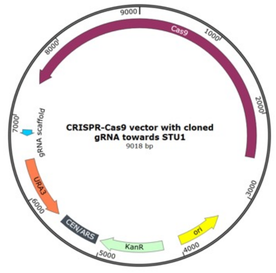

Figure 5: Christian Gombio's plasmid was derived from Biggins' lab pSB3218 plasmid with *Cas9* gene that codes from CRISPR-Cas9. gRNA scaffold is a sequence from the *STU1* gene that will code for the guide RNA, *URA3* allows us to select for this plasmid in yeast, CEN/ARS allows the plasmid to be replicated in yeast, *KanR* allows us to propagate and make copies of the plasmid in *E. coli* cells, and *ori* is the origin for replication for *E. coli*.

Results

- Results of the gel electrophoresis (Figure 6) showed that there was a successful PCR run.
- The sequencing results came back and were compared against a wild-type *STU1* sequence (Figure 7), and it was found that the IDLT MELT motif mutation to IDLE was successfully placed in the *Saccharomyces cerevisiae* DNA.
- There was a successful replacement of the threonine (T) amino acid at position 719 to glutamic acid (E) (Figure 7).

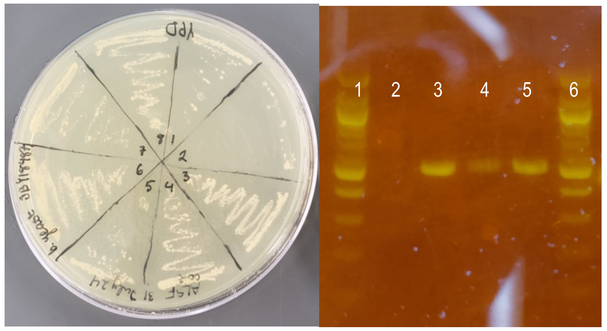

Figure 6: (Left) Plate from Figure 4 a week later with grown colonies. (Right) Gel electrophoresis results. Lane 1 was 1Kb DNA ladder; Lane 2 was colony 7 from the plate; Lane 3 was colony 5 from the plate; Lane 4 was colony 2 from the plate; Lane 5 was colony 1 from the plate; Lane 6 was 1Kb DNA ladder. The bands present in Lanes 3-5 indicate an approximately 500 base pair length of DNA, as expected.

```
Query  2    NSNTKDNNNVTKRKVSAPPSSTAATKVSENYTNFDDFPSNQIDLEDELSNSYSNPLIKKY  181
            NSNTKDNNNVTKRKVSAPPSSTAATKVSENYTNFDDFPSNQIDL DELSNSYSNPLIKKY
Sbjct  675  NSNTKDNNNVTKRKVSAPPSSTAATKVSENYTNFDDFPSNQIDLTDELSNSYSNPLIKKY  734

Query  182  MDKNDVSMSSSPISLKGSNKLGEYETLYKKFNDASFPAQIKDALQYLQKELLLTSQQS  355
            MDKNDVSMSSSPISLKGSNKLGEYETLYKKFNDASFPAQIKDALQYLQKELLLTSQQS
Sbjct  735  MDKNDVSMSSSPISLKGSNKLGEYETLYKKFNDASFPAQIKDALQYLQKELLLTSQQS  792
```

Figure 7: BLAST Alignment of the codon sequence derived from sequencing results for one of four DNA samples compared to the codon sequence of *Saccharomyces cerevisiae*; This shows only one difference at position 719 from threonine (T) to glutamic acid (E), as seen in the red box. The Query line is the codon sequence of the DNA that was transformed into the *Saccharomyces cerevisiae*, and the Sbjct (subject) line is the codon sequence for the Wild Type DNA.

Acknowledgements:

- Thank you to Sue Biggins and the Biggins Lab of the Fred Hutch Cancer Center for their support on the project.
- Thank you to Christian Gombio for his CRISPR vector, and Anh-Thu Truong and Son Nguyen for their HDR template.
- Thank you to my family and friends for their support and willingness to let me practice my presentation on them.

Conclusions and Current Research

- We were successful at creating a mutation of the *STU1* gene at codon 719 from Threonine to Glutamic Acid.
- The next steps in our research are to run phenotypic tests on the *Saccharomyces cerevisiae* colonies using Benomyl plates.
- The Benomyl plate will be used to test if the functions of the anaphase-promoting complex and the overall kinetochore are affected by the mutation we made. Benomyl is a drug that impacts the ability of the microtubules to properly attach to kinetochores at low doses (Schuyler et al. 2021).
- The Benomyl plates will be set up in a gradient, with one side having a higher Benomyl content. The plate will be topped off with a layer of YPD, and samples will be placed in rows, perpendicular to the gradient. The goal is to get a gradient of cell growth along the plate.

Figure 8: Colonies 3, 4, 5 and 8 replated onto a fresh YPD plate to ensure fresh *Saccharomyces cerevisiae* cells are available for future testing.

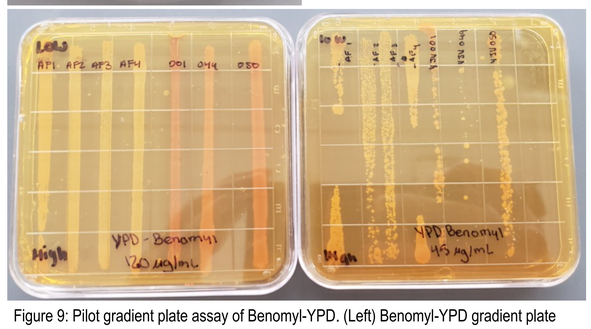

Figure 9: Pilot gradient plate assay of Benomyl-YPD. (Left) Benomyl-YPD gradient plate containing 120ug/mL of Benomyl. (Right) Benomyl-YPD gradient plate containing 45ug/mL of Benomyl. (Both) AF1 was from Colony 1, AF2 was from Colony 2, AF3 was from Colony 3, and AF4 was from Colony 4 on the plate seen in Figure 8. This experiment was done using old gradient plates, so we will have to repour and redo these plates for better results.

References