Boston University Kilachand Honors College

Characterization of DNA Double Strand Break Repair: A Novel Approach using CRISPR-Cas9

Daniel W. Dietrich, John L. Celenza, PhD

CAS/GRS BA/MA Biochemistry and Molecular Biology / Biotechnology

Department of Biology

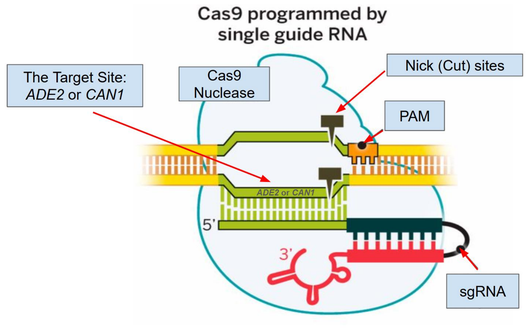
Introduction

CRISPR-Cas9 technology has revolutionized molecular genetics and has led to a better understanding of molecular mechanisms involved with human diseases. One key aspect of CRISPR-Cas9 lies in the ability to create targeted double-strand breaks (DSBs) in the DNA double helix, resulting in a hazardous lesion if left unrepaired. If DSBs are not repaired, then genetic instability and cell death can ensue. Further, DSBs naturally occur due to exogenous sources such as ionizing radiation like X-Rays or UV Radiation. Due to the nature of DSBs compared to single-strand breaks, there is no complementary template for repair, meaning that its repair can often be error prone, potentially leading to cancer. Therefore, organisms across all domains of life have conserved mechanisms of DSB repair to avoid these deleterious effects.

Here, we take advantage of CRISPR-Cas9 to generate DSBs in two *Saccharomyces cerevisiae* (brewer's yeast) genes, *ade2* and *can1*, and determine which DSB repair mechanisms are used. As yeast have many of the same proteins, or orthologs, as humans, this simple eukaryotic model system is commonly used to understand biological processes essential to species. To characterize the genes involved in DSB repair, the following mutant genotypes are employed: *rad52* and *rad59* implicated in homology directed repair (HDR), and *yku70* and *yku80* involved in nonhomologous end joining (NHEJ). When one of the proteins in either of these repair pathways is disrupted, DSB repair mechanisms must adjust and use alternative (and less preferred) repair pathways. Because DSB repair can result in mutations at the repair site, CRISPR-Cas9 targeted to the *ADE2* gene will result in *ade2* mutants that display a red pigment and CRISPR-Cas9 targeted to the *CAN1* gene will result in *can1* mutants that are resistant to the toxic compound canavanine. CRISPR-Cas9 induced mutations in *ADE2* or *CAN1* are quantified in wild-type yeast and the repair mutant genotypes. Next-Generation Sequencing of *ade2* and *can1* mutant populations characterizes the class of mutations (insertion/deletion or base substitution). By characterizing the DSB repair pathways used in *S. cerevisiae*, this project offers a novel approach to understanding these robust processes important to human disease and health. Key questions are addressed, including:

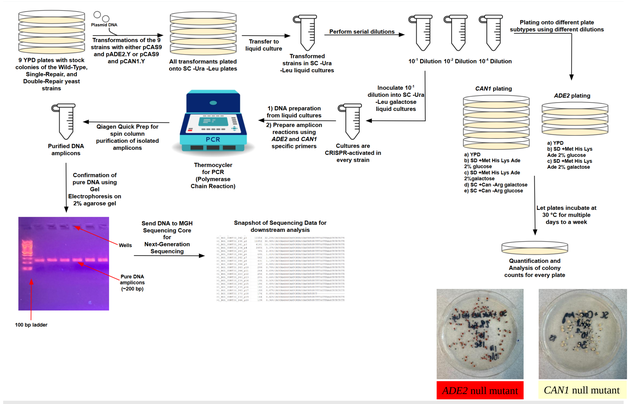
1. What happens to the mutation frequency at CRISPR-Cas9 targets *ADE2* and *CAN1* in the single and double repair mutant genotypes?
2. What classes of mutations are produced at the site of repair?
3. Which repair pathways remain active in each repair mutant genotype?

CRISPR-Cas9 Diagram

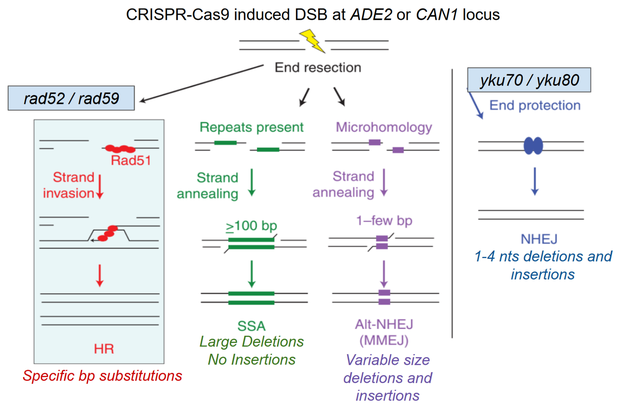


Modified diagram from Doudna and Charpentier, 2014

Methods



Proposed Mechanisms for Double Strand Break Repair



Modified diagram from Jasin and Rothstein, 2013

Results

Parameters for Statistical Analyses

- All cell counts and plating compiled in replicates of 5.
- 2-tailed T-TEST quantified differences in average mutation frequencies between yeast strains.
- p-values < 0.05 are denoted with * and p-values < 0.005 with **.

The *CAN1* gene shows an expected strong preference to undergo DSB repair using NHEJ

- *CAN1* allele shows a decrease in mutation frequency for all genetic mutants comparted to *WT*.
- All *yku70*/*yku80* single repair and double repair mutants displayed a strong significant difference (p < 0.005) in fold-change compared to *WT*.

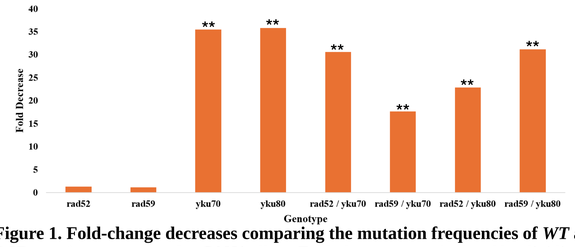


Figure 1. Fold-change decreases comparing the mutation frequencies of *WT* and DSB repair mutants at the *CAN1* allele. Fold-change was calculated by dividing the average mutation frequency of *WT* divided by the average mutation frequency of the appropriate genotype.

The *ADE2* gene indicates a different mutation frequency pattern from *CAN1*, suggesting *ADE2* utilizes both HDR and NHEJ

- For *ADE2*, each mutant with *rad52*, *rad59*, and *yku70* displays a significant decrease (p < 0.05) in fold-change compared to *WT*.
- All double repair mutants have a strong significant decrease (p < 0.005) in fold-change .
- Unexpected finding: this indicates that *ADE2* requires a double knockout of both gene types to produce the same strength of fold-decrease obtained in *CAN1*.

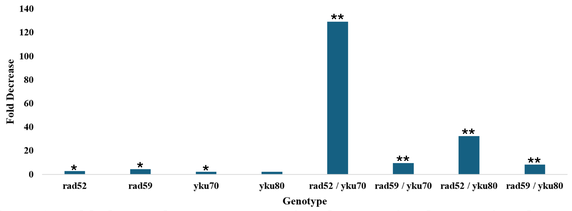


Figure 2. Fold-change decreases comparing the mutation frequencies of *WT* and DSB repair mutants at the *ADE2* allele. Fold-change was calculated by dividing the average mutation frequency of *WT* divided by the average mutation frequency of the appropriate genotype.

Double repair mutant analysis suggests that *CAN1* relies on *YKU70/YKU80*, while *ADE2* relies on both *RAD52/RAD59* and *YKU70/YKU80*.

- *CAN1* shows significant decreases (p < 0.005) in fold-change when going from the single *rad59* mutant to either of its respective double repair mutants.
- *ADE2* displays significant decreases (p < 0.05 and p < 0.005) in all comparisons when going from a single repair to a double repair mutant.
- This emphasizes differential regulation of *ADE2* and *CAN1* DSB repair.

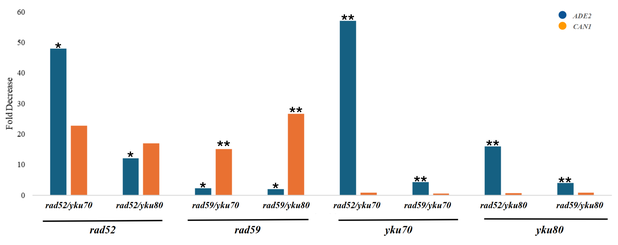


Figure 3. Fold-change decreases comparing the mutation frequencies of single repair mutants to double repair mutants in both *ADE2* and *CAN1*. Fold-change was calculated by dividing the average mutation frequency of the single repair mutant by the double repair mutant.

CAN1 sequencing data requires analysis at the Protospacer Adjacent Motif (PAM) Sequence.

- The PAM sequence defines CRISPR/Cas9 targeting and cut site.
- All sequence analysis requires the location of this conserved sequence to identify differences in base compositions as either, insertions, deletions, and/or substitutions.

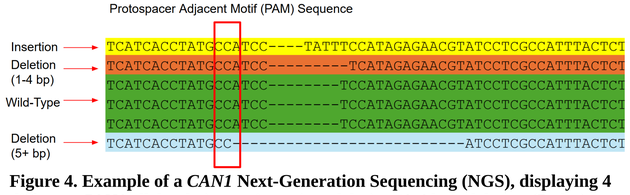


Figure 4. Example of a *CAN1* Next-Generation Sequencing (NGS), displaying 4 different *CAN1* alleles, being an insertion, deletion (1-4 bp), deletion (5+ bp), and *WT*.

CAN1 NGS displays a drastic decrease in mutant population when knocking out either *yku70* or *yku80*.

- The knockout of *yku70*/*yku80* resulted in at least 90.6 % *WT* sequences in all repair mutants.
- The knockout of *rad52*/*rad59* strains had more limited *WT* sequences, with none greater than 18.6 %.
- The types of alleles quantified were all drastically less in the *yku70*/*yku80* mutants than the *rad52*/*rad59* mutants. This further solidifies evidence of the *CAN1* dependence on NHEJ.

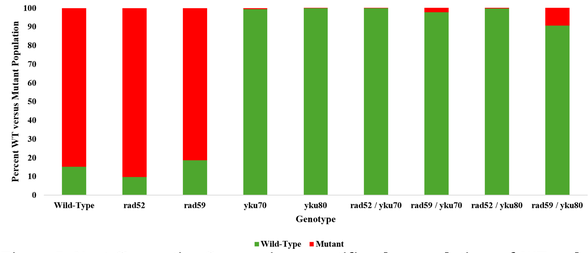


Figure 5. Next-Generation Sequencing quantifies the populations of *WT* and mutant alleles in the *CAN1* locus. This stacked bar chart shows the total population of every strain at 100 %, divided into two sections per genotype to represent the percent of mutant versus *WT* sequences.

Table 1. Quantification of *CAN1* alleles and breakdown by mutant type.

Genotype	Total Mutant NGS reads	% Insertion	Number of Insertion Alleles	% Deletion (1-4 bp)	Number of Deletion (1-4 bp) Alleles	% Deletion (5+ bp)	Number of Deletion (5+ bp) Alleles
Wild-Type	42848	46.4	21	45.7	8	7.94	10
rad52	48079	21.2	27	34.6	15	44.3	41
rad59	43728	45.1	24	37.6	11	17.3	17
yku70	333	3.60	1	90.1	2	6.31	1
yku80	46	0	0	63.0	1	37.0	1
rad52 / yku70	35	0	0	54.3	1	45.7	1
rad59 / yku70	983	9.16	2	70.6	3	20.2	4
rad52 / yku80	108	0	0	29.6	1	70.4	3
rad59 / yku80	3390	10.3	5	13.7	3	76.0	6

Conclusions and Future Directions

- Analogous human genes regulated in a similar manner as *CAN1* may use a therapeutic knockout of *Ku* (the human ortholog to yeast *yku*) to decrease mutant cell populations dramatically, helping with the prevention of cancerous cell production.
- The *CAN1* gene resolves DSBs with Nonhomologous End Joining (NHEJ), dependent on the *yku* genes.
- The *ADE2* gene displays a more complex DSB repair process, utilizing both NHEJ and HDR processes, dependent on both *yku* and *rad* genes.
- The large variation in mutant type from both the *CAN1* and *ADE2* sequencing reveals the production of many mutant alleles, produced by NHEJ, HDR, and other less used pathways, such as Single-Strand Annealing, to produce large (20+ bp) deletions at alleles.
- This data further motivates an investigation into the regulation of the more complex *ADE2* and the mutant alleles produced.

Acknowledgements

This research was made possible through the guidance and support of Dr. John Celenza both in the laboratory and throughout the BA/MA program. Additionally, thank you to Kilachand Honors College for funding the sequencing done by MGH Sequencing Core.

References

1. Chang, H., Pannunzio, N., Adachi, N., and Lieber, M. (2017). Non-homologous DNA end joining and alternative pathways to double-strand break repair. *Molecular Cell Biology, Nature Reviews*. 18, 495-506.
2. DiCarlo, J. E., Norville, J. E., Mali, P., Rios, X., Aach, J., and Church, G. M. (2013). Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. *Nucleic Acids Research*. 41, 4336-4343.
3. Doudna, J. A., and Charpentier, E. (2014). The new frontier of genome engineering with CRISPR-Cas9. *SCIENCE*. 346:6213.
4. Emerson, C., and Bertuch, A. (2016). Consider the workhorse: Nonhomologous end joining in budding yeast. *Biochem Cell Bio*. 94(5), 396-406.
5. Jasin, M., and Rothstein, R. (2013). Repair of Strand Breaks by Homologous Recombination. *Cold Spring Harb Perspect Biol*. 11, 1-5.
6. McVey, M., and Lee, S. (2008). MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings. *Cell Press*. 24, 529-538.
7. Symington, L. S., and Gautier, J. (2011). Double-Strand Break End Resection and Repair Pathway Choice. *Annu Rev Gen*. 45, 247-71.
8. Wong, E. D., Miyasato, S. R., Aleksander, S., Karra, K., Nash, R. S., Skrzypek, M. S., Weng, S., Engel, S. R., Cherry, J.M. (2023). *Saccharomyces* genome database update: server architecture, pan genome nomenclature, and external resources. *Genetics*. 1:224.