Introduction

Although there have been many advancements in genome editing with Cas proteins, there continues to be a need to optimize PAM site edits to enhance the efficacy of genome editing. This experiment intends to investigate the most effective PAM site edit to prevent recutting in the *Saccharomyces cerevisiae* genome and explore whether PAM site edits are added to colonies that do not lose function in the ADE2 gene. The ADE2 gene makes *S. cerevisiae* colonies white and when it loses function the colonies turn red. Edits to PAM sites are known to prevent recutting and increase cell survival. One study edited a PAM site and incorporated a start codon for a green fluorescent protein with an HDR, which resulted in increased production of green fluorescent protein in mouse embryonic stem cells which was a result of higher cell survival (Harmsen et al, 2022). Homology-directed repair (HDR) templates were designed to have specific edits to a PAM site and to create a loss of function for the ADE2 gene to be able to test the efficacy of the PAM site edits. HDR was effective in the experiment by Long et al. where they used HDR templates to change a loss of function mutation back to wildtype in the DMD gene in embryonic mice. The results showed that HDR was effective and precise at introducing the specific edits to the genome.

Results

Analysis of Colony Phenotypes and Sequences:

The experiment aimed to test the efficacy of edits to PAM sites to prevent the recutting of the genome. Yeast colonies were transformed with pML104+gRNA, encoding for Cas9 and gRNA that bind and cut in the ADE2 gene, along with HDR templates, which were designed with edits to the PAM site and loss of function edits that help identify the yeast colonies that were repaired with HDR templates. The four HDR templates differ in the position of PAM site basepair substitutions: HDR1 (second position), HDR2 (third position), HDR3 (both positions), and HDR4 (no edit) (Figure 9). The controls had the expected results: pML104 had high survival of entirely white colonies, pML104+gRNA had low survival of both red and white colonies, and water had no survival. The HDR4 transformation had low survival overall with few red colonies. Primarily, all results for HDR1, 2, and 3 are similar and the standard deviations overlap for all analyzed data. On average, HDR1 had 54 red colonies, HDR2 had 43 red colonies, and HDR3 had 41 colonies (Figure 1). The average frequency of red colonies for HDR 2 and 3 was 0.688 and HDR1 was 0.67 (Figure 2). The average normalization of colony count was 0.42, 0.40, and 0.34 for HDR 1, 2, and 3 respectively (Figure 3).

The alignment was used to verify the PAM edits were incorporated. The sequences were aligned with the HDR templates that helped design them to see what mutations were made. The HDR transformations had successful repairs with the HDRs. The HDR4 yeast colonies had both the edits from the HDR and deletions or insertions from NHEJ repair (Figure 7). HDR 1, 2, and 3 transformed yeast colonies had the PAM edits and at least one of the designed loss of function edits for all ten sequences (Figures 4-6).

PAM edits incorporation in white colonies:

The second research question explored the incorporation of PAM edits into the yeast genome when the loss of function edits were not incorporated. Yeast colonies were transformed

with pML104+gRNA, which encoded for Cas9 and a gRNA that would cut in the ADE2 gene, and various HDR templates (HDR templates 2, 3, 45, 35, 30, 25) that all had PAM site edits and loss of function mutations that are described in Figure 9. There were no PAM edits added to any of the sequenced genomes of the white colonies. This supports the expectations that the surviving white colonies were not edited and an increase in white colony survival does not mean that the PAM sites were incorporated to prevent recutting.

Discussion

Efficacy of PAM site edits:

Basepair substitution edits in the PAM site have equal efficacy at preventing the recutting of the genome by Cas9 with gRNA to increase survival. There is no significant difference in the number of surviving red colonies, frequency of red colonies, and normalized colony counts of transformations for HDR 1, 2, and 3 (Figures 1-3). The alignments for HDR 1, 2, and 3 verify that PAM site edits were successfully incorporated into the genome from the HDRs (Figures 4-6). Since these results are significantly different from the results from the control transformations pML104+gRNA and pML104+gRNA+HDR4, it proves that an edited PAM site does decrease the amount of genome recutting which increases survival. These results are supported by Harmsen et al., who showed increased cell survival with basepair substitutions altering the PAM site and creating a start codon(2022).

Based on the Dicarlo et al. paper, it is evident that Cas9 complexed with gRNA are very specific to what site they cut, and the results from the study showed that the Cas9 and gRNA only cut a gene, CAN1, and not within another gene, LYP1(2022). This shows that Cas9 will only cut a genome that it binds to well. So if the PAM site is edited, Cas9 may not be able to bind well enough to cut the genome. It is possible altering the PAM site, as was done in this experiment, prevents the amino acids within Cas9 from being able to recognize the PAM site. This is why all of the PAM site edits had a very similar effect. Cas9 was altered in an experiment by Chen et al. to be able to recognize a series of other PAM sites, which it successfully did, and to do so the Cas9 protein configuration and amino acids had to be altered (2019). This suggests that Cas9 which recognizes a PAM site 5'-NGG-3' can physically only recognize that precise PAM site. Any alteration to the PAM site would result in a sequence that would not fit within the Cas9 protein. Therefore, the Cas9 would not be able to bind to the repaired yeast genome with altered PAM sites and cut the genome again which results in more yeast colonies surviving.

A limitation of this experiment is the variation in conducting the procedure of the experiment and the counted results between groups; it is assumed there is perfect completion of every part of the experiment. The sample size is also not very large limiting the accuracy of the results. Another limitation is the low sample size of sequenced red colonies from every transformation, so there may be more NHEJ-repaired red colonies that were not sequenced and skew the results. More PAM site edits could be tested to find the most effective one. One option is to test which nucleotide is most effective at preventing recutting by substituting one position of the PAM site with A, T, and C. Given thymine and cytosine are pyrimidines and their structures significantly differ from that of guanine, they will most likely be more effective at preventing Cas9 binding and, therefore, genome recutting.

PAM edits incorporation in white colonies:

HDR templates do not incorporate PAM edits during repair without the other loss of function edits. There were no PAM edits that were incorporated into the ADE2 gene of white colonies that were transformed with the various HDR templates mentioned in Figure 8. The figure also shows that there were no other mutations occurred and all of the sequences matched perfectly to the wildtype ADE2 gene that they are aligned with. NHEJ repair could have occurred, but it typically repairs with deletions or insertions (Addgene). Since there are no mutations at all, NHEJ repair is not a probable explanation for the repair to wild type. However, the genomes were unedited likely because Cas9 did not cut their genomes or very few nucleotides broke off the genome once it was cut, which resulted in repairing to wildtype. It is common for genomes to not be cut for a variety of reasons including "a lack of gRNA and/or Cas9 expression or (2) a lack of efficient target cleavage in cells expressing both Cas9 and gRNA" (Addgene). If the genomes are not cut, the resulting sequence is wildtype.

The sequences available to analyze are very limited, so it is possible that some of the white colonies did have PAM edits, but they were not sequenced. More white colonies could have been or could be sequenced to see if there is a PAM edit within some of the genomes.

These results help prove that the red colonies are the only colonies that could have PAM edits and that is why white colonies were not considered to have PAM edits when analyzing the data to find the most effective PAM site edit.

References

(n.d.). CRISPR guide. Addgene. https://www.addgene.org/guides/crispr/

Chen, W., Zhang, H., Zhang, Y., Wang, Y., Gan, J., & Ji, Q. (2019). Molecular basis for the PAM expansion and fidelity enhancement of an evolved Cas9 nuclease. PLoS biology, 17(10), e3000496. https://doi.org/10.1371/journal.pbio.3000496

DiCarlo, J. E., Norville, J. E., Mali, P., Rios, X., Aach, J., & Church, G. M. (2013). Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems. Nucleic acids research, 41(7), 4336–4343. https://doi.org/10.1093/nar/gkt135

Harmsen, T.J.W., Pritchard, C.E.J., Riepsaame, J. et al. HideRNAs protect against CRISPR-Cas9 re-cutting after successful single base-pair gene editing. Sci Rep 12, 9606 (2022). https://doi.org/10.1038/s41598-022-13688-y

Long, C., McAnally, J. R., Shelton, J. M., Mireault, A. A., Bassel-Duby, R., & Olson, E. N. (2014). Prevention of muscular dystrophy in mice by CRISPR/Cas9-mediated editing of germline DNA. Science (New York, N.Y.), 345(6201), 1184–1188. https://doi.org/10.1126/science.1254445

Chat GPT and Perplexity were used to improve writing clarity and grammatical issues, as well as, search for articles about CRISPR-Cas9 and PAM site edits.

Figures:

Data for Graphs:
https://docs.google.com/spreadsheets/d/1TCvAMzU4dtlfMk82e3FUQEQ5Cy7tAhnhEdddUo1MHis/edit?usp=sharing (This is the data used for the creation of the above graphs. The raw data from "# red colonies," "freq red colonies," and "normalized colony count" sheets was copied into Excel to create the graphs.)

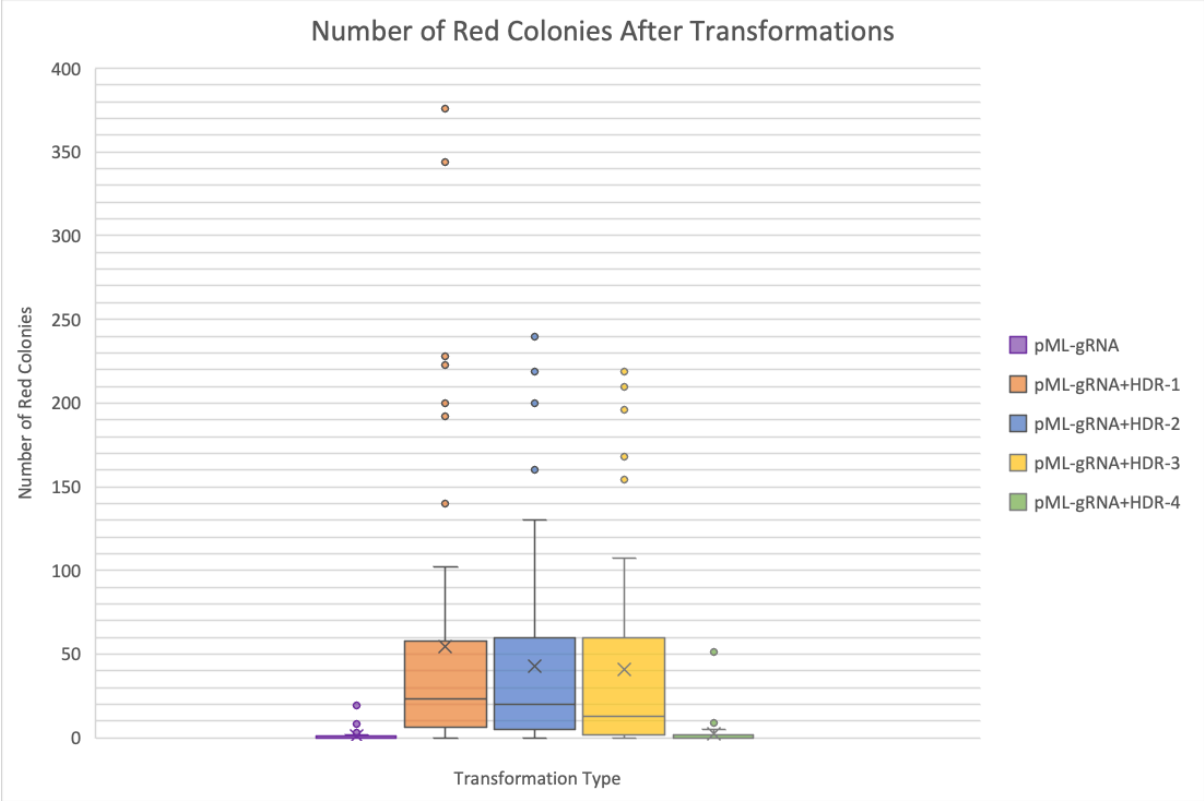

Figure 1: The figure shows the effectiveness of specific PAM edits to prevent recutting of the ADE2 gene in yeast. The number of red colonies illustrates the survival of yeast colonies following Cas9 cutting and repair with loss-of-function mutations, either through HDR or NHEJ, resulting in red colonies. Surviving colonies demonstrate immunity from Cas9 recutting. Red yeast colonies that grew on YC-ura plates after being transformed with pML104+gRNA, and with or without an HDR template, were counted. The error bars represent the standard deviation of the number of red colonies. The sample size is 55.

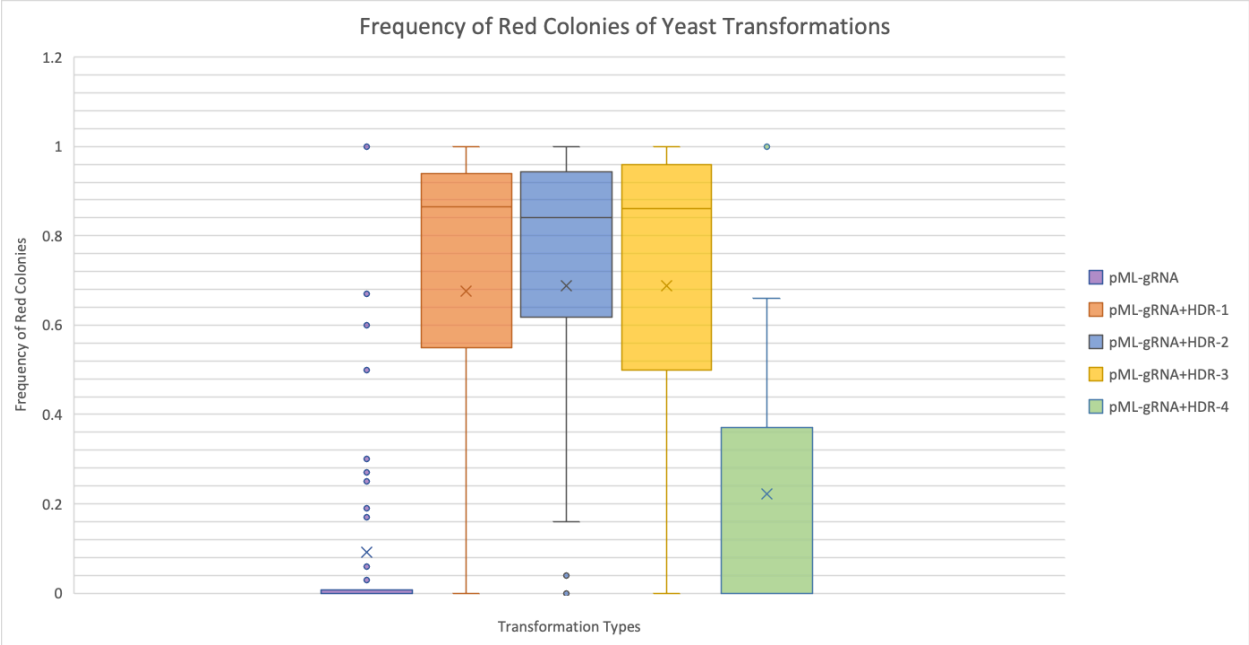

Figure 2: The figure shows that any PAM edit results in higher survival of edited colonies. Yeast colonies that grew on YC-ura plates were counted after being transformed with pML104+gRNA, and with or without an HDR template. The frequency of red colonies was obtained by dividing the number of red colonies by the total number of colonies for each transformation. The error bars are the standard deviation of the frequencies. The sample size is 49.

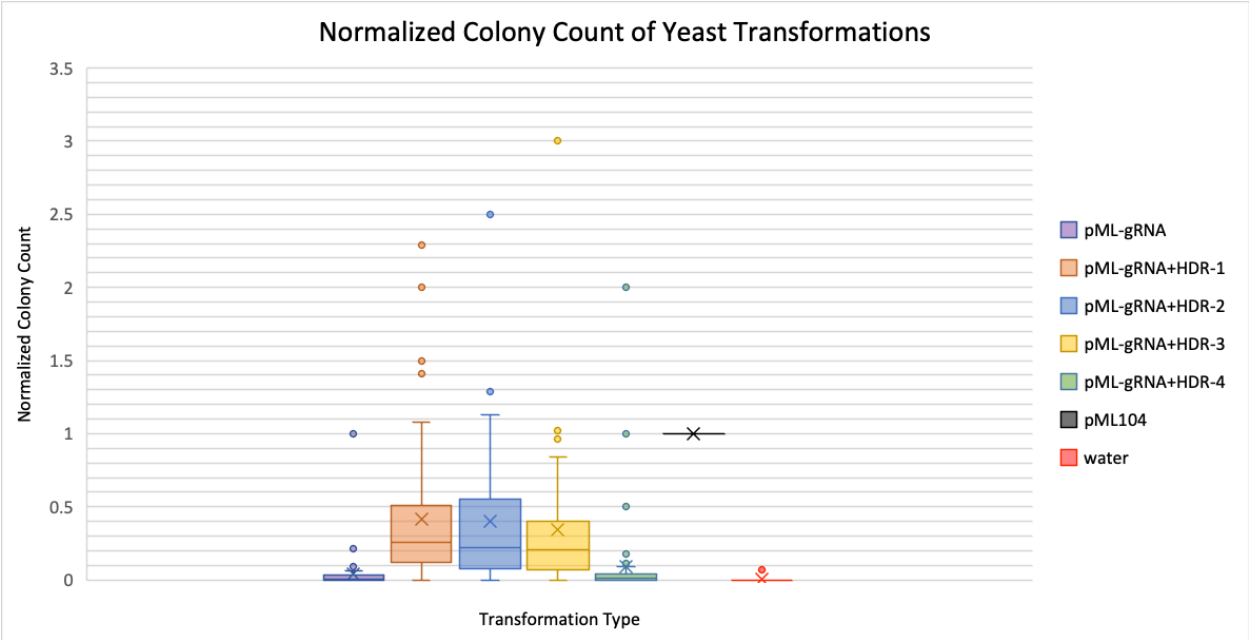

Figure 3: The figure shows the colonies that had PAM edits had overall higher survival than transformed colonies without. Yeast colonies that grew on YC-ura plates were counted after being transformed with pML104, pML104+gRNA (with or without an HDR template), or water. The normalization was obtained by dividing the total number of colonies for each transformation by the total number of colonies for the pML104 transformation. The error bars represent the standard deviation of the normalizations. The sample size is 49.

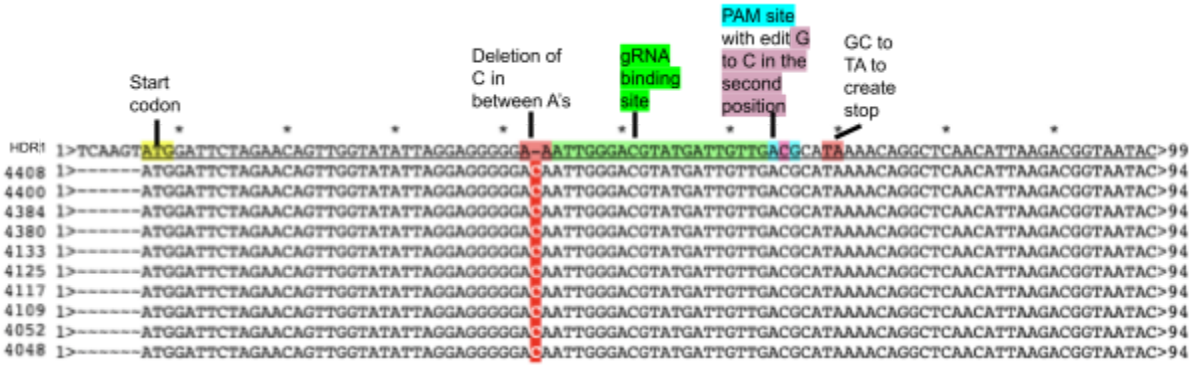

Figure 4: Each of the ten yeast colony sequences successfully incorporated the PAM site edit from the HDR1 template. This is an alignment of ADE2 gene sequences with the HDR1 template sequence (top sequence). The sequences are from red yeast colonies that were transformed with pML104+gRNA and HDR1, which enabled the repair of the double-stranded cut by Cas9+gRNA. Red highlighted nucleotides represent edits that were not incorporated.

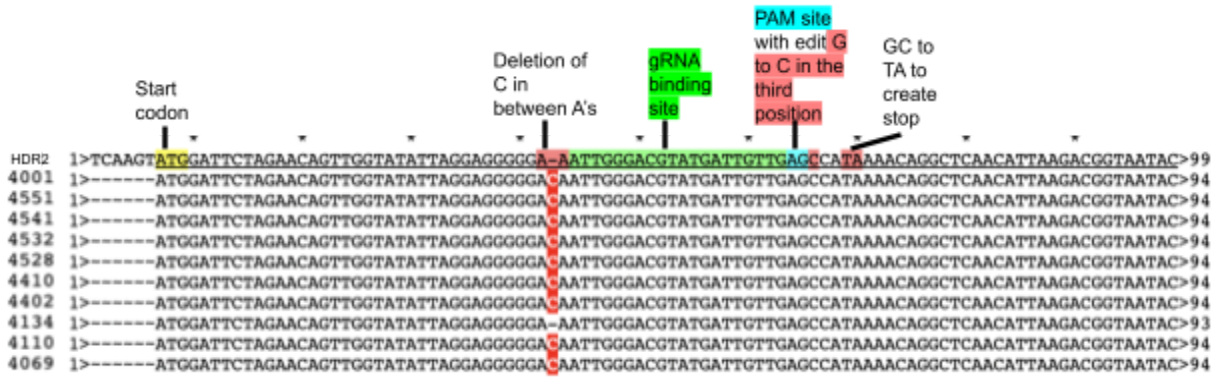

Figure 5: Each of the ten yeast colony sequences successfully incorporated the PAM site edit from the HDR2 template. This is an alignment of ten ADE2 gene sequences with the HDR2 template sequence (top sequence). The sequences are from red yeast colonies that were transformed with pML104+gRNA and HDR2, which enabled the repair of the double-stranded cut by Cas9+gRNA. Red highlighted nucleotides represent edits that were not incorporated.

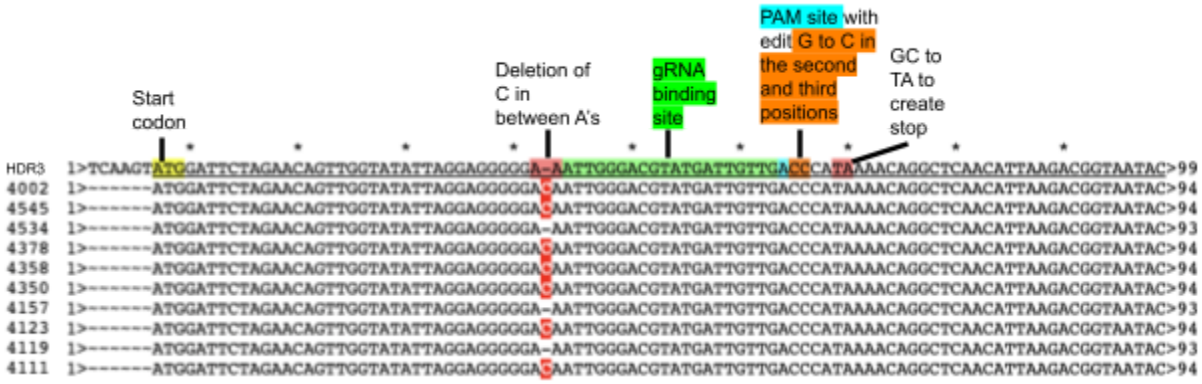

Figure 6: Each of the ten yeast colony sequences successfully incorporated the PAM site edit from the HDR3 template. This is an alignment of ten ADE2 gene sequences with the HDR3 template sequence (top sequence). The sequences are from red yeast colonies that were transformed with pML104+gRNA and HDR3, which enabled the repair of the double-stranded cut by Cas9+gRNA. Red highlighted nucleotides represent edits that were not incorporated and the red-highlighted minus sign is a deletion.

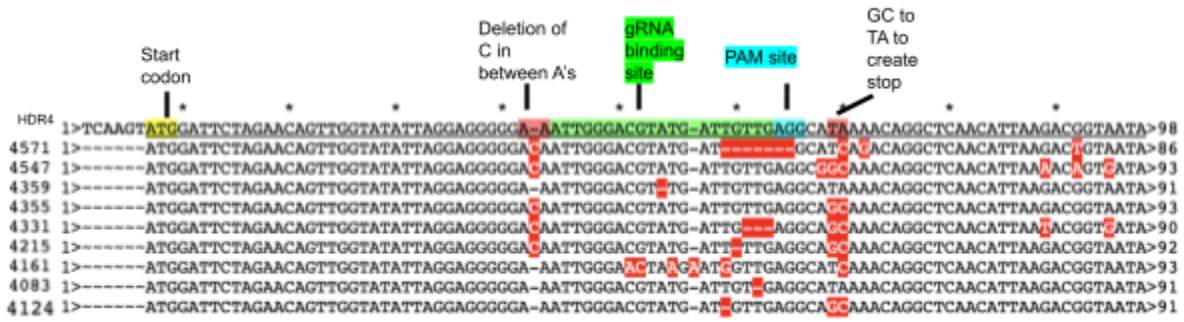

Figure 7: Eight of the above yeast colony sequences used NHEJ, HDR, or both repair mechanisms to fix the cut genome. This is an alignment of nine ADE2 gene sequences with the HDR4 template sequence (top sequence). The sequences are from red yeast colonies that were transformed with pML104+gRNA and HDR4 which enabled the repair of the double-stranded cut by Cas9+gRNA. Red-highlighted nucleotides are edits that were not incorporated, insertions, or basepair substitutions; the red-highlighted minus signs are deletions.

Figure 8: Alignment of ten ADE2 gene sequences with the wildtype ADE2 sequence (top sequence). The sequences are from white yeast colonies that were transformed with pML104+gRNA and various HDR templates used to repair the double-stranded cut by Cas9+gRNA. No edits were incorporated.
The HDR templates for the above sequences were transformed with HDR25 (4431, 4060), HDR30 (4437, 4391), HDR35 (4390, 4300), HDR45 (4389), HDR3 (4568, 4059), HDR2 (4530).

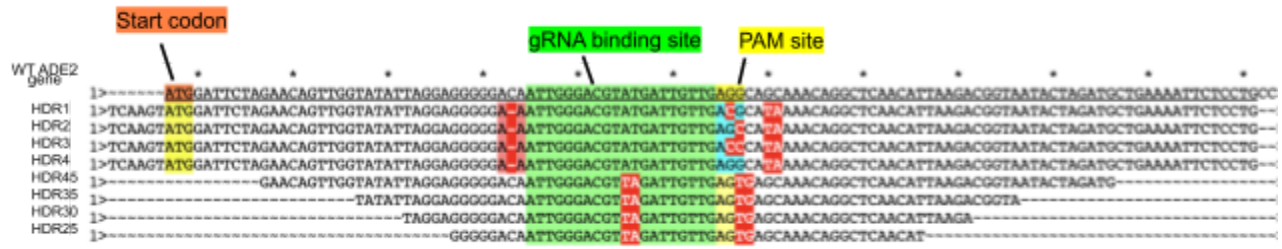

Figure 9: This figure represents all of the HDR template sequences that are aligned with the wild type of the ADE2 gene. HDR 1, 2, 3, and 4 all have a GC to TA basepair substitution that creates a premature stop downstream from the PAM site and a deletion of a C just upstream from the gRNA binding site. HDR 4 has no PAM edit. HDR 1, 2, and 3 have G to C base pair substitutions to the PAM site. HDR 1 has the substitution to the second position of the PAM site, HDR 2 has the substitution to the second position, and HDR 3 has the substitution to the second and third positions. HDR 45, 35, 30, and 25 all have the same PAM edit baspair substitution G to T and another substitution C to G that creates a stop codon and the the AT to TA substitution also creates a stop codon. They vary in the length of their homologous arm lengths (45 bp, 35bp, 30bp, and 25bp).