Gene Targeting and "Hit-and-Run" Transformation

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Introduction

Gene targeting is a genetic technique that uses homologous recombination to change an endogenous gene. It has been a fundamental technique in molecular genetics since its introduction in the late 1970s, for it allows scientists examine the function of each gene or of multiple genes by selectively deleting genes of interest and it has potential use in gene therapy. Although many studies identified key genetic requirements for gene targeting and it is being used in experiments every day to delete a gene, add a gene, or replace a gene, many aspects of the mechanism is still unknown. One phenomenon that results from gene targeting, referred as "hit-and-run" transformation, initially described by Kraus, Leung and Haber (2001) for budding yeast, was studied in detail in order to broaden the understanding of gene targeting. In Kraus's paper, it was found that some cells make plasmid-like circular DNA during intended gene targeting. For this to occur, the targeting fragment had to begin to recombine with the target locus but somehow end up by copying adjacent sequences, including a nearby origin of replication, and then become an autonomously replicating circle. How such circles are formed or what mechanism drives the formation of circular DNA is not well understood. In order to study more about the "hit-and-run" events, gene targeting in *Saccharomyces cerevisiae* will be examined.

The budding yeast, *Saccharomyces cerevisiae,* is an experimental organism that is used in many labs. It is an ideal model organism to study because it is a simple unicellular organism,

which possesses characteristics of more complex organisms. Its genome is made up with 16 chromosomes, which is about four times the complexity of *Escherichia coli* (Chan & Tye, 1980). Because of the simplicity to introduce new DNA into *S. cerevisiae* by transformation and alter its genetics, the use of yeasts in laboratories as a model organism grew in the 1980, and it was used often to study DNA repair mechanism, since yeast, as all eukaryotes, have acquired many different mechanisms to repair DNA double strand breaks (DSBs) in chromosomes, similar to what occurs in mammals.

Cells are routinely exposed to potentially lethal lesions, called DNA double-strand breaks that are generated during normal cell cycle or by external DNA-damaging agents, such as ionizing radiation, oxidative free radicals, and chemotherapeutic drugs. The repair of double-strand breaks (DSBs), in which both strands of the DNA helix are broken, is essential for the viability of the cells and for maintenance of its genomic integrity because they stop DNA replication and may lead to chromosome rearrangements, thereby promoting tumorigenesis. There are two major pathways that cells use to repair DSBs: homologous recombination and non-homologous end joining.

Homologous recombination is a mechanism that a cell uses to repair DNA sequences by using the homologous (identical) or nearly identical genetic information contained in another DNA molecule, usually a sister chromatid. Within homologous recombination, there are several different pathways which cell uses to repair DSBs. Pâques and Haber (1999) summarized four possible mechanisms of homologous DBS repair in their review: gene conversion, break-induced replication, single-strand annealing, and gene targeting. Even though there are differences in these mechanisms such as when certain mechanisms are chosen by cell or whether the repair ends with crossing over or not, they all use certain proteins to aid in homology search and

recombination. For example, genes of the *RAD52* epistasis group are required for the repair of DNA damage-induced mitotic recombination events (Symington, 2002). Also, to begin the repair, an exonuclease generates single-stranded ends at the break by resection of the complementary DNA strands. For this 5' to 3' resection of DSB ends cyclin-dependent kinase CDK1, in budding yeast, is required (Ira et al., 2004). After the resection with the help of specialized enzymes such as Rad51 in *Saccharomyces cerevisiae*, one of these single strands then 'invades' the homologous DNA duplex by forming base pairs with its complementary strand (Haber, 1999).

Gene targeting, even though it occurs by homologous recombination, is in a way corruption of the DSB repair. When DNA double-strand break repair by other homologous recombination mechanisms is high-fidelity process that restores broken sequence using the undamaged template, gene targeting, rather than restoring, replaces the broken sequence using exogenous DNA and results in loss of the targeted chromosomal region (Symington, 2002; Rothstein, 1983). In early studies, when the ends-in gene targeting was first developed, it used homologous recombination to integrate homologous DNA sequence of the foreign plasmid DNA into the yeast chromosome genes (Hinnen et al., 1978). However, because the ends-in gene targeting inserted foreign plasmid DNA at the target chromosomal site without deleting the targeted gene, there were some limitations to it, and in order to address them, gene replacement strategies were developed in yeast (Rothstein 1983). The new ends-out gene targeting used recombinant linear DNA that came from cleaving a plasmid in which the two ends of the fragment are homologous to the regions flanking the targeted gene, and it facilitated replacement of the targeted gene with the selectable marker (Hastings et al., 1993). When the gene targeting was studied in more detail, it was found out that stable integrations in targeted site is significantly deficient in *rad52* mutant strains, and Schiestl concluded that the mechanism of

ends-out gene targeting in yeast should require different recombination machinery other than simple gene conversion accompanied by crossing over (1994).

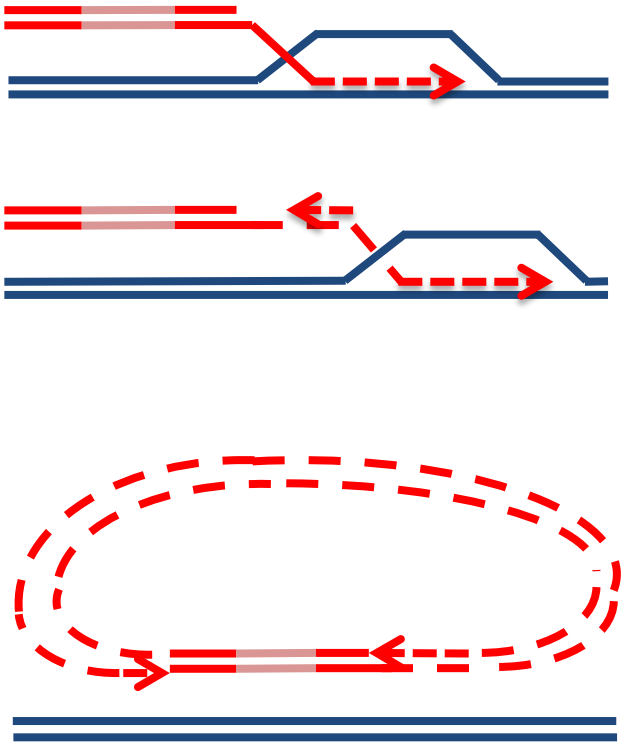

Figure 1. Hypothesis on BIR-dependent hit-and-run event. dsDNA fragment with a marker would have strand invasion and new DNA synthesis via BIR-like mechanism, and by copying nearby ARS followed by ligation at two ends would result in formation of the circular dsDNA fragment that could be retained.

When Kraus observed "hit-and-run" events (formation of circular DNA fragment during intended gene targeting), he concluded that it appears to be Break-induced replication-dependent that can have extended DNA synthesis (2001). Break-induced replication (BIR) is a nonreciprocal recombination-dependent replication mechanism that is facilitated when only one end of the DSB shares homology with a template, and it is used to establish a unidirectional replication fork that can copy the homologous template to the end of the chromosome (McEachern and Haber, 2006). BIR can repair double strand breaks caused by replication fork collapse or by the erosion of telomere. Only one of the broken ends is used to invade the neighboring homologous strand to replicate the repair the break, and because of this, heterozygosity gets lost from double strand break repair by BIR. Unlike in Gene Conversion, Rad51-dependent BIR requires the nonessential subunit of Polδ, Pol32, to initiate new BIR (Lydeard et al. 2010). BIR also can give new, elongated telomere to a broken chromosome end as proposed by Dunn et al. (1984), and this is seen in transformed mammalian cells and even in human cancer cells (Lydeard et al. 2007).

Other than its relevance to BIR, “hit-and-run” circular DNA sequences from gene targeting transformations should contain autonomously replicating sequence. It is known that DNA require autonomously replicating sequence, or ARS, in order for it to be retained since it contains origin of replication in the yeast genome (Chan & Tye, 1980). Without the ARS, the number of the gene in the DNA would stay the same while the number of cells increase exponentially since the genomic sequence in the nucleus cannot be replicated, and the phenotype would not be seen. Therefore, it is critical that any piece of DNA, including plasmids or a circular DNA fragments, contains ARS to be propagated.

It is also known that for linear DNA fragments to become circular, it would require DNA ligase, possibly DNA ligase IV which is used in non-homologous end joining, and illegitimate integration into random chromosomal locus may also be the result of non-homologous end joining. Non-homologous end joining (NHEJ) is another major DSB repair mechanism along with homologous recombination where the broken ends ligate without the regions of sequence similarity (homology) (Lieber et al., 2003). Even though it is possible for NHEJ to utilize short homologous DNA sequences called microhomologies to perform accurate repair, usually, NHEJ results in loss of nucleotides with imprecise repair (Moore & Haber, 1996).

In order to study about the "hit-and-run" events, gene targeting in *Saccharomyces cerevisiae* was performed, using *ade2::URA3* targeting cassette fragment to delete the wild type *ADE2* gene. There are three possible outcomes from the gene targeting: 1) crossover after homologous strand invasion resulting in stable integration of the targeting cassette into the ADE2 sequence; 2) non-crossover after the strand invasion and ligation at the ends of the cassette resulting in formation of the circular DNA fragment, “hit-and-run”; 3) illegitimate integration resulting in the integration of the cassette into random chromosomal locus.

Materials and Methods

Yeast Strains

The *S. cerevisiae* strain BY4741 was used in this study (Brachmann et al., 1998). It is *MATa* haploid which contains complete deletions of *his3*, *leu2*, *met15*, and *ura3*. In complete deletion of *URA3*, only the ORF was deleted leaving promoter and terminator regions intact. By using BY4743, a diploid of BY4741, with *pol32::KAN* from Research Genetics (Record #36841, YJR043C), *MATa* haploid with *pol32Δ* deletion was derived by sporulation. Random sporulation was used to screen for *MATa* phenotype, and the correct integration was confirmed by PCR flanking the target area and internal KAN sequence.

Generation of the targeting cassettes

Three kinds of targeting cassettes were constructed to target *ADE2* locus with *URA3* marker. PCR amplification was used with A207 strain, which contained wild-type URA3, as a template. The 50bp *ade2*::*URA3* targeting cassette was made with mixed primers containing 50bp of homology to *ADE2* and 20bp of the *URA3* promoter and terminator sequences which are 227bp and 4bp away from *URA3* ORF, respectively. 500bp targeting cassette was created from using one of the stable integrants, resulted from transformation using 50bp *ade2::URA3* targeting cassette, with primers which had homology to ADE2 locus 500bp away from inserted *URA3* marker. Another 50bp targeting cassette was constructed by using the same 5' end primer from the first 50bp targeting cassette but using 3' end primer which targeted only 23bp away from ARS1516. The first 50bp targeting cassette was 568bp away from the ARS1516.

Transformation

The procedure for transformation of PCR product followed that of Gietz and Schiestl (1995). The targeting cassettes created by PCR were run on a 1% agarose gel with markers with

known molecular weight. The markers were used as standards to quantitate the concentration of PCR products, and 10ng of targeting cassette was used per transformation.

Determination of integration site usage and targeting cassette circularization

Transformants were selected on synthetic complete –URA plates. The colonies that grew on URA plates were patched and then replica plated on an FOA plate, in order to distinguish the colonies that contained the circularized targeting cassette, which can be lost and permit grow on 5-FOA containing media. In addition, the isolates were replica plated on to synthetic complete – ADE plates to determine disruption of the *ADE2* locus.

Results

Gene targeting using the ade2::URA3 targeting cassette results in multiple outcomes

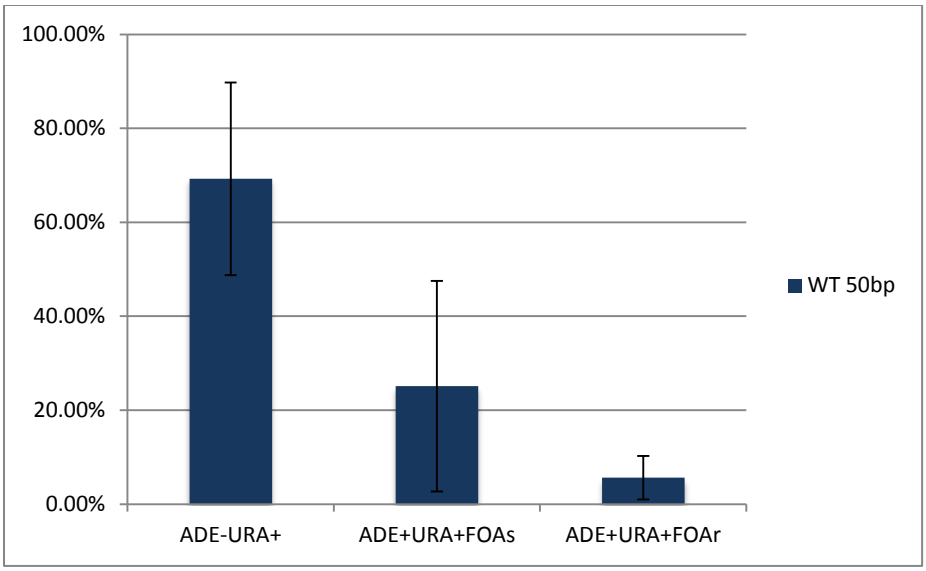

Figure 2. Targeting site preference for WT using 50bp targeting cassette. The percentages were calculated by summing the total number of isolates per experiment and dividing each category (ADE^-URA^+, $ADE^+URA^+FOA^s$, and $ADE^+URA^+FOA^r$) by total number of isolates. Total of 446 isolates were screened.

First, in order to see the targeting site preference, BY4741, WT strain, was transformed with *ade2::URA3* targeting cassette. The targeting cassette was constructed with 50bp of homology to *ADE2* on each side of the *URA3* ORF. When gene targeting transformation was performed using the 50bp *ade2::URA3* PCR product, three different results were observed: (1) out of 446 isolates, 309 isolates (69%) were stable ADE^-URA^+ transformants that represent normal successful gene targeting; (2) 112 isolates (25%) were ADE^+URA^+ transformants where the *URA3* sequences apparently integrated at another location by illegitimate integration and (3) 25 isolates (6%) were ADE^+URA^+ transformants in which the *URA3* marker could be easily lost by selection on 5-FOA, which is the result expected for a "hit-and run" event. Results from five experiments were combined, and error bar was inserted using standard deviation. The error bars are large because in very first transformation I had about 29% of stable legitimate integration and

67% of illegitimate integrations. Transformations showed over 55% of stable legitimate integration ever since.

Increasing homology length increases targeting to ADE2 locus, but doesn't alter frequency of hit-and-run events

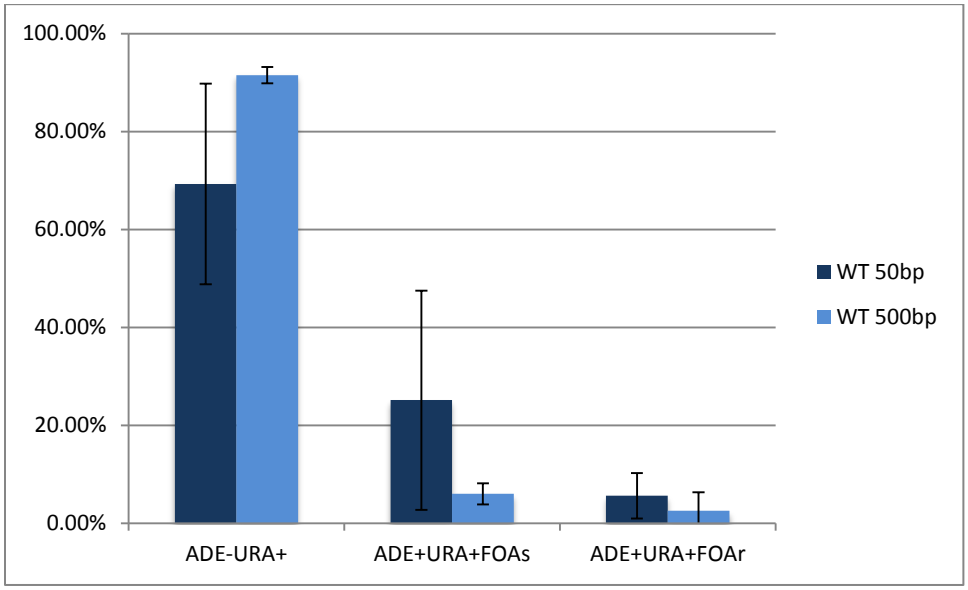

Figure 3. Targeting site preference in WT using 50bp and 500bp targeting cassette. The result from gene targeting using 500bp targeting cassette was graphed next to the result using 50bp targeting cassette for comparison.

In order to see how the increase in homology length of the targeting cassette affects the integration and formation of the circle, different *ade2::URA3* targeting cassettes, using 500bp of homology to *ADE2*, was constructed and was transformed in WT strain. Using the 500bp cassette, 183 isolates out of 200 (91.5%) were stable $ADE^{-}URA^{+}$ integrants, 12 out of 200 isolates (6%) were stable but illegitimate $ADE^{+}URA^{+}$ FOA-sensitive transformants, and 5 out of 200 isolates (2.5%) were hit-and-run $ADE^{+}URA^{+}$FOA-resistant transformants. Comparing results from two transformations, using 50bp and 500bp targeting cassette, the stable integration increased from 69% using 50bp of homology to 91% using 500bp of homology; illegitimate integration decreased from 25% to 6%; and hit-and-run event also decreased from 6% to 3%.

The increase in stable integration in correct locus, *ADE2*, was expected from previous studies (Coic et al., 2011). However, the increase in correct targeting on *ADE2* locus did not have an effect on formation of the circles.

Targeting site preference in WT and *pol32Δ* strains

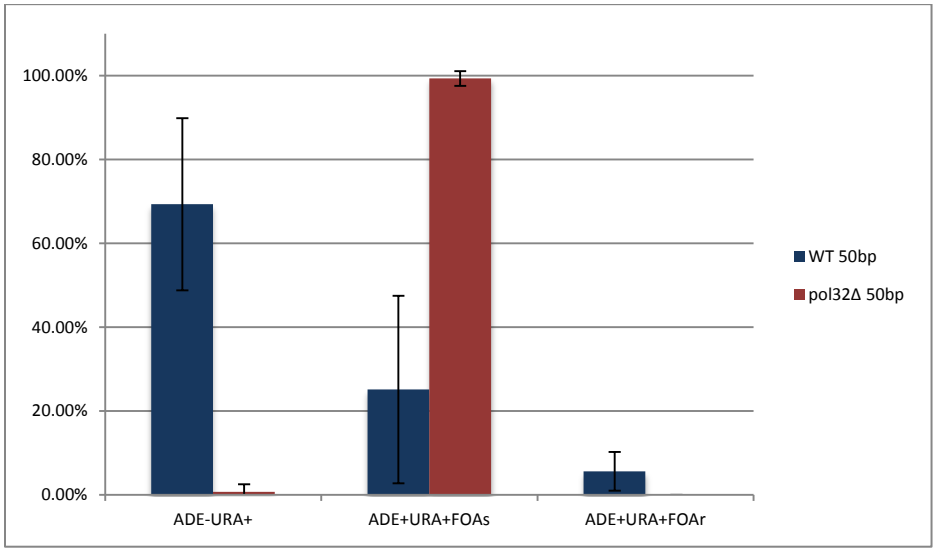

Figure 4. Targeting site preference with WT and *pol32Δ* mutant using 50bp targeting cassette.

One of the assumptions provided by previous study (Kraus et al., 2001) was that this "hit-and-run" event may be BIR-dependent. To confirm this assumption, *POL32*, a gene required for BIR, was deleted from WT and same gene targeting transformation using the 50bp *ade2::URA3* targeting cassette was performed. Total of 141 isolates were screened: 1 isolate (1%) was stable $ADE^{-}URA^{+}$ transformants; 140 isolates (99%) were illegitimate $ADE^{+}URA^{+}$ FOA-sensitive transformants; and none of the transformants was $ADE^{+}URA^{+}$ FOA-resistant, the hit-and-run event. Compared to WT, targeting in *pol32Δ* strain resulted in decreased usage of legitimate locus, significant increase in illegitimate integration, and no formation of circles.

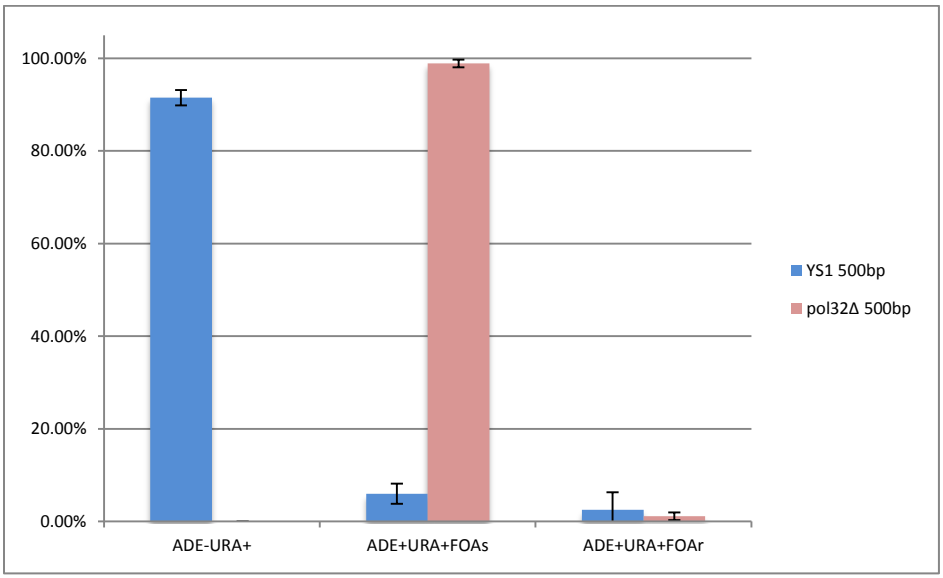

Figure 5. Targeting site preference with WT and *pol32Δ* mutant using 500bp targeting cassette.

In order to see whether increasing homology length would have same effect on *pol32Δ* as in WT, I performed transformation with *pol32Δ* using 500bp targeting cassette. Comparing the WT and *pol32Δ*, it seemed increasing homology length increased correct targeting in WT but not in *pol32Δ*. In *pol32Δ*, there was not much difference between results from 50bp and 500bp targeting cassettes: there was no stable integrants; 359 out of 363 isolates (99%) were illegitimate integration, which is similar as in 50bp; and 4 out of 363 isolates (1%) was hit-and-run event. In WT, there was significant difference in targeting site preference between 50bp and 500bp more accurately directing the targeting cassette with longer homology to *ADE2* locus; however, there was no difference in *pol32Δ* between 50bp and 500bp targeting cassette. Unlike the results using 50bp targeting cassette, gene targeting using 500bp targeting cassette in *pol32Δ* showed 4 isolates (1%) of hit-and-run event.

Increased proximity to ARS1516 does not significantly increase the rate of hit-and-run events

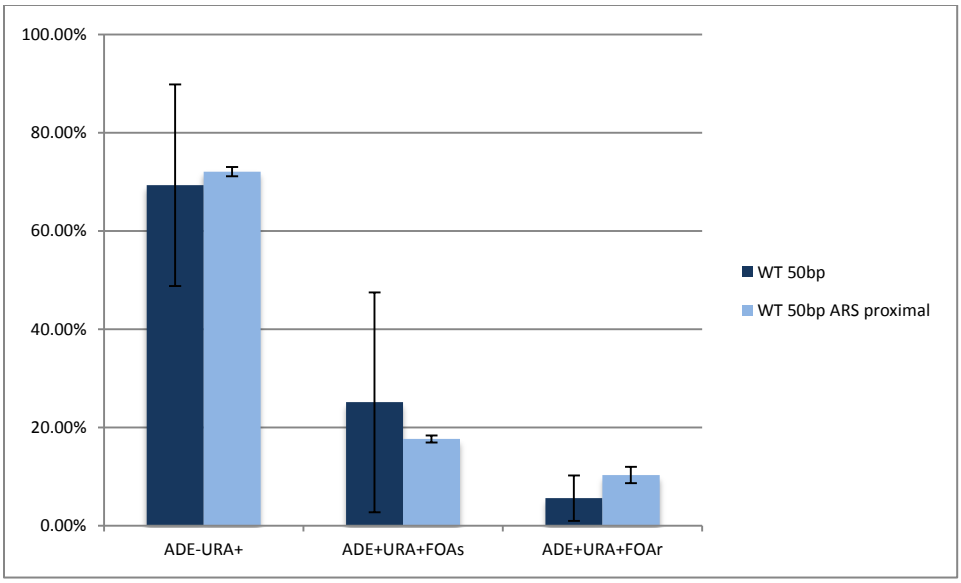

Figure 6. Targeting site preference in WT using 50bp and 50bp proximal to ARS targeting cassette. The result from gene targeting using 50bp ARS proximal targeting cassette was graphed next to the result using 50bp targeting cassette for comparison.

Another targeting cassette was constructed to see if the proximity to ARS would affect the formation of the circles. The targeting cassette also had 50bp or homology to *ADE2* to control the effect of homology length on gene targeting, but the upstream homology was located only 23bp from ARS1516; whereas the first 50bp *ade2::URA3* targeting cassette targeted 568bp from ARS1516. Transformation was performed using this ARS1516 proximal targeting cassette, and in WT, 49 out of 68 isolates (72%) were stable integrants, 12 isolates (18%) were illegitimate integrants, and 7 isolates (10%) were hit-and-run events. The percentage was not significantly different than using 50bp targeting cassette 568bp from ARS1516.

The 50bp targeting cassette proximal to ARS1516 was also transformed in *pol32Δ* strain, and it again showed over 90% of illegitimate integration with no accurate stable integration and

5% of hit-and-run events. Compared to the result with 50bp targeting cassette, 50bp ARS proximal targeting cassette showed increase in hit-and-run event (0% versus 5%, respectively).

Examination of Circularized Recombination Using Inverse PCR

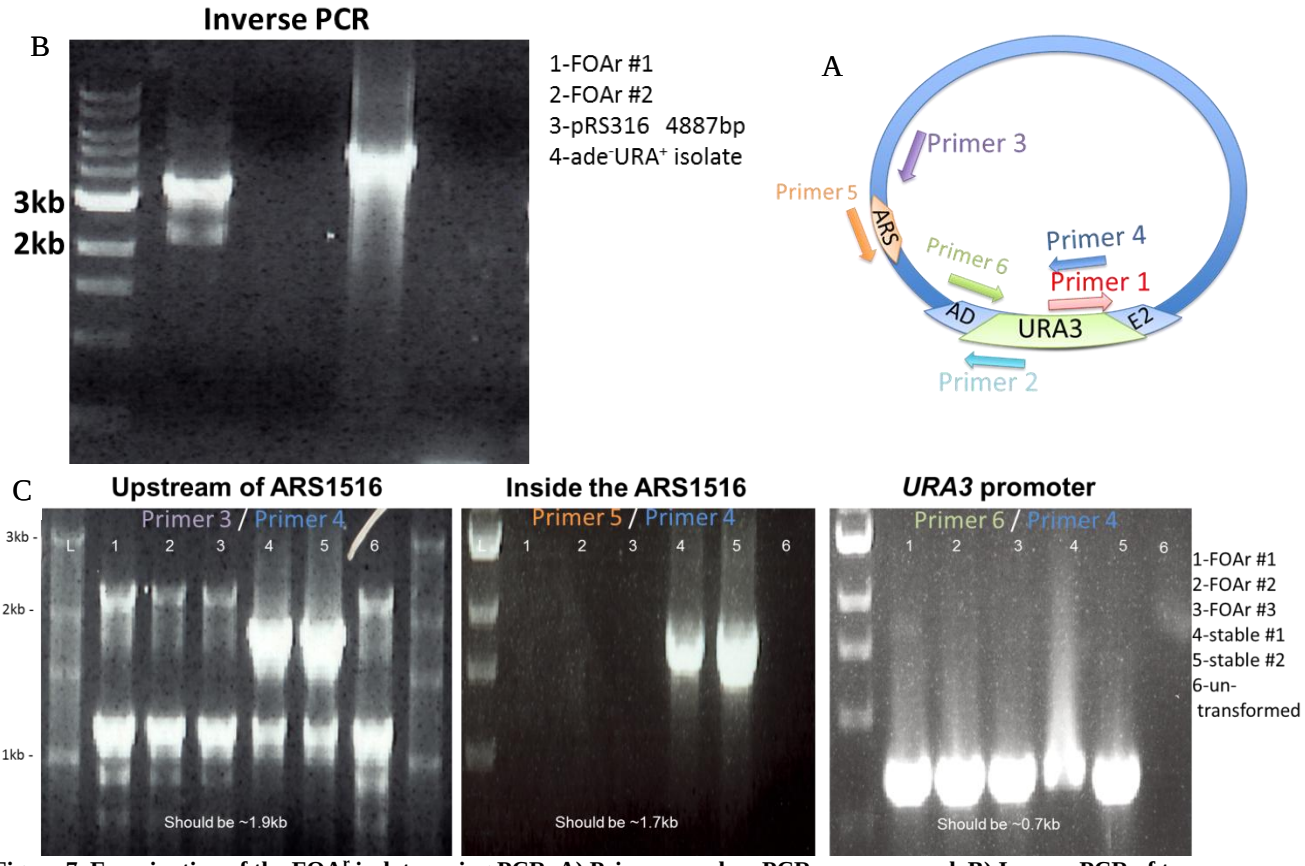

Figure 7. Examination of the FOA^r isolates using PCR. A) Primers used on PCR were mapped. B) Inverse PCR of two FOA^r isolates, *URA3* plasmid, and an ADE$^-$URA$^+$ isolate using Primer 1 and Primer 2. C) Confirming PCR for ARS1516 usage with three sets of primers.

In order to figuring out the size of the circles, I performed inverse PCR on two of the FOA-resistant, circularized isolates (#1 and #2) using a *URA3* plasmid (pRS316) and a stable integrant (*ade2::URA3)* as positive and negative controls. Inverse PCR using Primer 1 and Primer 2 showed correct size band in the positive control (between 4 and 5kb), and it also showed a ~3.5kb band in one of the FOA^r isolates, FOAr #1. Since there is distance between Primer 1 and Primer 2, about 0.7kb, the actual size of the circle in FOAr #1 is around 4.2kb.

With positive control showing band that is almost a limit for Taq DNA polymerase used in lab, FOAr #2 would likely to have size larger than 5kb, and it is not showing up on PCR due to its size.

Examining the usage of ARS1516 using PCR

In Figure 7C, I also performed set of PCRs to check if the circles actually contained ARS1516. Using an anti-sense primer that is located inside *URA3* ORF, I paired with a primer which is located upstream of ARS1516, a primer located inside the ARS, and a primer located in *URA3* promoter region. Three out of 25 circularized isolates were tested, and surprisingly, I could not see any band from the circles when I used upstream of ARS1516 sense primer with anti-sense *URA3* internal primer and when I used internal ARS1516 primer with internal *URA3* primer, meaning that the circles did not contain ARS1516. I have not tested circularized isolates from 500bp or 50bp ARS proximal targeting cassettes to see the usage of ARS1516.

Discussion

Our initial model for *ade2::URA3* gene targeting included three possible outcomes. First, it could strand invade in *ADE2* locus and after crossing over, the targeting cassette would stably integrate within the targeted area in *ADE2* locus. Second, it could integrate at an alternate locus by strand invasion or by another mechanism. Third, it could strand invade in *ADE2* locus, copying nearby ARS1516, not going through crossover by BIR-like mechanism, and form exogenous autonomously replicating circular dsDNA.

We studied these possible gene targeting outcomes by using three different *ade2::URA3* targeting cassettes to perform transformation on BY4741. The three outcomes included stable legitimate integration in *ADE2* locus replacing *ADE2* gene with *URA3*, illegitimate integration of *URA3* in unknown locus, and hit-and-run event forming circularized dsDNA containing *URA3*. First, stable legitimate integration was the result we would expect for regular gene targeting replacing *ADE2* gene with *URA3* marker. The majority of the isolates (69%) were stable legitimate integrations; however 1 out of 4 was unexpected, illegitimate integration (Figure 2).

It is unknown yet what the origin of the illegitimate integration is and further research should examine where it is integrating. It could be result of completely random integration in different loci, or it could be using its internal homology to *URA3* promoter region and direct *URA3* containing targeting cassettes back to *URA3* locus. When BY4741 was constructed, the *URA3* ORF was completely deleted, but this deletion left the promoter and terminator regions intact (Brachmann et al., 1998). In order to test the usage of *URA3* locus, I could use PCR primers around *URA3* locus where the ORF was or use Southern blot with *URA3* proximal probe on *URA3* locus. Another way I could test the usage would be to delete promoter region in WT and perform gene targeting on the mutant.

Another unexpected result from gene targeting was the formation of an unstable circular dsDNA by hit-and-run event with 6% of probability using 50bp targeting cassette. The isolates that formed circle were examined further using inverse PCR (Fig. 7B). The result showed that they varied in size, from 4.2 kb to larger than 5kb, and this result coincided with previous research by Kraus et al., in which the Southern blot examination was done on circles and showed that circles are highly heterogeneous in terms of size and copy number (2001). Also I tried to examine its source by checking to see if the circularized dsDNA contained ARS1516, since our initial assumption was that hit-and-run event happens in *ADE2* locus utilizing ARS1516 as its source of replication origin (Fig. 7C). However, several PCR results showed that the assumption is not entirely correct. When three of the FOA^r isolates were tested, PCR result showed that the circles did not contain ARS1516. This result was surprising since Kraus wrote that 2 out of 13 circular isolates contained ARS1516, and he also used PCR to confirm (2001). Since the circularized dsDNA fragment has to contain some ARS sequence in order for it to be retained, it seems that some circles are formed through another locus utilizing another ARS sequence, while some copied ARS1516 in *ADE2* locus. In order to completely understand the circles, further research should be conducted to assay all of the circles with Southern blot using *URA3* probe and sequencing of the circularized dsDNA fragment.

There were two other targeting cassettes that were used in this experiment to study three outcomes of gene targeting. When 500bp targeting cassette was used, the expected result was that increased homology would increase the accuracy of the gene targeting to correct locus, *ADE2*, as shown in Coic et al. (2011), and that was exactly what we found. The usage of *ADE2* locus increased and the usage of unknown locus by illegitimate integration and formation of the circles decreased, compared to 50bp (Fig. 3). When another targeting cassette, 50bp proximal to

ARS1516, was used to examine how it would affect gene targeting and hit-and-run event, the result was not much different from using the first 50bp targeting cassette (Figure 6). This result was comprehensible from the examination of the circles, for circles might or might not utilize ARS1516 as its replication origin. Even though there was slight increase in formation of circles, it was not significant; indicating that proximity to ARS1516 does not affect the formation of the circles.

Another assumption we had was that hit-and-run events are dependent on BIR. In order to test this, *pol32Δ* strain was made, and the same gene targeting transformation using three different targeting cassettes were used. When gene targeting with 50bp targeting cassette was performed on *pol32Δ* and when compared to WT, the result showed only 1% of stable legitimate integration, significant increase in illegitimate integration (99%) compared to WT, and no hit-and-run events (Figure 4). From our assumption we expected to see no circularized isolates, however the sudden increase in illegitimate integration was unexpected. Again, as in WT, further research should be conducted with Southern blot, PCR, or by deleting *URA3* promoter region in order to study the where the illegitimate integration happens. From the result of 50bp targeting cassette on *pol32Δ*, at first it seemed that hit-and-run events were dependent on *POL32* since there was no circularized isolates; however, gene targeting with different targeting cassettes had different results actually showing FOA-resistant isolates.

When 500bp of homology was used, it was surprising to see the percentage for illegitimate integration was still around 99% with no stable legitimate integration but 1% of hit-and-run event (Figure 5). This result was very different than the results from WT, where there was significant increase in accurate stable integration in *ADE2* locus. While previous study by Coic et al. in 2011 and gene targeting on WT in this study showed that increasing homology

length directs the targeting cassette in more accurate targeting to correct locus, increasing homology in *pol32Δ* had no significant difference in accurate targeting between 50bp and 500bp targeting cassette. In terms of hit-and-run event, even though it was only from 0% to 1%, there was formation of circles when 500bp targeting cassettes used meaning that the formation of the circularized dsDNA during gene targeting is not dependent on *POL32*.

Gene targeting transformation with 50bp ARS proximal targeting cassette also showed similar result to 500bp with over 95% of illegitimate integration, no stable legitimate integration, and some (~4%) hit-and-run event. By the percentage of illegitimate integration using three different targeting cassettes in *pol32Δ* and comparing the result to WT, it seems that *POL32* is required for targeting to *ADE2* in this context; however I cannot exclude the possibility that *POL32* is somehow facilitating usage of the 50bp outer homology, and in the absence of *POL32*, the inner *URA3* usage is preferred.

While I see no circles using my original 50bp targeting cassette in a *pol32Δ*, I do see between 1-5%, respectively using the 500bp or 50bp ARS targeting cassettes. Therefore, it seems that in these hit-and-run events, it is possible that *POL32* and BIR is only partially responsible for these circles, or that the increased proximity to ARS1516 is enough to promote circle formation in the absence of *POL32*. To test the hypothesis that ARS1516 is being used to make the circles I see with the 500bp and 50bp ARS targeting cassettes, I will need to check for the presence of ARS1516 by either my PCR assay or using a Southern blot.

Even though our initial assumption about hit-and-run event being BIR dependent event was not true since there was formation of the circles in *pol32Δ*, by the lowered percentage of hit-and-run event of *pol32Δ* compared to WT, it is possible that *POL32* is partially responsible for the formation of the circles. Further research should be done, however, on characteristics of the

circles and on another mutant that disrupts BIR, in order to conclude BIR dependency or *POL32* dependency of hit-and-run events.

After collecting and examining all the data, our initial model for *ade2::URA3* gene targeting changed. It still includes three possible outcomes: first, it could successfully target *ADE2*, strand invade in *ADE2* locus and after crossing over, replace *ADE2* with the targeting cassette in *ADE2* locus; second, it could use its inner homology to target *URA3* promoter region, strand invade in *URA3* locus and after crossing over integrate the targeting cassette into *URA3* locus; third, it could go through hit-and-run event by possibly strand invading *ADE2* locus to copy ARS1516 or strand invading in *URA3* locus to find another autonomously replicating sequence in order to be retained. I plan on testing whether the stable, but illegitimate integrants use the *URA3* homology as well as whether the circular isolates that do not use ARS1516 use the region flanking the *URA3* locus to begin to test my new model. These results will provide new insights into the mechanism of hit-and-run events and the usage of BIR in gene targeting.

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