ADAPTATION OF CRISPR/CAS9 TO IMPROVE THE EXPERIMENTAL UTILITY OF THE MODEL SYSTEM *SCHIZOSACCHAROMYCES POMBE*

By

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ABSTRACT OF THE THESIS

ADAPTATION OF CRISPR/CAS9 TO IMPROVE THE EXPERIMENTAL UTILITY OF THE MODEL SYSTEM *SCHIZOSACCHAROMYCES POMBE*

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Thesis Director:

Dr. Mikel Zaratiegui

Schizosaccharomyces pombe is a model organism that is utilized in several areas of research including DNA and chromatin biology. However, methods for genomic manipulation of the organism are outdated and cumbersome. The CRISPR/Cas9 toolset was recently developed from the prokaryotic type II CRISPR-associated system (a form of adaptive immunity) and has been shown to be successful at site-specific mutagenesis and modulation of gene expression in multiple eukaryotic organisms. We sought to modify the two components of this instrument, Cas9 and short-guide RNA (sgRNA), for effective use in *S. pombe*. As proof of concept, the *ade6* gene was targeted for CRISPR/Cas9 mutagenesis by employing two constructs: one with an *adh1* expressed Cas9 and one with sgRNA expressed by *rrk1*, the promoter for K RNA, and precisely terminated by a Hammerhead ribozyme. The *ade6* gene, a long terminal repeat (LTR) sequence of the

retrotransposons Tf1 and Tf2, and TER1 were also targeted by the *rrk1* sgRNA and the catalytically null Cas9 (dCas) for site-specific enrichment of DNA sequence through chromatin immunoprecipitation. We found some Cas9 expressing vectors were inactivated by random mutation and studied this phenomenon using the inducible nmt1 promoter (no mRNA transcription in thiamine). Cultures with either nmt1:Cas9 or nmt1:dCas9 repressed by thiamine grew 4.4 to 7 times (p-value= 2.1×10^{-15} and 2.2×10^{-16}, respectively) faster than those with full-expression. Toxicity was not attributable to the catalytic activity of Cas9 and further investigation is required. Cas9 and sgRNA were combined into a single expression vector to reduce exposure to inactivating mutations and yielded mutagenesis efficiencies of 86-92%. Also, by reducing expression of nmt1:dCas9 in thiamine, we achieved 50% to 300% enrichment of genomic target sites. The described *rrk1* expressed sgRNA, with Cas9 and dCas9, will enable more efficient genomic manipulation of *S. pombe*.

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Results and corresponding experiments from sections 3.1 through 3.4 have previously been reported in: Jacobs JZ, Ciccaglione KM, Tournier V, Zaratiegui M. 2014. Implementation of the CRISPR-Cas9 system in fission yeast. Nature Communications 5: 5344.

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1. Introduction

1.1 *Schizosaccharomyces pombe* as a model organism

S. pombe has been utilized as the model biological system for various cellular phenomena since the 1950s. Fission yeast are a single-celled archiascomycete fungus which were first isolated from East African millet beer in the 1890s. They are most closely related to the budding yeast, *Saccharomyces cerevisiae*, but significant divergence has occurred since their split from a common ancestor 1000 million years ago (Heckman et al., 2001, Hedges, 2002). *S. pombe* have compact chromosomes and share conserved cellular processes with metazoans, such as factors involved in chromatin biology and RNA interference (Wood et al., 2002). Their ease of use in the laboratory makes them an ideal model organism and enables researchers to explore biological processes of higher eukaryotes. Experiments with fission yeast have been performed in the fields of cell cycle control (Gutierrez-Escribano and Nurse, 2015), mitosis (Tay et al., 2013), meiosis (reviewed in Lam and Keeney, 2015), DNA damage repair (Wang et al., 2015), and chromatin biology (Kuscu et al., 2014). However, new genetic tools exist which may expedite and simplify these investigations into *S. pombe*.

1.2 Discovery and function of CRISPR-associated systems

CRISPRs (clustered regularly interspaced short palindromic repeats) were first discovered in *Escherichia coli* K12 and classified as a family of short repeat sequences with unknown function (Ishino et al., 1987, Nakata et al., 1989). Subsequent research found that these sequences were common and found in 40% of bacteria and 90% of archaea (Makarova et al., 2011). Using *in silico* analysis, researchers also identified various CRISPR-associated (Cas) genes which were only located near CRISPR sequences (Jansen et al., 2002). Indeed, both Cas genes and CRISPRs were later discovered to interact (Mojica et al., 2009) and be necessary to provide resistance to viruses (Barrangou et al., 2007) and plasmid DNA (Marraffini et al., 2008). CRISPRs and Cas genes are now understood to serve as a rudimentary immune system for prokaryotes.

Immunity is achieved through the activity of the CRISPR/Cas system within a cell. Depending on the organism, one of three CRISPR-associated systems is used, but they follow a similar pathway (Figure 1; see review by Gasiunas et al., 2014 or Sorek et al., 2013). The acquisition stage starts when foreign DNA is introduced into the cell and cleaved by general nucleases. The nucleases Cas1 and Cas2, among other Cas proteins, digest the DNA into small segments called protospacers. These protospacers are then introduced into the CRISPR array and bordered by repeat sequence; they are now spacers. Protospacer sequences are selected by the Cas proteins only if a short and specific nucleotide sequence – otherwise known as a protospacer adjacent motif (PAM) – is directly next to the sequence. Then, the expression stage occurs when the CRISPR array of spacers is transcribed into pre-CRISPR RNA (pre-crRNA) and the pre-crRNA is processed to become CRISPR RNA (crRNA) or guide RNA (gRNA). Lastly, the interference stage begins when crRNAs become bound by a complex of Cas proteins and base pair to DNA sequences with homology, or near homology, to the spacer sequence and have a PAM. Subsequently, nucleases within the complex initiate cleavage and create a double-strand break (DSB), thereby exerting the cell’s immune response. The CRISPR/Cas system is elegant and improves recognition and degradation of foreign DNA in many prokaryotic organisms.

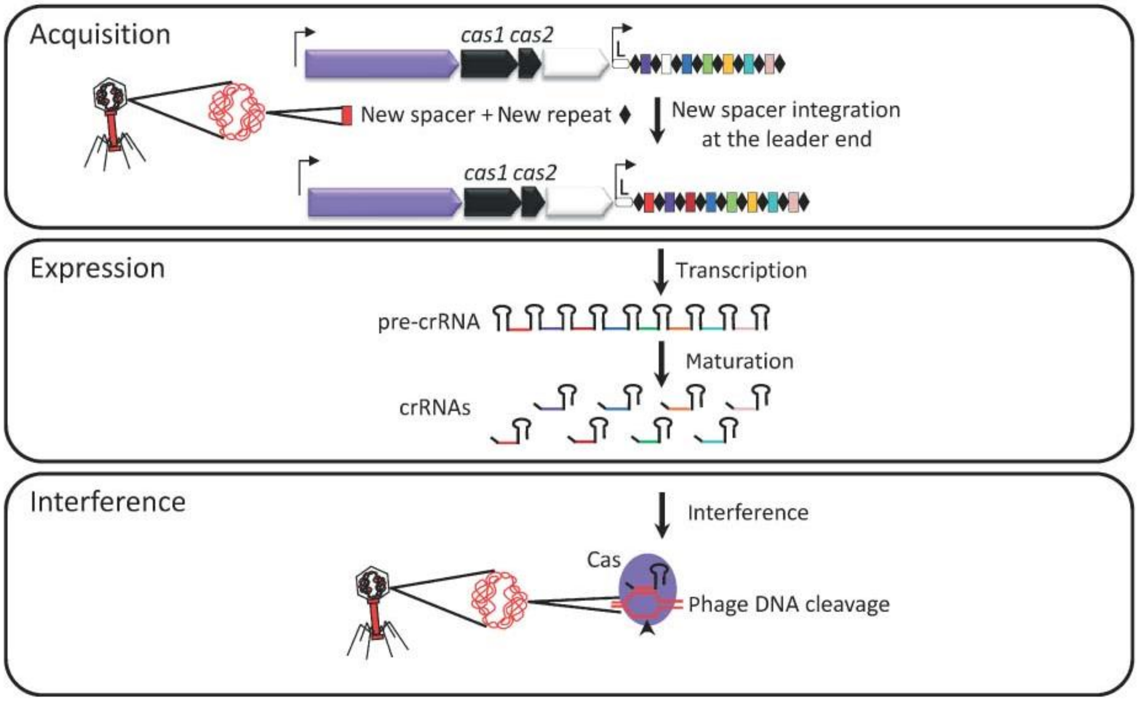


Figure 1. Illustration of the three stages of CRISPR-based immunity. Acquisition: a new spacer from exogenous DNA is incorporated into the CRISPR locus. Expression: spacers are expressed and processed into crRNAs. Interference: mature crRNAs are bound by Cas proteins and create DSBs in recognized DNA sequences. Adapted from Barrangou, Copyright (2013), with permission from John Wiley & Sons.

1.3 Usage of type II CRISPR-associated systems in eukaryotic organisms

CRISPR-associated systems serve as a defense against invading DNA molecules in native prokaryotic organisms, however, a subset of components have been identified that are now used transgenically for genome editing purposes (see review by Barrangou, 2015). In contrast with other systems, expression of the type II system, as in *Streptococcus pyogenes*, occurs with the separate production of pre-crRNA and trans-activating crRNA (tracrRNA; figure 2). These RNAs hybridize to form dsRNA which is recognized by RNase III and cleaved. The RNA-guided endonuclease Cas9 complexes with the mature crRNA and carries out interference through recognition and cleavage by the HNH nuclease domain and a RuvC-like domain of Cas9 (Jinek et al., 2012). Jinek et al. (2012) describe the distillation of this process by combining crRNA and tracrRNA into a chimeric single-guide RNA (sgRNA). Together with Cas9, the sgRNA can be programmed to target any dsDNA with a PAM site and generate a DSB. DSBs are highly deleterious to the cell (Kasparek and Humphrey, 2011) and can be resolved through homologous recombination (HR) or the error prone non-homologous end joining (NHEJ). Exploiting the cells preference for HR, studies in bacteria (Jiang et al., 2013), mouse (Wang et al., 2013), human cells (Cho et al., 2013, Cong et al., 2013, Mali et al., 2013, Jinek et al., 2013), zebrafish (Chang et al., 2013, Hwang et al., 2013), and *S. cerevisiae* (DiCarlo et al., 2013) have introduced homologous sequences that contain specific mutations to successfully introduce these mutations into the genome (figure 3). Thus, we seek to implement this programmable sgRNA, catalytic Cas9, and other Cas9 tools for efficient genome editing and examination of *S. pombe*.

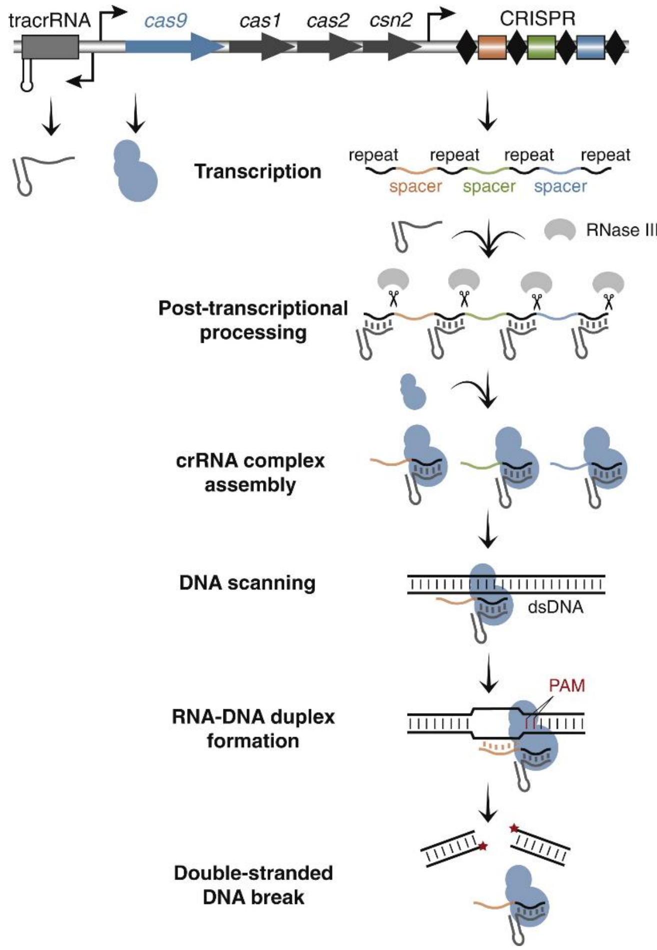


Figure 2. Schematic of a *S. pyogenes* type II CRISPR-associated system. Encompasses the expression phase through the interference phase. Reprinted from Qi et al., Copyright (2013) with permission from Elsevier.

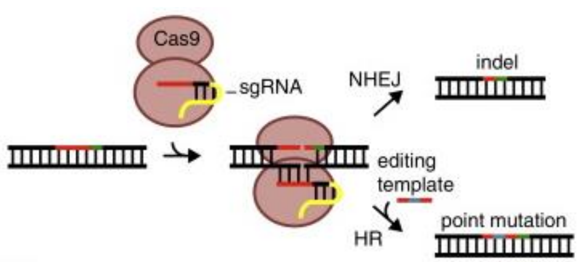


Figure 3. Resolution of Cas9 generated double-strand breaks. Homologous recombination, with an editing template, results in the incorporation of specific mutation. Non-homologous end joining results in random mutation. Adapted from Charpentier and Marraffini, Copyright (2014), with permission from Elsevier.

2. Materials and Methods

2.1 Constructs

Select plasmids are available from the Addgene repository (http://www.addgene.org) and all plasmids are organized in Table 1. All oligonucleotide information can be found in Table 2. All amplification reactions were performed using high fidelity Phusion DNA polymerase (NEB) in the supplied 1xHF buffer. Gibson assembly (Gibson et al., 2009) was used to produce the Cas9 expression plasmid pMZ222 by combining two Cas9 fragments amplified from plasmid p414-TEF1p-Cas9-CYC1t (Addgene; oM447/oM448 and oM446/oM449; served to introduce a silent mutation into the CspCI site) with plasmid pART1 (McLeod et al., 1987) marked with *S. cerevisiae LEU2* and digested with PstI. The *adh1* promoter was replaced with nmt1 by following the Gibson assembly procedure to combine pART1 (digested by BamHI/SphI), Cas9 fragment (oM777/oM778), and nmt1 promoter (oM801/oM802), which generated pMZ373. A catalytically inactive Cas9 (dCas9) expression vector was synthesized by Gibson assembly of pART1 (digested by SmaI), a dCas9 fragment amplified from plasmid pTDH3-dCas9 (Addgene #46920; oM729/oM721), and a synthetic double-stranded DNA containing a 3x HA tag and end processing signal, this became pMZ382. The dCas9 vector was further modified by removing the *adh1* promoter via digestion with SphI and NcoI and the addition of nmt1 amplified from pMZ293 (oM905/oM906) or nmt41 amplified from pMZ294 (oM905/oM906) through Gibson assembly resulting in pMZ455 and pMZ456, respectively. Gibson assembly was used to build a sgRNA expression vector by ligating a synthetic double-stranded DNA containing the sgRNA, the CspCI placeholder, and *rrk1* promoter and leader RNA sequences (Figure 4) into plasmid fragment pUR19 (Barbet et al., 1992; OM473/OM474), marked with *ura4*, to produce pMZ252. A Hammerhead ribozyme sequence was amplified from the satellite RNA of Tobacco Ringspot Virus using oM552 and oM553 and inserted into the pMZ252 fragment (oM550/oM551) directly 3' of the sgRNA by Gibson assembly, resulting in pMZ283. All sgRNA plasmids were assembled by digesting pMZ283 with CspCI and ligating phosphorylated double-stranded oligonucleotides with specific target sequences (oM450/oM456 (*ade6-wt*); oM452/oM457 (*ade6-M210*); oM454/oM458 (*ade6-L469*); oM1002/oM1003 (scrambled LTR sequence); oM1004/oM1005 (scrambled TER1 sequence); oM1006/oM1007 (LTR consensus sequence); oM1008/oM1009 (TER1 sequence)) using T4 ligase (NEB), generating pMZ284, pMZ285, pMZ286, pMZ471, pMZ472, pMZ473, and pMZ474, respectively. A single vector containing Cas9 and sgRNA sequences was built by amplifying the Cas9 sequence from pMZ222 (oM554/oM555) and using Gibson assembly to insert it into pMZ283 digested by ZraI, yielding pMZ374. Single plasmids with specific target sites were generated by following the same procedure as pMZ283, but with pMZ374 to produce pMZ288 (*ade6-wt*), pMZ289 (*ade6-M210*), pMZ381 (*ade6-L469*), pMZ453 (oM566/oM567; *clr4-y451*), pMZ395 (oM572/oM573; *set1-y810*), and pMZ394 (oM602/oM603; *set1-y897*).

The DNA provided as an HR donor template was produced by PCR amplification of *reb1* gene as control (oM100/oM101) and *ade6-wt* and *ade6-M210* as targets (oM12/oM13). Additional templates were generated by mutagenesis PCR with oM564/oM563 and oM565/oM562 (*clr4-Y451A*), with oM570/oM569 and oM571/oM568 (*set1-Y810A*), and with oM606/oM605 and oM607/oM604 (*set1-Y897A*; the former primer of each pair being the outside primer for each mutagenesis). PCR products were purified with silica membrane columns (Epoch Life Science) and used directly for transformation.

2.2 Transformation and CRISPR/Cas9 mutagenesis

All *S. pombe* strains used had the *ura4-D18* and *leu1-32* alleles and were otherwise wild-type unless specifically noted. Yeast were grown in 5mL of MB media (Sunrise Science, Product #2016), with the addition of necessary supplements, overnight at 32°C. Cells were then seeded into 20mL of the same MB media per transformation (at 32°C) and harvested during mid-log phase (OD_{600}=0.5). Transformation was achieved by applying the lithium acetate procedure used in Bahler et al. (1998) and adding 1µg of plasmid. Strains to be mutagenized were similarly treated as those undergoing transformation, but 1µg of PCR template (for homologous recombination) was also added to the transformation mix. Heat-shocked cells were plated to Edinburgh Minimal Media (EMM, US Biological, #E2205) that selected for transformants and complemented biochemical deficiencies of the yeast strain – unless otherwise noted. Plates were placed at 32°C for a minimum of four days before individual colonies were selected for DNA sequence analysis or used for another round of transformation or mutagenesis. Colony PCR was conducted by streaking individual colonies in patches, boiling in 0.02 M NaOH for 10 minutes, and amplifying the region of interest.

2.2.1 *ade6* (Proof of Concept)

In the split-vector mutagenesis experiments, four *S. pombe* strains containing one of four *ade6* alleles (wt, M210, L469 or M216) were transformed with 1µg pMZ222 (Cas9; marked with *LEU2*) and plated to EMM with 150mg/L uracil and 150mg/L adenine for 4 days at 32°C. Colonies synthesizing Leu2p were grown in the media without leucine and transformed with 1µg of repair template (fragments from the unrelated gene *reb1* (control), or a fragment from *ade6-wt* or *ade6-M210* capable of HR) and a *ura4* marked sgRNA – 1µg pMZ284 (sgRNA directed against *ade6-wt*), pMZ285 (sgRNA directed against *ade6-M210*), or pMZ286 (sgRNA directed against *ade6-L469*) and. In the single vector experiments, the same four strains were transformed with 1µg pMZ288 (Cas9/sgRNA directed against *ade6-wt*), pMZ289 (Cas9/sgRNA directed against *ade6-M210*), or pMZ381 (Cas9/sgRNA directed against *ade6-L469*) and 1µg of repair template (*reb1*, *ade6-wt*, or *ade6-M210*). Both the split and single vector mutagenesis experiments were plated to EMM with 10mg/L adenine (which leads to red colored colonies revealing mutant *ade6* strains and white colonies revealing wild-type *ade6*) and appropriate supplements for 4 days at 32°C prior to colony color counts. Colonies were restreaked twice maintaining selection for the mutagenic plasmid, to remove unmutated contamination, and the targeted *ade6* allele was amplified by colony PCR. Both single vector and sgRNA vector transformations were replicated for a total of three transformations where the sgRNA targeted the *ade6* allele present (pMZ288 and pMZ284 for *ade6-wt* and *ade6-M216* strains, pMZ289 and pMZ285 for the *ade6-M210* strain, pMZ381 and pMZ286 for the *ade6-L469* strain).

2.2.2 *set1* and *clr4* (Additional Mutagenesis Sites)

A *S. pombe* strain with an *ade6-M210* genotype was mutagenized with 1µg pMZ395 (Cas9/sgRNA directed against *set1-y810*) and 1µg *set1-Y810A* HR template or 1µg pMZ394 (Cas9/sgRNA directed against *set1-y897*) and 1µg *set1-Y897A* HR template and plated to EMM with 150mg/L leucine and adenine at 32°C. 1µg pMZ453 (Cas9/sgRNA directed against *clr4-y451*) and 1µg *clr4-Y451A* repair template were used to mutagenize a strain with a Position-Effect-Variegation (PEV) reporter gene embedded within pericentric heterochromatin which is silenced in wild-type cells (otr1R(SphI)::*ade6-wt*, *ura4*-DS/E, *ade6-M210*, and *leu1-32*) and the resulting cells were plated to EMM with 150mg/L leucine and 10mg/L adenine at 32°C to reveal the silencing state of the *ade6* gene. Single colonies (specifically colonies with the *ade6-wt* expressing phenotype from the *clr4* mutagenesis) were selected and restreaked for colony PCR and DNA sequencing after 4 days.

2.3 Chromatin Immunoprecipitation of targeted dCas9 and Quantitative PCR

S. pombe cells were transformed with pMZ455 (nmt1dCas9), plated to EMM with 150mg/L uracil and 10μM thiamine, subsequently transformed with pMZ283 (sgRNA with null target), pMZ284 (sgRNA directed against *ade6-wt*), pMZ471 (sgRNA directed against scrambled LTR), pMZ473 (sgRNA directed against LTR), pMZ472 (sgRNA directed against scrambled TER1), or pMZ474 (sgRNA directed against TER1) and plated to EMM with 10μM thiamine. Single colonies were chosen from each transformation and were grown to log phase of OD_{600} .65-1.2 in 100mL of EMM with 10μM thiamine at 32°C. Cultures were fixed in 1% formaldehyde for 20 min while gently shaking at room temperature and quenched in .125M glycine for 5 min while gently shaking at room temperature. Cells were collected by centrifugation at 3000 rpm for 3 min, then washed in 20mL ice-cold PBS, collected as previously mentioned, and set at -80°C overnight. Cell treatments continue approximately as per Pidoux et al. (2004). Frozen samples were thawed on ice and suspended in 10mL of 0.4mg/mL zymolyase 100T (USBiological) in PEMS (100mM PIPES, 1.2M sorbitol, 1mM $MgCl_2$, 1mM EDTA, pH = 7.5) for 30 min at 37°C or when cells have mostly become spheroblasts as detected by the loss of refringence through light microscopy. Chromatin samples were collected by centrifugation, suspended in 1mL ChIP lysis buffer (50mM HEPES-KOH, 140mM NaCl, 1mM EDTA, 1% TritonX-100, 0.1% sodium deoxycholate, pH=8) with freshly added protease inhibitors (Merck/Millipore 539136) and 2mM PMSF, and sonicated for nine minutes with alternating 30 sec cycles of sonication and cooling. Sonication was achieved using a bath sonicator (Diagenode) at 4°C with the resulting chromatin sheared to ~300bp. Samples were clarified by two spins (one 5 min and another 15 min) at 16,300g at 4°C. The supernatant was collected and a Bradford assay was performed to quantify chromatin concentrations and each lysate was standardized so the final concentration for each sample was 4.6µg/µL. 980µL was pre-cleared with 10µL of Protein A Dynabeads (Life Technologies) for 30 min at 4°C and anti-HA rabbit antibodies (Abgent AP1012a) were conjugated to Protein A Dynabeads for 1 hr at 4°C. Three technical replicates per sample were used with 300µL of chromatin per immunoprecipitation being added to 7.5µL of bound beads (1.88µL antibody/7.5µL beads) and rotated overnight at 4°C; 50µL of chromatin was stored as whole cell extract at -20°C. Immunoprecipitations were washed once with 1mL ChIP lysis buffer, once with 1mL ChIP lysis buffer with 0.5M NaCl, once with 1mL ChIP wash buffer (10mM Tris-HCl, 250mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1mM EDTA, pH=8) and once with 1mL TE (10mM Tris-HCl, 1mM EDTA, pH=8). Whole cell extract samples and immunoprecipitated beads were suspended in 250µL TES (50mM Tris-HCl, 10mM EDTA, 1% SDS, pH=8) and placed at 65°C overnight to reverse the crosslinking. 220µL TE with 30µL proteinase K (20mg/mL; Fisher Scientific) was added to the beads of each immunopreciptation and 170µL TE with 30µL proteinase K was added to each whole cell extract; proteinase K treatment was performed for 2-3 hours at 50°C. Samples underwent phenol/chloroform/iso-amyl alcohol (pH=8) extraction before being treated with 100µg/mL RNase A (Amresco) at 37°C for 30 min. Immunoprecipitated and whole cell extracted DNA was column purified (Qiagen) and eluted into 50µL elution buffer (10mM Tris-HCl, pH=8). Quantitative PCR was performed on an Eppendorf Realplex Mastercycler using KAPA SYBR FAST qPCR mix (Kapa Biosystems) with appropriate oligo pairs (oM1010/oM1011 for *ade6*; oM534/oM535 for TER1; oM16/oM17 for LTR; oM532/oM533 for 6F6 [ChIP normalization]). ChIP enrichment was calculated with the $\Delta\Delta C_t$ method.

2.4 5' RACE Analysis

Cells transformed with pMZ222 (Cas9) and subsequently transformed with pMZ285 (sgRNA directed against *ade6-M210*) were selected from single colonies and grown in EMM until mid-log phase. Yeast were harvested and the hot phenol method was used to extract total RNA. 5µg of that RNA underwent 5' RACE utilizing the 5' RACE System Version 2.0 kit (Life Technologies) with the gene-specific primer oM546 and the nested gene-specific primer oM547. The resulting cDNA was cloned by Topo-TA (Life Technologies) and sequenced.

2.5 Northern Blot Analysis

S. pombe cells were transformed with pMZ222 (Cas9) and some were later transformed with pMZ285 (sgRNA directed against *ade6-M210*). Single colonies with the Cas9 vector only and both vectors were grown in EMM with 150mg/mL uracil and EMM, respectively, until mid-log phase. The cells were then harvested and total RNA was isolated using the hot phenol method. 5μg from each treatment was run in separate lanes on an 8% polyacrylamide gel electrophoresis (PAGE) with 8M urea and next to a radioactively labeled Decade Marker (Life Technologies). RNA was blotted onto charged nylon membrane by semi-dry transfer overnight and hybridized with radioactively labeled probes oM546 and oM599.

2.6 Growth Rate Assays

Cells transformed with pMZ285 (sgRNA directed against *ade6-M210*) or pMZ373 (nmt1Cas9) or pMZ455 (nmt1dCas9) or pMZ456 (nmt41dCas9) were plated on EMM with the necessary supplements that either contained or lacked 10μM thiamine. Plates were grown at 32°C for four days and photographed. Single colonies from the pMZ455 and pMZ456 transformations were selected for a second transformation with pMZ285, plated as above, and photographed after four days. Three colonies from each plate containing thiamine were selected and grown on EMM plates with supplements and 10μM thiamine for five days at 32°C. Cells were washed with EMM and grown in 5mL EMM with supplements for 14 hours at 32°C. An equivalent amount of cells was taken from each transformation and suspended in 200μL of EMM with supplements and with or without 10μM thiamine. These cultures were grown in a BioTek ELx808IU absorbance microplate reader at 32°C set to make an OD reading and shake the culture every 3 min.

2.7 Mutation Analysis Assays

2.7.1 In *E. coli*

Frozen cultures of *E. coli* containing pMZ222 (Cas9) and pMZ288 (Cas9/sgRNA directed against *ade6-wt*) were plated separately to single colonies and grown at 37°C overnight. One colony was selected from each to seed 5mL LB with 100mg/L Carbenicillin (Sigma) cultures which were placed at 37°C overnight. 5µL was removed from each culture and used to seed six 5mL cultures per plasmid. Serial cultures were maintained for seven days by using 5µL from one culture to seed the next culture; cultures were miniprepped in the end. *S. pombe* cells transformed with pMZ284 (sgRNA directed against *ade6-wt*) were mutagenized with the six miniprepped pMZ222 plasmids or pMZ222 miniprepped from glycerol and plated individually. Yeast cells transformed with pMZ285 (sgRNA directed against *ade6-M210*) were mutagenized with 1µg pMZ222 and 1µg of yeastmaker salmon sperm DNA (Clontech) or 1µg of *ade6-M210* PCR template. *S. pombe* cells containing no plasmids were mutagenized with the six miniprepped pMZ288 plasmids or pMZ288 miniprepped from glycerol or pMZ289 (Cas9/sgRNA directed against *ade6-M210*) miniprepped from glycerol and 1µg of yeastmaker salmon sperm DNA or 1µg of *ade6-M210* PCR template. EMM plates were made with 10mg/L adenine in addition to necessary supplements.

2.7.2 In *S. pombe*

Cells transformed with pMZ284 (sgRNA directed against *ade6-wt*) were mutagenized by transformation with 1µg pMZ222 (Cas9) and 1µg *ade6-M210* template. Transformations were plated to EMM with the necessary supplements and placed at 32°C for four days. Five phenotypically white and five phenotypically red colonies were selected from each transformation and patched to EMM plates with 225mg/L uracil or 225mg/L leucine and grown for four days at 32°C. Each colony underwent DNA analysis of the *ade6* gene (oM12/oM13). Only white colonies with wild-type sequence and all red colonies continued through the procedure. Total DNA was extracted from each colony individually approximately as prescribed by Rose et al. (1990). *S. pombe* cells were taken from plate and suspended in 250µL Lysis Buffer (2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris-HCl pH=8.0, 1mM EDTA pH=8.0). 300µL of 0.5mm glass beads (BioSpec Products) and 250µL of phenol/chloroform were also added and samples were bead beat by a bead beater (Scientific Industries) for 30 min at 3000rpm. 250µL TE was added and samples were spun for 10 min at 16,300g. 400µL of the aqueous phase was removed, ethanol precipitated, and suspended in 100µL of 100µg/mL RNase A for 30 min at 37°C. DNA was column purified, eluted in 50µL elution buffer, electroporated into electrocompetant *E. cloni* cells (Lucigen), and plated to LB with 100mg/L Carbenicillin plates. Single colonies were selected from each transformation. Each was grown separately in 5mL LB with 100mg/L Carbenicillin and miniprepped. Restriction fragment length polymorphism (using DraI (NEB) and HindIII (NEB)) and DNA sequence analyses were performed on recovered plasmids.

2.8 Statistical Analysis

Statistical significance was evaluated using Split-plot two-way analysis of variance (ANOVA) using R.

3. Results

3.1 Synthesis of sgRNA vector for *S. pombe*

The purpose of the current program of research was to create a sgRNA system of expression in fission yeast. The major difficulty to overcome in fission yeast was the lack of promoters that permit expression of RNAs with an arbitrary 5' end. Most CRISPR/Cas9 systems adapted for use in metazoan systems utilize the U6 type III promoter. However, as is the case in *S. cerevisiae*, *S. pombe* RNA Polymerase III promoters contain promoter elements in the transcribed region, so the expressed RNA is constrained by the presence of these sequences and would yield inactive sgRNA. This difficulty was overcome in *S. cerevisiae* using a RNA polymerase III promoter whose RNA is later processed, removing a leader RNA that contains the promoter elements and leaving a precise 5' end (DiCarlo et al., 2013). Thus, we experimented with the *rrk1* gene which is transcribed by RNA polymerase II into K RNA, a component of the RNAse P ribonucleoprotein (Krupp et al., 1986), and most likely has a cleavable leader RNA. While there is a lack of consensus in the literature, high-throughput RNA sequencing data suggests that in mutants of *rrp6*, part of the exosome that breaks down byproducts of RNA maturation, a leader RNA of approximately 250 nucleotides is upstream of mature K RNA (Wilhelm et al., 2008). Operating under this hypothesis, the *rrk1* promoter and leader sequence were assembled in an expression cassette preceeding a CspCI restriction site, sgRNA sequence (DiCarlo et al., 2013, Mali et al., 2013), and Hammerhead ribozyme sequence (Dower et al., 2004, Gao and Zhao, 2014), 5' to 3' respectively (Figure 4). The CspCI site was used for easy insertion of target sequence upstream of sgRNA following digestion by CspCI and the Hammerhead ribozyme sequence is present for the precise processing of the 3’ end. A Hammerhead ribozyme was necessary because processing by RNA polymerase II (due to *rrk1*) results in polyadenylation of the 3’ end of the mRNA. Northern blotting and 5’ rapid amplification of cDNA ends (RACE) analysis were performed to confirm proper processing of the sgRNA (Figures 5 and 6). As expected, we found a sgRNA sequence of ~100bp via northern blotting. Also, 5’ RACE analysis showed the *rrk1* promoter and leader sequences are successfully removed and precise cleavage at the 5’ end of the sgRNA targeting sequence for *ade6-M210* (TCTATTGTTCAGATGCTTCG) was achieved.

ttttgcttatgttggtggtagttggcatgcgtagactgatgactagtcagcaaggagcgtagaacagtcacactcgttatatatgtgcttccaaga
aaactcaagaatttaccattagcaaacactttttgaaatgttagacatttaaatgacgaaggcatatagaagctttgaataggtgttgtaaagtgtt
gatttatgtgacgctgagggtgcgcatgaaaggaatgttgggtcacgattattaaacagtttgctagcttggacacttgagtattggaagttgttg
aattctaaaaaactttcagttgatttgaatagttgctgttgccaaaaaacataacctgtaccgaagaatgggcttaactcaattcttgtgggttatct
ctctGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAA
AAAAGTGAGTGGCACCGAGTCGGTGGTGCCCTGTACCGGATGTGCTTTCCGGTCTGA
TGAGTCCGTGAGGACGAAACAGG

Figure 4. sgRNA expression sequence from pMZ283. *rrk1* promoter and leader RNA sequences (lowercase); CspCI placeholder (lowercase and underlined); sgRNA sequence (uppercase); Hammerhead ribozyme sequence (uppercase and underlined).

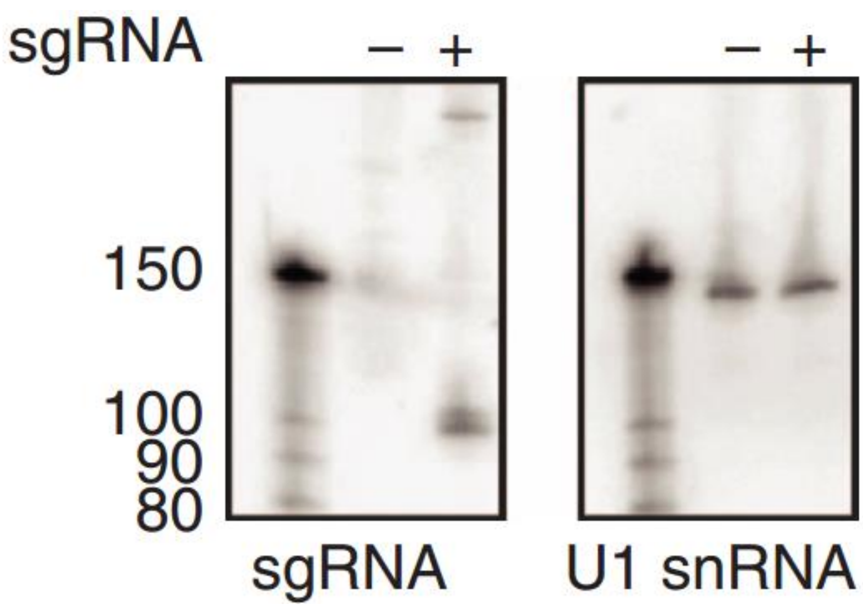


Figure 5. Northern Blotting of sgRNA targeting *ade6-M210* (pMZ285) in *ade6-wt* strain with Cas9 expression. Decade marker lane one, no sgRNA lane two, and with sgRNA lane three; RNA size to the left (bp) and probe at the bottom (sgRNA or small nuclear RNA).

Figure 6. 5' RACE analysis of sgRNA targeting *ade6-M210* (pMZ285). Four guanine bases resulted from RACE procedure.

3.2 CRISPR/Cas9 mutagenesis of *ade6*

Our lab next sought to test the newly generated Cas9 and sgRNA expression plasmids for a proof of concept experiment. The *ade6* gene was selected as the site of CRISPR/Cas9 mutagenesis because mutations within the gene result in the observable accumulation of a red colored precursor when cells are grown on media containing low adenine (Szankasi et al., 1988; Schar et al., 1993). Four *ade6* alleles were specifically chosen for this study. These alleles were *ade6-wt*, *ade6-M210*, *ade6-L469*, and *ade6-M216* which are phenotypically white, dark-pink to red, dark-pink to red, and light pink, respectively, when strains are grown on low adenine media. The *ade6-M210* and *ade6-L469* alleles result from point mutations located 1bp from each other. These point mutations are conveniently near a NGG protospacer adjacent motif (PAM) and disrupt a XhoI restriction site (Figure 7). The *ade6-M216* allele also arises from a point mutation, but the mutation is located over 1kb upstream from the other mutations. Three sgRNA plasmids were generated to target *ade6-wt*, *ade6-M210*, and *ade6-L469* strains and test the specificity of the Cas9 and sgRNA constructs. DNA templates of the same size were generated via PCR from *ade6-wt*, *ade6-M210*, and a non-homologous gene (control) for use in DNA repair by homologous recombination.

```
1,443              ade6           PAM    1,477
…TCTTCACTCTATTGTTCAGATGCCTCGAGGTGTCC…
        TCTATTGTTCAGATGCCTCG α-WT
        TCTATTGTTCAGATGCTTCG α-M210
        TCTATTGTTCAGATGCCTTG α-L469
```
Figure 7. sgRNA target site and alleles of *ade6*.

To ensure that Cas9 does not produce changes in the absence of the targeting sgRNA, only the Cas9 expression plasmid was transformed into the four strains previously mentioned. One colony was selected from each transformation and transformed with one of three sgRNA plasmids and one of three repair templates (36 combinations). Each transformation was plated to EMM low in adenine and lacking leucine (Cas9 selection) and uracil (sgRNA selection). The resulting colonies were recorded by phenotype and individual colonies were sequenced at the sgRNA target site.

Mutagenesis efficiencies were found to be specific to the sgRNA and PCR template used. Of the possible combinations, 8 out of 36 transformations resulted in a majority of cells (50 to 90%) changing phenotypes from white to red to white, dark pink/red, or light pink to red (Figure 8). The transformation with the greatest mutagenesis efficiency for each strain was observed to be those where the sgRNA targeted the *ade6* allele present and a homologous repair template for an alternate *ade6* allele was co-transformed (75-98%). Specifically, strains that were initially *ade6-M210* and *ade6-L469* (red phenotype) became white and resembled the *ade6-wt* strain when the sgRNA plasmid provided the corresponding targeting sequence, and a wild-type homologous recombination template was co-transformed with it. Strains with the *ade6-wt* or *ade6-M216* alleles (white and light pink phenotype, respectively) instead grew with a dark pink to red phenotype to appear like *ade6-M210* or *ade6-M216* strains when transformed with an *ade6-wt* targeting sgRNA (the *ade6-M216* allele has wild-type sequence in the targeted region) and a M210 template. The remaining four transformations, with sgRNA targeting the genomic *ade6* allele, appeared to be resistant to the CRISPR/Cas9 mutagenesis. In these samples, mutation efficiencies ranged from 0 to 5% and the *ade6-M210* and *ade6-L469* strains remained mostly phenotypically red. The lowest efficiencies (at or near 0%) were observed in transformations with a sgRNA not targeting the present *ade6* allele even when the target site only differed by one base (24 of 36 combinations).

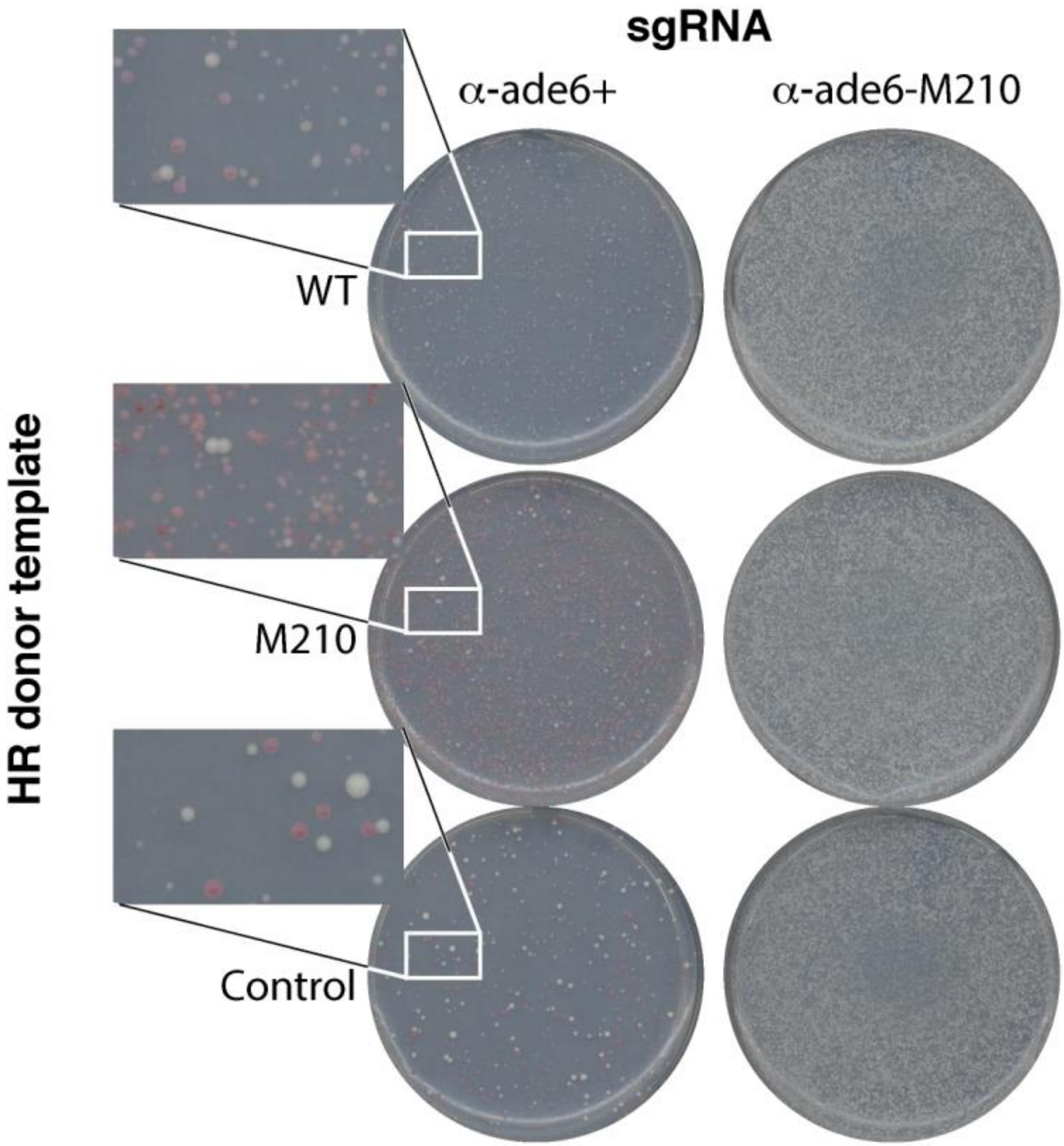


Figure 8. Visual representation of transformed *ade6-wt* strain. PCR template is indicated on the left (*ade6-wt*, *ade6-M210*, *reb1* (negative control)) and the sgRNA is indicated at the top (α-*ade6+* cleaves the *ade6-wt* strain and α-*ade6-M210* cleaves the *ade6-M210* strain).

Transformation efficiencies were observed to be dependent on the sgRNA present only. The lowest efficiencies (between 10 and 10^2 cfu/µg) occurred when the *ade6* allele present was targeted by the sgRNA. By contrast, strains not of the same genotype as the sgRNA had very high transformations efficiencies – around 10^4 cfu/µg. This evidence suggests that expression of Cas9 and a homologous targeting sgRNA reduces the number of cells that survive transformation.

Individual colonies from each transformation, where the sgRNA targets the strain's *ade6* allele, were sequenced at the *ade6* gene. When possible, representative colonies that underwent a color change (mutation) or were phenotypically the same (likely no mutation) were selected (Figure 9). Analysis of the sequencing results demonstrate that predictable or unpredictable mutations occur as a result of CRISPR/Cas9 mutagenesis. The nature of the mutation is largely correlated with the repair template that is transformed with the sgRNA. When a completely non-homologous template or a homologous template (contains the target of the introduced sgRNA) is co-transformed with the sgRNA, then there is a high likelihood of unpredictable point mutations within the target region – mostly consisting of microdeletions or microinsertions at the cleavage site. Conversely, introduction of a mostly homologous template (contains a slightly altered target of the introduced sgRNA) resulted in 22 out of 23 mutated colonies replicating the mutation contained in the template. Both categories of mutation confer resistance to double-strand breakage by Cas9, but likely arise by different cellular mechanisms. The unpredictable mutations are characteristic of DNA repair by NHEJ (small insertions or deletions) and the predictable mutations likely occur by HR with the PCR template. In addition to various mutations, the sequencing results also show that the *ade6* gene sequence of some colonies was preserved and no mutation or phenotype change occurred. Furthermore, if the genomic target site did not change, then one of the trans-acting factors, Cas9 or sgRNA, was likely altered and unable to perform its function (catalyze DSBs and base-pair with homologous sequence, respectively). Together, the *ade6* sequencing results show that predictable and unpredictable mutations are generated by CRISPR/Cas9 mutagenesis, but some colonies are resistant to the mutagenesis and do not mutate.

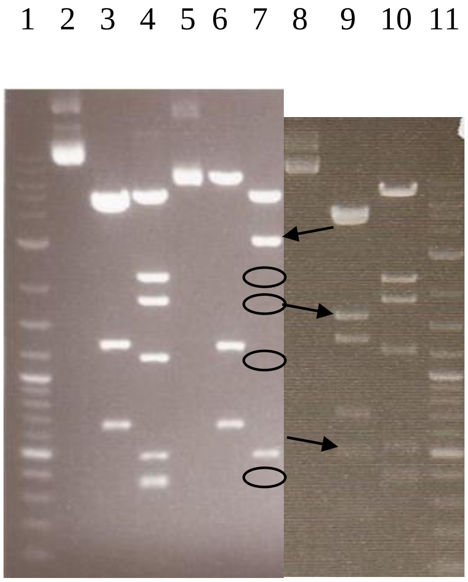
original genotype	Template	Phenotype		target PAM CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC WT CTCTTCACTCTATTGTTCAGATGC-TTCGAGGTGTCCCTGTCGCCACTGTTGC M210 CTCTTCACTCTATTGTTCAGATGC-CTTGAGGTGTCCCTGTCGCCACTGTTGC L469
WT	+	White	1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
		Red	1442>	CTCTTCACTCTATTGTTCAGATGCCCTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-C-CGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC--TCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC--TCGAGGTGTCCCTGTCGCCACTGTTGC
	210	White	1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
		Red	1442>	CTCTTCACTCTATTGTTCAGATGC-TTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-TTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-TTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-TTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-TTCGAGGTGTCCCTGTCGCCACTGTTGC
	Control	White	1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
		Red	1442>	CTCTTCACTCTATTGTTCAGATGCCCTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-TTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC--TCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
M216	+	Pink	1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
		Red	1442>	CTCTTCACTCTATTGTTCAGATGC-C-C-AGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCA------CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGCCCTCGAGGTGTCCCTGTCGCCACTGTTGC
	M210	Pink	1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
		Red	1442>	CTCTTCACTCTATTGTTCAGATGC-TTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-TTCGAGGTGTCCCTGTCGCCACTGTTGC
	Control	Pink	1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
		Red	1442>	CTCTTCACTCTATTGTTCAGAT---TTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATG---TCGAGGTGTCCCTGTCGCCACTGTTGC

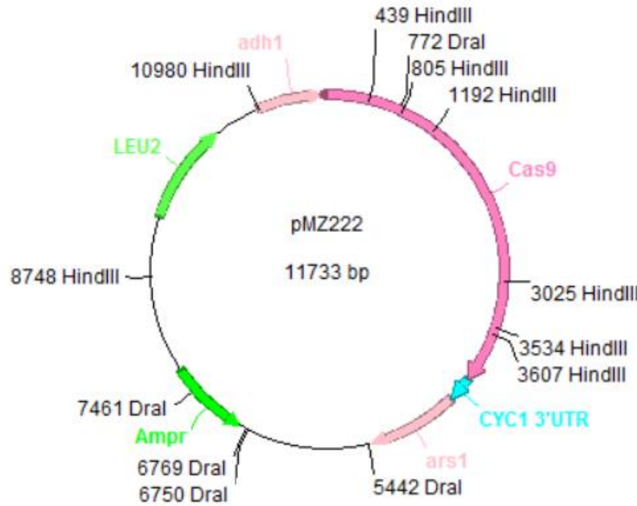
M210	+	Red	1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
		White	1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
	M210	Red	1442>	CTCTTCACTCTATTGTTCAGATGC-TTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGCTTTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC--------TGTCCCTGTCGCCACTGTTGC
		White	1442>	CTCTTCACTCTATTGTTCAGATGCTTTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC--TCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGA-TTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATG---TCGAGGTGTCCCTGTCGCCACTGTTGC
	Control	Red	1442>	CTCTTCACTCTATTGTTCAGATGC-TTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-TTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-TTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGAT----TCGAGGTGTCCCTGTCGCCACTGTTGC
		White	1442>	CTCTTCACTCTATTGTTCAGATGC-TTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGCTTTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-TTCGAGGTGTCCCTGTCGCCACTGTTGC
L469	+	White	1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-TTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTCGAGGTGTCCCTGTCGCCACTGTTGC
	M210	Red	1442>	CTCTTCACTCTATTGTTCAGATGC-TTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-TTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-TTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-TTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-TTTGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTTGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-TTCGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-TTCGAGGTGTCCCTGTCGCCACTGTTGC
	Control	Red	1442>	CTCTTCACTCTATTGTTCAGATGC-CTTGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGAT---CTTGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTTGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTTGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTTGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTTGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTTGAGGTGTCCCTGTCGCCACTGTTGC
			1442>	CTCTTCACTCTATTGTTCAGATGC-CTTGAGGTGTCCCTGTCGCCACTGTTGC

Figure 9. Summary of *ade6* gene sequencing results. Sequence data start from nucleotide 1442 in *ade6* gene and covers the sgRNA target sites.

3.3 Expression of Cas9 negatively affects *S. pombe* growth

Next, we investigated how colonies became non-responders to CRISPR/Cas9 mutagenesis. Two possible sources of resistance that were explored were silent mutations in the target site region and mutations that inactivated the Cas9 or sgRNA sequences (the latter being observed in DiCarlo et al. (2013) with the implementation of Cas9 in *S. cerevisiae*). Various small deletions or insertions were observed, but silent mutations were not (Figure 9). These small mutations were not seen in phenotypically white cells that remained white after mutagenesis, but did occur in some visibly red cells that remained red after mutagenesis. Most mutations within the *ade6* gene result in a red phenotype so it is unsurprising that some colonies remained red after mutagenesis. In the absence of a template for HR, NHEJ performed in these colonies for DNA repair would be sufficient to disrupt the target site and arrest cleavage by Cas9. Cas9 and sgRNA expression vectors were recovered from non-responding wild-type cells and mutagenized (red) cells to determine if the mutation of Cas9 or sgRNA was prevalent among resistant colonies. Digestion and sequencing analysis of the transformed plasmids showed that plasmids expressing Cas9 were mutated causing the inactivation of Cas9 expression or truncation of its synthesis (Figure 10). However, sgRNA sequences and Cas9 from mutated clones were not found to be mutated. Both sources of resistance are potentially troublesome for the efficacy of site-specific mutagenesis by CRISPR/Cas9, but the detection of inactivating mutations specific to Cas9 shows that problems observed in *S. cerevisiae* (DiCarlo et al., 2013) exist in *S. pombe* as well.






```
                   R  K  N  R  I  C  Y  L  Q  E  I  F  S  N  E  M  A  K  V     93
Cas9+             AGAAAGAATCGGATCTGCTACCTGCAGGAGATCTTTAGTAATGAGATGGCTAAGGTG   278
Cas9mut1          AGAAAGAATCGG--CTGCTACCTGCAGGAGATCTTTAGTAATGAGATGGCTAAGGTG
                   R  K  N  R    L  L  P  A  G  D  L  *

                   E  E  D   K  K  H  E  R  H  P  I  F  G       120
Cas9+             GAGGAGTCC-TTTTTGGTGGAGGAGGATAAAAAGCACGAG       369
Cas9mut2          GAGGAGTCCATTTTTGGTGGAGGAGGATAAAAAGCACGAG
                   E  E  D  I  F  G  G  G  G  *
```

Figure 10. pMZ222 (Cas9 plasmid) and observed mutations. Lanes 1 and 11 – 2log ladder (NEB #N3200). 2 – 4 pMZ222: undigested; DraI; HindIII. 5 – 7 Cas9mut3: undigested; DraI; HindIII. 8 – 10 Cas9mut4: undigested; DraI; HindIII. Circled regions indicate missing restriction fragments and regions specified by an arrow indicate new or altered restriction fragments.

Cas9 and sgRNA are equally essential for CRISPR/Cas9 mutagenesis, but several resistant colonies were found to only contain mutated Cas9 plasmids and active sgRNA plasmids. This suggests that *S. pombe* undergoes positive selection for Cas9 inactivation by sporadic mutation and Cas9 expression by *adh1* is somehow deleterious to the cell. We challenged this hypothesis by developing a Cas9 regulated by the inducible nmt1 promoter. In this new system, Cas9 is expressed at levels similar to the *adh1* regulated Cas9 when thiamine is absent from the growth medium, but its expression is reduced (about 1/160th of *adh1*) when thiamine is present (Forsburg, 1993). Fission yeast transformed with this plasmid were plated onto medium containing thiamine (repression of Cas9) and onto medium lacking thiamine (full expression of Cas9). Resulting colony size was evaluated and was found to differ between treatments. Several large colonies were observed under conditions repressing nmt1 whereas only small colonies were observed under conditions where nmt1 remained active (Figure 11). Consistent with this, cells grown in liquid medium with full Cas9 expression grew at a lower rate than cells growing in medium containing thiamine and had Cas9 repressed (Figure 12). Statistical analyses confirm that the addition of thiamine has an approximately 4.4 fold positive effect on growth rate. Together, these observations support the conclusion that expression of Cas9 is toxic to *S. pombe* and prolonged expression of Cas9 plasmids will likely result in the accumulation of mutations and disruption of CRISPR/Cas9 mutagenesis.

No Thiamine 10µM Thiamine

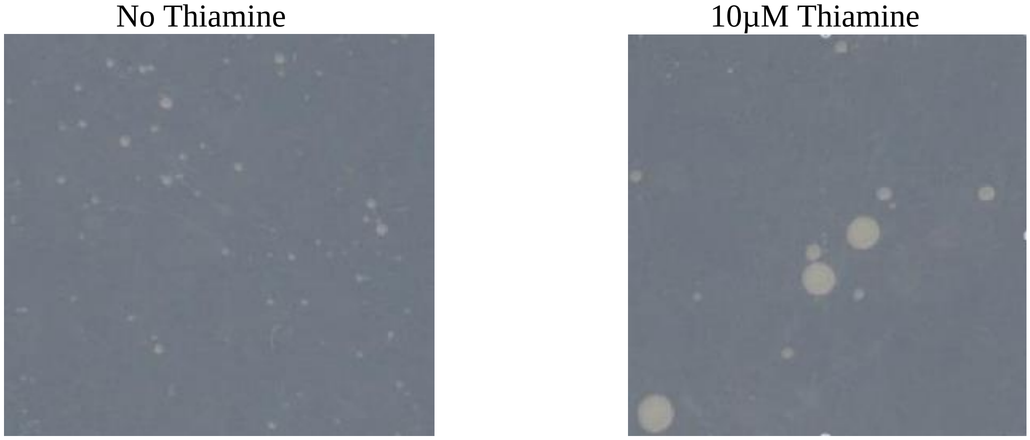


Figure 11. Growth with differential expression of Cas9 on solid media. Left, growth with full Cas9 expression. Right, growth with Cas9 repression. All surviving colonies contained the plasmid pMZ373 (nmt1Cas9).

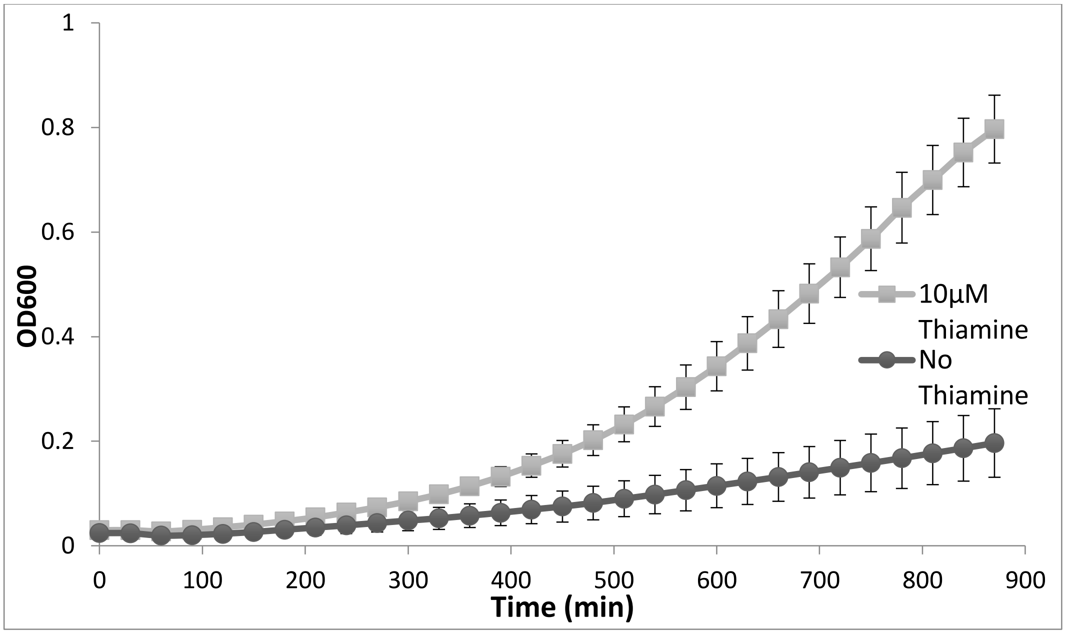


Figure 12. Growth with differential expression of Cas9 in liquid media. Growth with thiamine (Cas9 repression) and growth without thiamine (full Cas9 expression) of pMZ373 (nmt1Cas9) containing clones. Error bars represent +/- one standard deviation.

3.4 Single Cas9/sgRNA plasmid improves mutagenesis efficiency

We generated a single plasmid expressing sgRNA and Cas9 to reduce or prevent the positive selection of Cas9 mutations during the first step of the split vector CRISPR/Cas9 mutagenesis. Cas9 regulated by the *adh1* promoter was inserted into the sgRNA plasmid. Targeting sequences for *ade6-wt*, *ade6-M216*, *ade6-M210*, and *ade6-L469* alleles were later cloned into the CspCI site. One of the four single Cas9/sgRNA plasmids were co-transformed with one of three homologous recombination templates (*ade6-wt*, *ade6-M210*, and control) into the same one of four strains from the dual plasmid approach (*ade6-wt*, *ade6-M216*, *ade6-M210,* and *ade6-L469*). Fission yeast were plated to plates with low adenine, as before, with the results summarized in Figure 13. Consistent with prior observations, sgRNA targeting is specific and a proper HR template is essential for the generation of desired genomic mutations. The greatest mutagenesis efficiencies (86-92%) occurred in strains targeted by the sgRNA, received a template able to participate in DNA repair via homologous recombination, and the repair template contained a sequence different from the sgRNA target site.

Genotype	gRNA	pcrDNA	Mutagenesis efficiency (%)	Std.dev.	*n*
WT	α-WT	WT	69.70	3.84%	3
		M210	88.85	3.78%	3
		Control	59.95	4.21%	3
	α-M210	WT	1.34	NA	1
		M210	0	NA	1
		Control	2.22	NA	1
	α-L469	WT	0.82	NA	1
		M210	0.47	NA	1
		Control	0	NA	1
M216	α-WT	WT	79.70	1.95%	3
		M210	91.13	3.96%	3
		Control	65.40	4.76%	3
	α-M210	WT	4.35	NA	1
		M210	3.61	NA	1
		Control	4.52	NA	1
	α-L469	WT	0.43	NA	1
		M210	0.59	NA	1
		Control	1.20	NA	1
M210	α-WT	WT	0	NA	1
		M210	0%	NA	1
		Control	0	NA	1
	α-M210	WT	86.21	6.38%	3
		M210	6.80	3.96%	3
		Control	0	0%	3
	α-L469	WT	0.24	NA	1
		M210	0	NA	1
		Control	0	NA	1
L469	α-WT	WT	1.84	NA	1
		M210	1.58	NA	1
		Control	1.50	NA	1
	α-M210	WT	1.82	NA	1
		M210	1.23	NA	1
		Control	1.64	NA	1
	α-L469	WT	87.62	4.82%	3
		M210	1.80	4.44%	3
		Control	4.55	2.30%	3

Figure 13. Summary of CRISPR/Cas9 mutagenesis with single vector. Error bars represent +/- one standard deviation.

Non-responders to CRISPR/Cas9 mutagenesis were also evaluated for mutation of the single vector. Colonies expressing wild-type *ade6* (phenotypically white) which remained white after mutagenesis with a sgRNA directed against *ade6-wt* and an *ade6-M210* HR template were selected and the Cas9/sgRNA plasmids recovered. Restriction patterns were found to be altered in the four recovered vectors indicating that mutation of these plasmids was the source of resistance to CRISPR/Cas9 mutagenesis (Figure 14). Thus, combining Cas9 and sgRNA into a single vector succeeded in shortening the mutagenesis protocol and reducing inactivating mutations to the mutagenesis mediating plasmid, but complete elimination of these mutations was not accomplished.

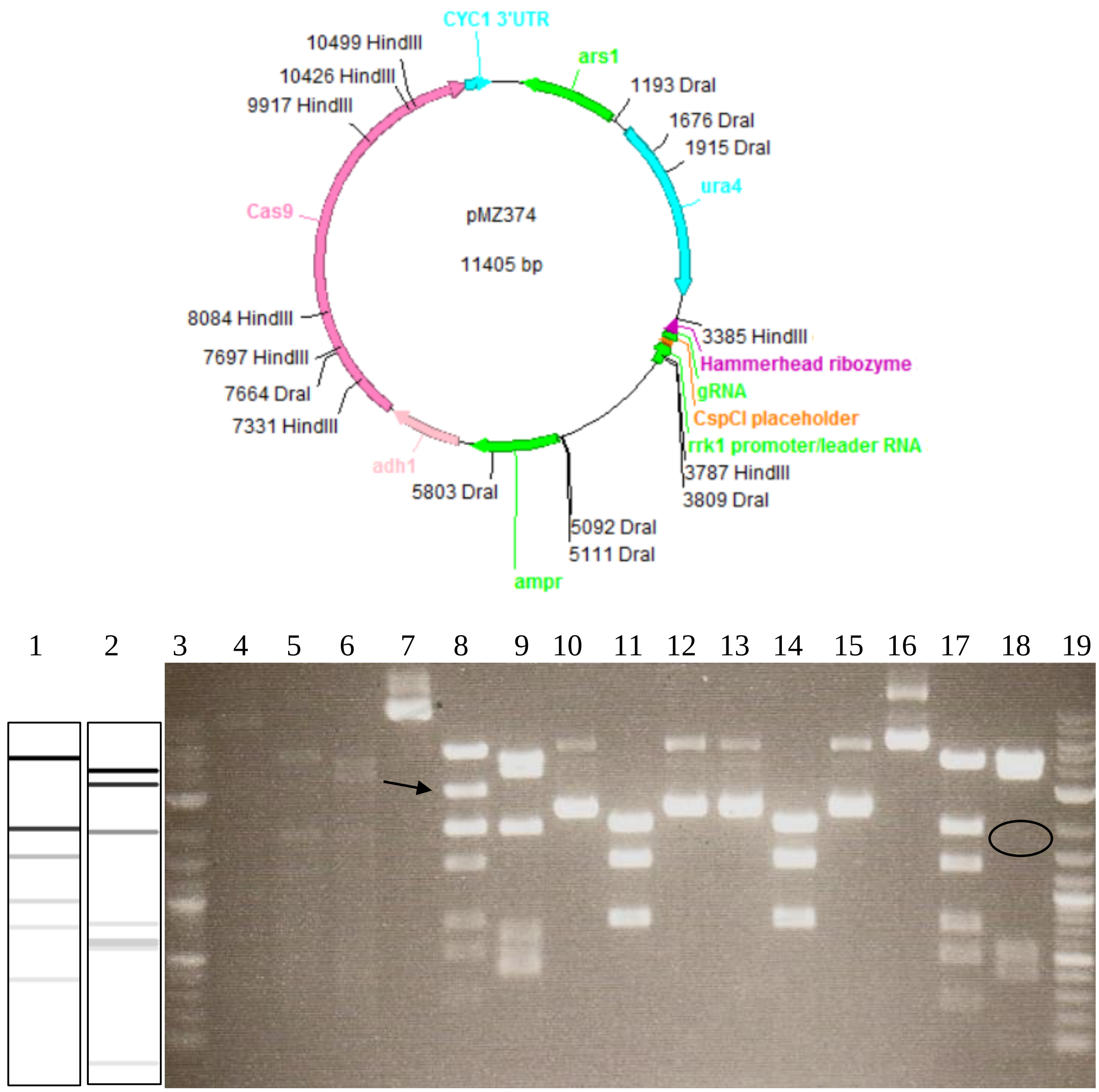
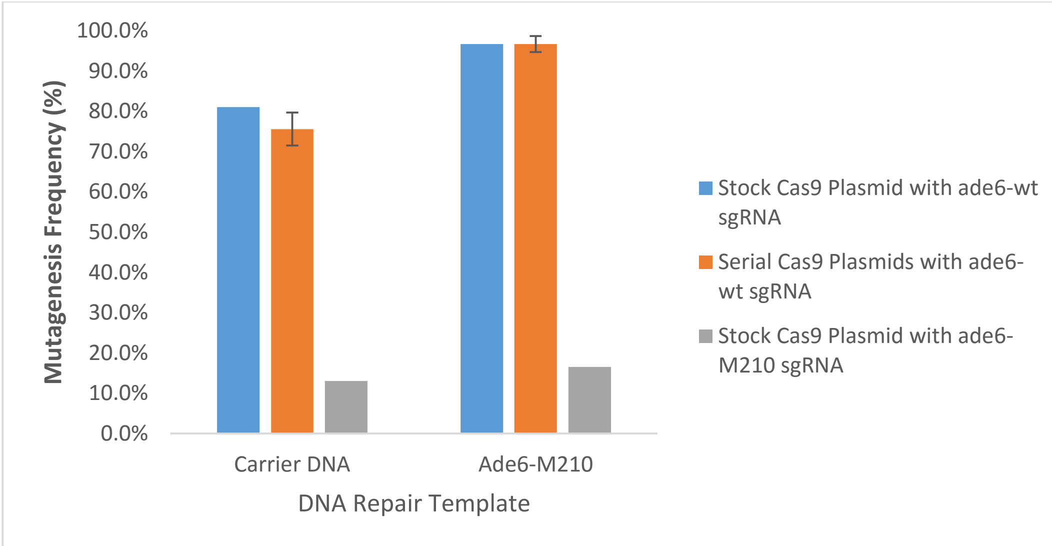


Figure 14. pMZ374 (Cas9/sgRNA plasmid) and observed mutations. Lanes 1 – 2 pMZ374 (expected): DraI; HindIII. 3 + 19 2log ladder (NEB #N3200). 4 – 6 pMZ374: undigested; DraI; HindIII. 7 – 9 Cas9/sgRNAmut1: undigested; DraI; HindIII. 10 – 12 Cas9/sgRNAmut2: undigested; DraI; HindIII. 13 – 15 Cas9/sgRNAmut3: undigested; DraI; HindIII. 16 – 18 Cas9/sgRNAmut4: undigested; DraI; HindIII. The circled region indicate missing restriction fragment and the region specified by an arrow indicate a new restriction fragment.

3.5 Cas9 inactivation is not observed in *E. coli*

An alternative source of Cas9 plasmid (Cas9 and Cas9/sgRNA) mutation that we explored was the replication step in *E. coli*. Cas9 and sgRNA expression vectors are multiplied many times within *E. coli* cells prior to lysis and purification of the vectors. As has been observed by other groups, expression of transgenic proteins (Cas9 in this case) can poison the cells (Miroux and Walker, 1996). This would select for cells that mutate the Cas9 promoter or coding sequence during the plasmid replication process. To test this, both Cas9 plasmids were serial cultured for seven days in sextuplicate with 5µL taken to start each culture. Evaluation in *S. pombe* involved CRISPR/Cas9 mutagenesis with sgRNA targeting the present *ade6-wt* allele and carrier DNA or *ade6-M210* for repair template. Comparison of serial cultured Cas9 plasmids to stock Cas9 plasmids with sgRNA targeting *ade6-wt* or *ade6-M210* alleles indicate that serial vectors behave most similarly to functional Cas9 and retain the ability to utilize sgRNA to mutagenize the *ade6* gene of *S. pombe* (Figure 15). Fixation of inactivating mutations in Cas9 expressing plasmids within the *E. coli* population was not found to occur after seven days and mutagenesis rates did not revert to background *ade6* gene mutation rates as approximated by Cas9 with a sgRNA targeting the *ade6-M210* allele. The DNA repair template also remained important as the highest frequency of phenotypically red colonies was observed in cells receiving the *ade6-M210* template. Therefore, it is unlikely that replication of Cas9 plasmids in *E. coli* is a significant source of resistance to CRISPR/Cas9 mutagenesis or Cas9 expression, if it occurs, is toxic to the cell. The absence of significant selection for inactivation of Cas9 in *E. coli* suggests that toxicity is specific to *S. pombe*.



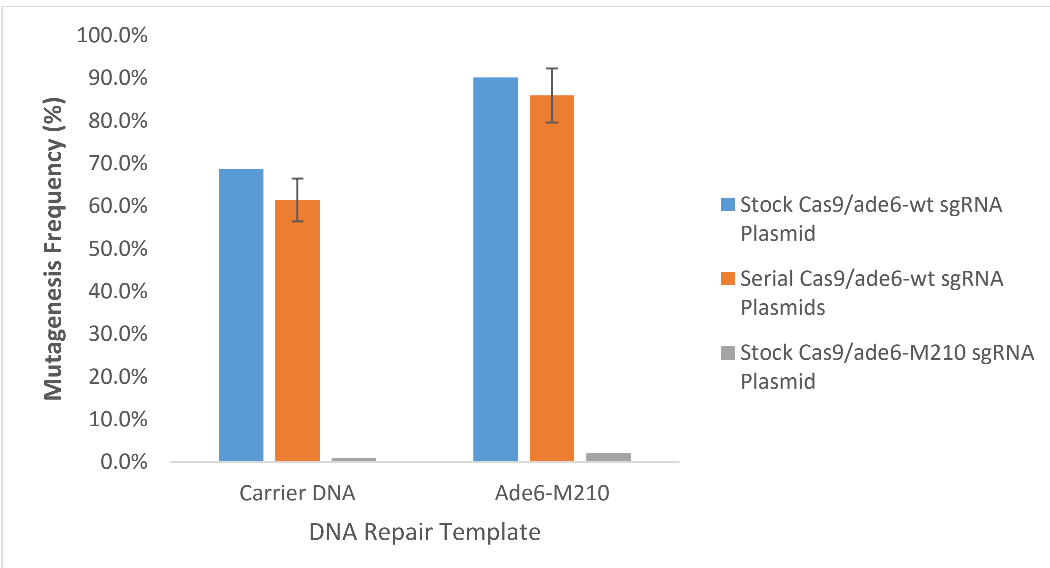


Figure 15. Results of CRISPR/Cas9 mutagenesis with serial cultured Cas9 plasmids. pMZ222 (Cas9) data shown above and pMZ288 (Cas9/sgRNA) data shown below. Mutagenesis frequency approximated by proportion of red colonies. Error bars for mutagenesis frequency of serial Cas9 plasmids generated by +/- one standard deviation.

3.6 Catalytic activity of Cas9 does not cause toxicity

With *E. coli* no longer the likely source of Cas9 mutation, we returned our focus to expression in *S. pombe*. Although unlikely, the Cas9 enzyme, when unbound by sgRNA, may randomly create double-strand breaks or nicks in the genome of the host cell. Treatment with MMS or bleomycin also creates DSBs and causes cells to undergo cell cycle arrest and perform DNA repair before replication is resumed (Kostrub et al., 1997; Memisoglu and Samson, 2000). Toxicity through Cas9 expression would be triggered by these checkpoint activities and would cause cultures to grow more slowly than cells not expressing Cas9; these conditions also select for Cas9 inactivation. This is consistent with observations showing slower growth during Cas9 expression and specific inactivation of the Cas9 plasmid (Figures 10, 11, and 12). However, direct evidence was lacking.

The postulation that non-specific creation of DSBs by Cas9 causes toxicity was tested by generating a vector coding for the catalytically inactive Cas9, dCas9. Four expression levels of dCas9 were produced by regulating dCas9 with the inducible nmt1 promoter or a weaker nmt promoter, nmt41, in two different plasmids. The nmt41 promoter functions similarly to nmt1 (repressed by thiamine), but the induced expression level is 1/30th of *adh1* and the repressed expression level is 1/790th of *adh1* (Forsburg, 1993). Utilizing these varying expression intensities of dCas9, we transformed *ade6-wt* cells with sgRNA targeting *ade6-M210*, dCas9 regulated by nmt1 or nmt41, or both vectors and grown on media containing 10µM thiamine (repression of dCas9) or no thiamine (full expression of dCas9). Colony size was then compared within each plasmid treatment set (sgRNA only, dCas9 only, and both). Large colonies formed in the presence of 10µM thiamine for each expression vector group and also in the absence of thiamine for the sgRNA group (Figure 16). Smaller colonies grew when the media was lacking thiamine and a dCas9 plasmid was present, regardless of the presence or absence of sgRNA (Figure 16). Similar results were observed in liquid media with the sgRNA only colonies growing independent of thiamine concentration and both nmt1/41 regulated dCas9 sets growing poorly without thiamine (dCas9 expressed) or growing well in 10µM thiamine (dCas9 repressed; Figure 17). Growth rates were found to be significantly different between cultures expressing dCas9 and cultures with dCas9 expression inhibited. Also, fission yeast transformed with a sgRNA vector and a dCas9 vector appear to exhibit dosage dependent toxicity with *adh1* levels of dCas9 expression being the most toxic (nmt1dCas9 and no thiamine) and 1/790th *adh1* levels being the least toxic (nmt41dCas9 with 10µM thiamine). Statistical analyses largely confirmed these observations, but show that thiamine had a ~1.2 fold effect on the growth of the sgRNA only culture (thiamine and sgRNA are not independent). This is likely due to the marginal benefit of the cells not needing to synthesize thiamine in the presence of exogenous thiamine. However, the interaction between sgRNA and thiamine was non-significant so the effect of thiamine would exist regardless of the presence of sgRNA. As expected, addition of thiamine to the media represses dCas9 and increases the growth rate ~7 fold (intercept=2.731e-4, thiamine=1.908e-3), which is highly significant (t=23.684, p<2e-16). Additionally, the nmt41 increases the growth rate ~1.5 fold over nmt1 (intercept=2.731e-4, promoter=1.581e-4), which is very significant (t=2.774, p=0.00717) and confirms dosage dependence. Lastly, the presence of sgRNA does not influence growth as it had no significant effect (t=-0.731, p=0.46742). Residuals from the analyses look reasonably parametric and uncorrelated, and the model captures most of the variability (R square=0.9358). Hence, thiamine is minimally responsible for the observed variations in growth rate and expression of Cas9 in *S. pombe* is toxic through a mechanism independent from its catalytic activity.

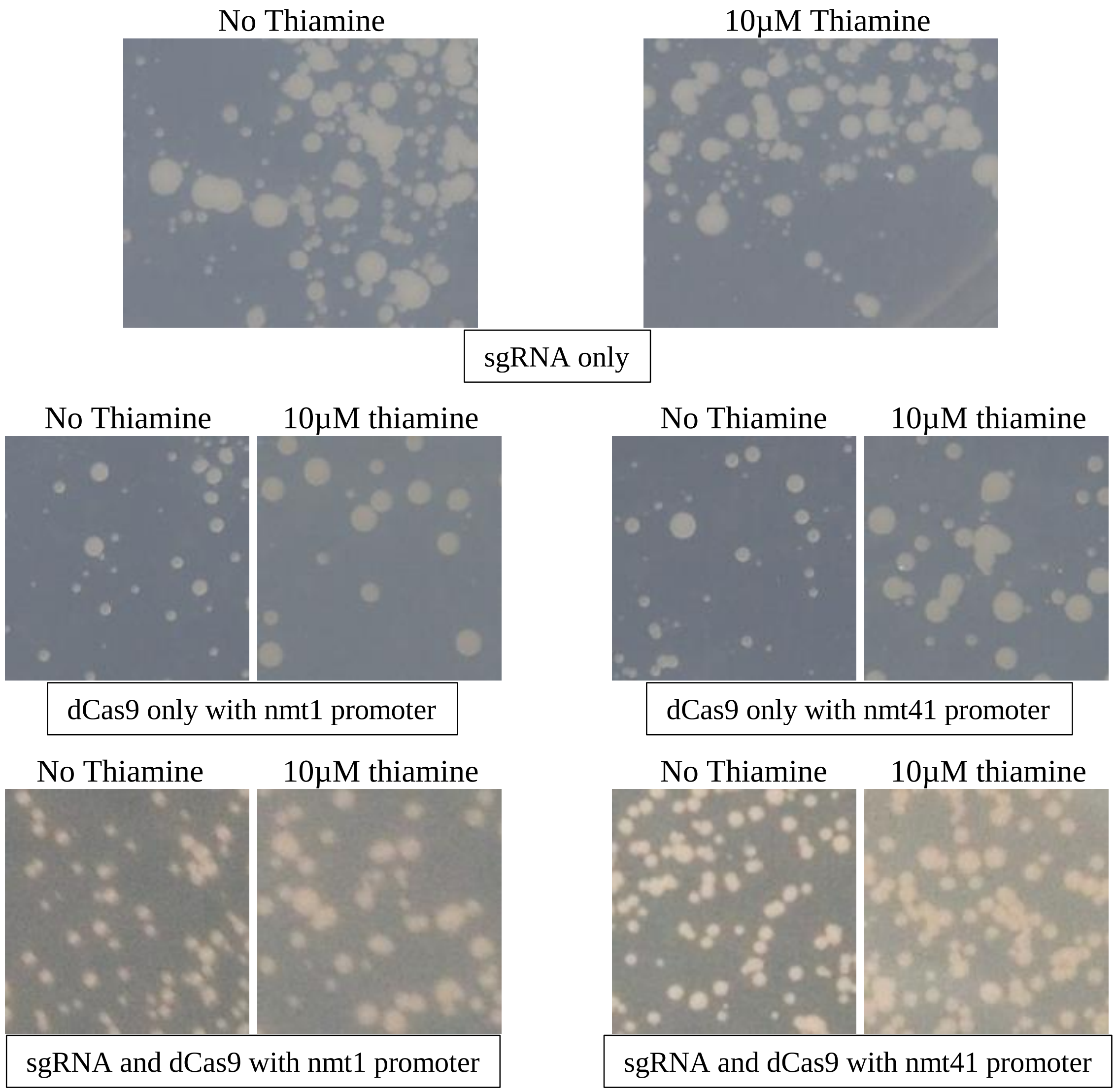


Figure 16. Growth of *ade6-wt S. pombe* while expressing various CRISPR components on solid media. Top left: culture growing without thiamine and expressing pMZ285 (sgRNA targeting *ade6-M210*). Top right: cultures growing with 10µM thiamine and expressing pMZ285. From middle left to right: pMZ455 (dCas9 with nmt1 promoter) culture grown without thiamine and expressing dCas9; pMZ455 culture grown with 10µM thiamine and dCas9 repressed; pMZ456 (dCas9 with nmt41 promoter) culture grown without thiamine and expressing dCas9; pMZ456 culture grown with 10µM thiamine and dCas9 repressed. From bottom left to right: pMZ455 with pMZ285 culture grown without thiamine and expressing dCas9 and sgRNA; pMZ455 with pMZ285 culture grown with 10µM thiamine and expressing sgRNA, but dCas9 is repressed; pMZ456 with pMZ285 culture grown without thiamine and expressing dCas9 and sgRNA; pMZ456 with pMZ285 culture grown with 10µM thiamine and expressing sgRNA, but dCas9 is repressed.

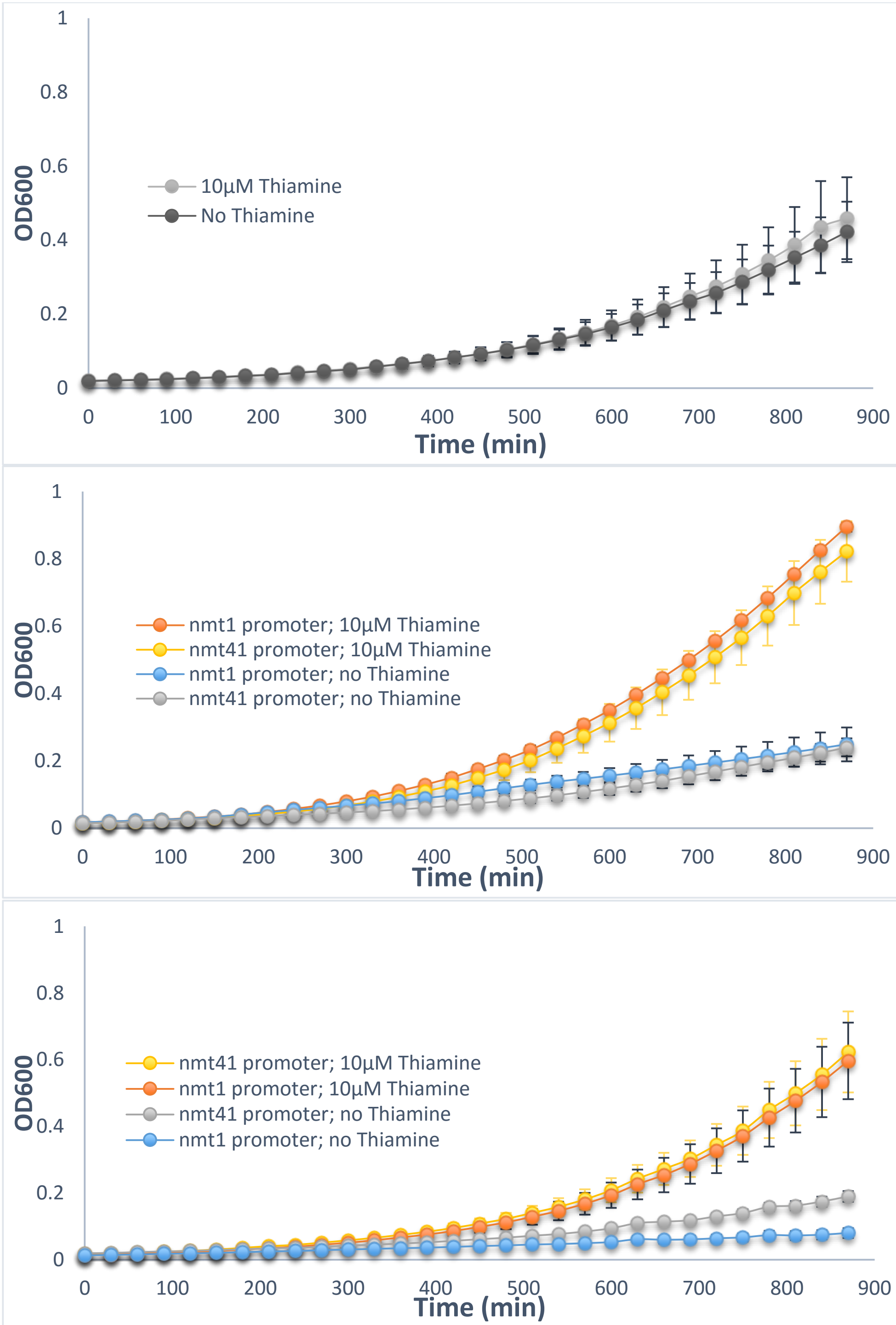


Figure 17. Growth of *ade6-wt S. pombe* while expressing various CRISPR components in liquid media. Top: cultures expressing pMZ285 (sgRNA targeting *ade6-M210*) and growing with or without 10µM thiamine. Middle: cultures expressing dCas9 (no thiamine) and cultures with dCas9 repressed (10µM thiamine); dCas9 regulated by the nmt1 promoter (pMZ455) or the nmt41 promoter (pMZ456). Bottom: cultures expressing pMZ285 and expressing dCas9 (no thiamine) or not expressing dCas9 (10µM thiamine); same dCas9 expression plasmids (pMZ455 and pMZ456). Error bars represent +/- one standard deviation.

3.7 Mutagenesis of additional sites

We attempted to confirm that this newly developed CRISPR/Cas9 mutagenesis system is effective at alternate target sites in different genes. The *clr4* and *set1* genes were selected for mutagenesis due to their roles in epigenetics. Each gene codes for a lysine methyltransferase (KMTs) which specifically methylates histone 3 lysine 9 (CLR4; Nakayama et al., 2001) or histone 3 lysine 4 (SET1; Roguev et al., 2003). These histone modifications are associated with condensed and transcriptionally inactive chromatin (heterochromatin) and relaxed and transcriptionally active chromatin (euchromatin), respectively. Each enzyme contains a SET domain which is necessary for the catalysis of lysine methylation (Jenuwein et al., 1998). Our goal was to disrupt the SET domain and abrogate chromatin reorganization by altering the peptide sequence from tyrosine to alanine at amino acid 451 of *clr4* and 801 and 897 of *set1* (Figure 18). To achieve this, CRISPR/Cas9 mutagenesis was performed by co-transforming the single plasmid system (Cas9/sgRNA) with the respective mutant HR template into an *ade6-M210* strain. DNA sequencing analysis showed 1 out of 3 colonies being *set1-Y810A* and 7 out of 10 colonies being *set1-Y897A*, but a *clr4-Y451A* colony was more difficult to generate (0 out of 10). The KMT CLR4 is responsible for the condensation and formation of pericentric heterochromatin which is essential for chromosome stability during chromatid separation (Kallgren et al., 2014, Alper et al., 2012). Without functional CLR4, chromosome breakage could occur and many cells would die or develop compensatory mutations. A reporter strain expressing the *ade6-M210* allele and also having an *ade6-wt* allele repressed (within a heterochromatin region) was applied to specifically identify a desired mutant. Fission yeast with an unmutated *clr4-wt* allele would remain phenotypically red (*ade6-M210* allele) due to the silencing of the ade6-wt reporter in heterochromatin, but colonies with the *clr4-Y451A* mutation would be unable to maintain heterochromatin and the *ade6-wt* allele would become expressed – they would be phenotypically white. DNA sequencing analysis of three phenotypically white colonies revealed that two contained the desired *clr4-Y451A* mutation. Thus, the approach can be successfully applied to several genomic loci and occasionally it may be useful to have a reporter when performing CRISPR/Cas9 mutagenesis in *S. pombe*.

Figure 18. Targeted nucleotide changes by CRISPR/Cas9 mutagenesis. Change of amino acid sequence from tyrosine to alanine at amino acid 810 and 897 of *set1* and 451 of *clr4*.

3.8 Precipitation of specific DNA sequences by dCas9

Another goal of our research was to develop a catalytically dead Cas9 that was capable of site-specific DNA targeting and could be immunoprecipitated with proteins transiently associated with a specific DNA sequence. A dCas9 expression vector was produced by combining the nmt1 promoter, published Cas9 sequence with mutant HNH and RuvC-like catalytic domains (Gilbert et al., 2013), an in frame C-terminal 3x HA tag, and the pART1 backbone. Targeting sgRNAs were created for a consensus long terminal repeat (LTR) sequence of the retrotransposons Tf1 and Tf2 and the replication terminator found in ribosomal DNA arrays (TER1); the sgRNA directed against *ade6-wt* was also used. Plasmids with sgRNA not targeting TER1, LTR, or the *ade6* gene were also generated. Cells were transformed with the nmt1dCas9 plasmid and one targeting sgRNA or one non-specific sgRNA, and plated on a medium containing 10µM thiamine (dCas9 repressed). Separate cultures for the six tranformations were established in a medium also with 10µM thiamine and were fixed with formaldehyde. Subsequently, crosslinked chromatin were sheared via sonication and chromatin-dCas9 complexes were immunoprecipitated by anti-HA antibodies. Quantitative PCR analysis of the bound DNA sequences show there is some enrichment of targeted sites over the nonspecific sites for TER1, LTR, and the *ade6* gene (Figure 19). However, there are multiple LTR and TER1 sites in the *S. pombe* genome, but only one *ade6* gene. The successful enrichment of *ade6* sequence demonstrates that dCas9 is sensitive enough to target unique sites and selectively immunoprecipitate them, but is unlikely suitable to identify other complexed proteins.

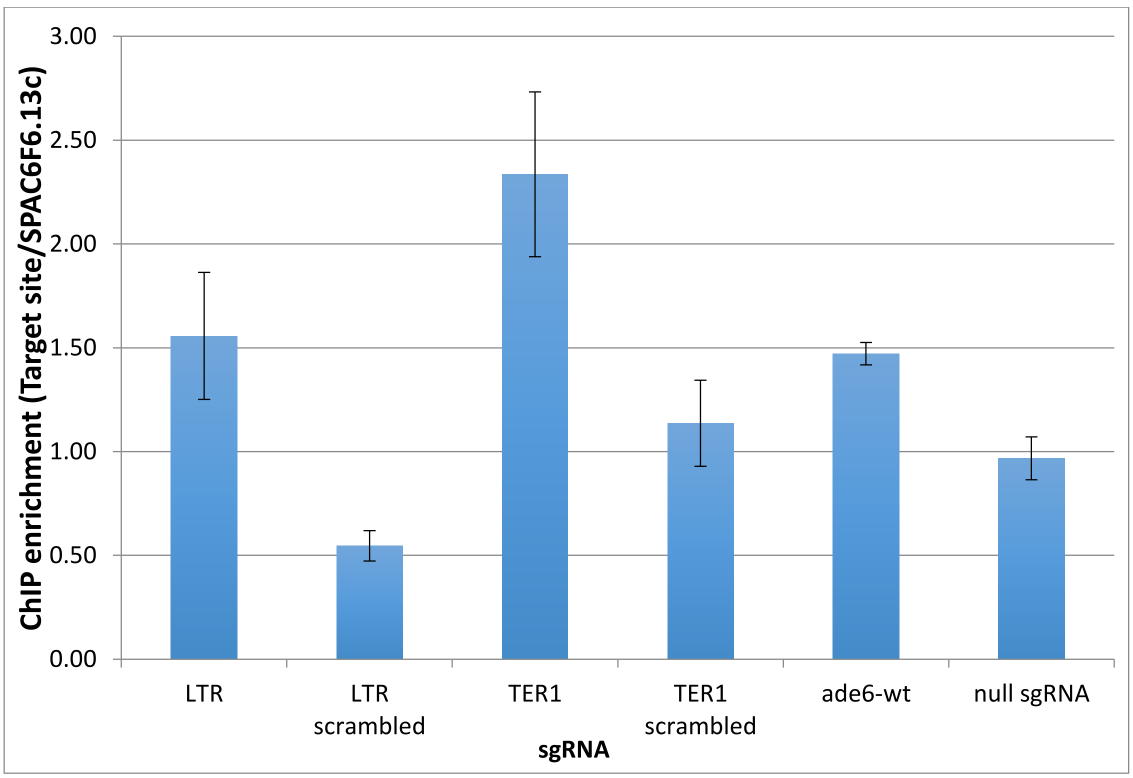


Figure 19. HA ChIP performed on various sites targeted by sgRNA. Target site enrichment with site specific sgRNA (LTR, TER1, *ade6-wt*) and corresponding non-specific sgRNA (LTR scrambled, TER1 scrambled, null sgRNA). Error bars represent +/- one standard deviation.

4. Discussion

4.1 Improving upon current methods of *S. pombe* mutagenesis

Several approaches are used for site-directed mutagenesis of the *S. pombe* genome. Numerous techniques have been developed that utilize homologous recombination of endogenous alleles with mutant alleles retained on selectable plasmids (Mudge et al., 2012; Gao et al., 2014). However, HR is more efficient in *S. cerevisiae* than in *S. pombe* and excision of plasmid marker genes requires a secondary homologous recombination event. This necessitates the cloning of large homologous regions to even achieve modest efficiencies (13-77%; Gao et al., 2014). Oligonucleotides of marker genes may also be inserted in a site-specific manner through HR, but then there is the risk of attributing marker-based effects to the introduced mutations. Alternately, several selection steps and mass screenings may be performed to employ *delitto perfetto* approaches and remove these marker sites (Storici et al., 2001). CRISPR/Cas9 mutagenesis requires no marker as DSBs are produced until, at high efficiency, mutations of the target sequence are produced. The single vector also reduces the time for potential inactivation and expresses the counterselectable marker *ura4* for simplified removal of the plasmid, with 5-fluoroorotic acid, prior to downstream studies.

4.2 Implementation of catalytically inactive Cas9 for DNA-specific binding in *S. pombe*

The three most recent approaches to assessing DNA-protein interactions are proteomics of isolated chromatin (PICh), insertional chromatin immunoprecipitation (iChIP), and engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP). PICh targets specific genomic regions through hybridization with biotinylated, locked nucleic acid (LNA) probes and pulldown using streptavidin beads (Déjardin and Kingston, 2009). Probes from PICh have some specificity and have been reported to successfully identify proteins that associate with repetitive sequences like satellites and telomeres, but not low copy number or unique genomic sequences. iChIP was initially developed to target specific genomic sites by tagging the location of interest with the DNA-binding protein, LexA, prior to pulldown of sheared chromatin (Hoshino and Fujii, 2009). This technique was later shown to successfully identify some DNA-protein interactions (Fujita and Fujii, 2011), but there is the possibility of LexA altering surrounding chromatin. The most recent approach is enChIP which utilizes a dCas9, tagged with 3x FLAG, that binds to endogenous DNA sequence specified by a sgRNA (Fujita and Fujii, 2013). Employment of enChIP is advantageous because it is sensitive enough for unique sites and does not need a tag at the target site. Without these handicaps, enChIP assists in the non-biased isolation of proteins associated with DNA and can be readily combined with mass spectrometry to identify these proteins. Our dCas9 system follows a similar approach, but makes use of a 3x HA fusion and is expressed in *S. pombe* instead of human 293T cells. Enrichment values approaching eight were observed while using enChIP indicating there is room for improvement over the maximum 3-fold enrichment seen by our construct. Nonetheless, our system has potential if modifications, such as an N-terminal tag, can increase enrichment.

4.3 Summary

The model biological system, *S. pombe*, is the ideal tool for use in basic research to explore aspects of the cell cycle, chromosome biology (including pericentric heterochromatin and telomeres), and DNA repair due to having significant homologies with higher eukaryotic cells. Through our studies we described two approaches which researchers may use to enhance their experimental capabilities for this organism. The CRISPR/Cas9 mutagenesis platform assists in the efficient engineering of the fission yeast genome and, by altering the length of the sgRNA, can perform transcriptional regulation concurrently with its editing function (Kiani et al., 2015). The catalytic null Cas9 toolset enables tethering of the enzyme to specific endogenous DNA sequence without digestion. Researchers have exploited this activity through a multitude of dCas9 modifications such as fusion of an antibody tag for the un-biased pulldown of sheared chromatin and associated proteins (Fujita and Fujii, 2013), fusion of an acetyltransferase to increase the expression of targeted genes (Hilton et al., 2015), and fusion of chromatin-condensing protein domains or targeting of enhancer sites to reduce gene expression (otherwise known as CRISPR interference or CRISPRi; Gilbert et al., 2013, Qi et al., 2013). However, access to Cas9 and dCas9 methods in *S. pombe* is predicated on our development of the flexible, RNA polymerase II-expressed, *rrk1* sgRNA system.

In addition to simply achieving expression in *S. pombe*, generation of this sgRNA cassette has advantages over the earlier *S. cerevisiae*-based sgRNA described in DiCarlo et al. (2013). RNA polymerase II expression allows for targeting of sequences with six consecutive thymines, which is inaccessible to the RNA polymerase III-expressed SNR52 promoter that was used – six consecutive thymines is a terminator of RNA polymerase III transcripts (Braglia et al., 2005, Wang and Wang, 2008). Also, our inclusion of a Hammerhead ribozyme facilitates the precise processing of sgRNA that is necessary to apply the transcriptional regulation approach reported in Kiani et al. (2015).

Our research also suggests that enhancements may be made to improve and expand the Cas9 toolset that we developed. CRISPR/Cas9 mutagenesis exhibited high efficiency and specificity for genome editing, but dCas9 demonstrated only marginal specificity for targeted genomic sites. Additionally, generation of dCas9 was not sufficient to prevent the toxicity of Cas9 and recapitulate the wild-type growth phenotype. This shows that catalytic activity is not responsible for toxicity and further experimentation will be necessary to discover its source. Subsequent engineering of non-toxic Cas9 and dCas9 may increase dCas9 specificity and further improve the efficiency of CRISPR/Cas9 mutagenesis. In conjunction with the increase in mutagenesis efficiency, implementation of alternate protospacer adjacent motifs would expand the number of sgRNA target sites available to Cas9/dCas9 (Esvelt et al., 2013) and usage of the homology-integrated CRISPR (HI-CRISPR) system, described in Bao et al. (2015), would enable mutagenesis of multiple target sites simultaneously. Regardless of the potential for future innovation, the methods and constructs described by our lab will serve as a convenient and powerful platform for use by *S. pombe* researchers to investigate phenomena in the field of genome biology and beyond.

Table 1. Description of plasmids used.

lab ID	expression cassette	sgRNA target	marker/ replication origin	backbone	Addgene number
pMZ222	*adh1*:Cas9	N/A	*LEU2/ars1*	pART1	52223
pMZ283	*rrk1*:sgRNA	Null	*ura4/ars1*	pUR19	52224
pMZ284	*rrk1*:sgRNA	*ade6-wt*	*ura4/ars1*	pUR19	52225
pMZ285	*rrk1*:sgRNA	*ade6-M210*	*ura4/ars1*	pUR19	52226
pMZ286	*rrk1*:sgRNA	*ade6-L469*	*ura4/ars1*	pUR19	52227
pMZ288	*adh1*:Cas9/ *rrk1*:sgRNA	*ade6-wt*	*ura4/ars1*	pUR19	59897
pMZ289	*adh1*:Cas9/ *rrk1*:sgRNA	*ade6-M210*	*ura4/ars1*	pUR19	59898
pMZ373	nmt1:Cas9	N/A	*LEU2/ars1*	pART1	N/A
pMZ374	*adh1*:Cas9/ *rrk1*:sgRNA	Null	*ura4/ars1*	pUR19	59896
pMZ381	*adh1*:Cas9/ *rrk1*:sgRNA	*ade6-L469*	*ura4/ars1*	pUR19	59899
pMZ382	*adh1*:dCas9	N/A	*LEU2/ars1*	pART1	N/A
pMZ394	*adh1*:Cas9/ *rrk1*:sgRNA	*set1-y897*	*ura4/ars1*	pUR19	N/A
pMZ395	*adh1*:Cas9/ *rrk1*:sgRNA	*set1-y810*	*ura4/ars1*	pUR19	N/A
pMZ453	*adh1*:Cas9/ *rrk1*:sgRNA	*clr4-y451*	*ura4/ars1*	pUR19	N/A
pMZ455	nmt1:dCas9	N/A	*LEU2/ars1*	pART1	N/A
pMZ456	nmt41:dCas9	N/A	*LEU2/ars1*	pART1	N/A
pMZ471	*rrk1*:sgRNA	Scrambled LTR sequence	*ura4/ars1*	pUR19	N/A
pMZ472	*rrk1*:sgRNA	Scrambled TER1 sequence	*ura4/ars1*	pUR19	N/A
pMZ473	*rrk1*:sgRNA	Consensus LTR sequence	*ura4/ars1*	pUR19	N/A
pMZ474	*rrk1*:sgRNA	TER1 sequence	*ura4/ars1*	pUR19	N/A

Table 2. Description of DNA oligonucleotides used.

Lab ID	DNA sequence
oM12	CAAAGATCCTGTCGAATCACCTG
oM13	CAGTTATGTCTATGGTCGCCTATGC
oM16	TGATAGGTAACATTATAACCCAGT
oM17	ACGCAGTTTGGTATCTGATT
oM100	CTGCGGAACATTGGGACTAT
oM101	TTCTTTGCTCCACACACAGC

oM446	TTAAGCAAGAGAATTGCTGCAGGTCGACTCTAGAGATGGACA AGAAGTACTCCATTG
oM447	GGAAGGTTGGAATTCGAGCTCGGTACCCGGGGATCGCAAATT AAAGCCTTCGAGCGTC
oM448	TGGGATCCGAGATAAGCAGTCTGGAAAGACAATCCT
oM449	AGGATTGTCTTTCCAGACTGCTTATCTCGGATCCCA
oM450	TCTATTGTTCAGATGCCTCGGT
oM452	TCTATTGTTCAGATGCTTCGGT
oM454	TCTATTGTTCAGATGCCTTGGT
oM456	CGAGGCATCTGAACAATAGATT
oM457	CGAAGCATCTGAACAATAGATT
oM458	CAAGGCATCTGAACAATAGATT
oM473	CGAGTCGGTGGTGCTTTTTTTCGGTACCGTGGGGATCCTCTAG AGTCGAC
oM474	ACTACCACCAACATAAGCAAAAGGTACCCGGGGTACCGAGCT CGAATTC
oM532	TTGTGCTCTTCATCCTGTGC
oM533	GAATCCGAGATTTCGTCCAA
oM534	GGTAAGGTAGGTCGTGAATCG
oM535	ATTTGAAAAGGGGGAACCAC
oM546	ACTCGGTGCCACTCACTTTT
oM547	ACGGACTAGCCTTATTTTAACTT
oM550	GAAAGCACATCCGGTGACAGGGCACCACCGACTCGGTGCCAC TC
oM551	GAGTCCGTGAGGACGAAACAGGGGTACCGTGGGGATCCTCTA GAG
oM552	CCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGA CGAAACAGG
oM553	CCTGTTTCGTCCTCACGGACTCATCAGACCGGAAAGCACATCC GGTGACAGG
oM554	CCCGAAAAGTGCCACCTGACGCCCTACAACAACTAAGAAAAT G
oM555	GATAATAATGGTTTCTTAGACGGGGATCGCAAATTAAAGCC
oM562	TTACATTTGATGCTGCTGGTGCAAAAG
oM563	TTTTGCACCAGCAGCATCAAATGTAAG
oM564	TGTTTGATGATGCTAGCGAGT
oM565	CCGATAATTGTTCGGCAAAT
oM566	GAGCTTACATTTGATTACGCGT
oM567	GCGTAATCAAATGTAAGCTCTT
oM568	GTCATAGAGGCTATTGGAGAAATC
oM569	GATTTCTCCAATAGCCTCTATGAC
oM570	GCCGTATCACATCACGAATG
oM571	TAGGAGCGCATGAATGATTG
oM572	GATATGGTCATAGAGTATATGT
oM573	ATATACTCTATGACCATATCTT

oM599	AGCTGACCTTAGCCAGTCCA
oM602	TACTTATGATTACAAGTTTCGT
oM603	GAAACTTGTAATCATAAGTATT
oM604	TTATGATGCCAAGTTCCCGGAAGA
oM605	TCTTCCGGGAACTTGGCATCATAA
oM606	TTGCCATGGAAAACATTGAT
oM607	TCCTGAATGCAATCCACTGA
oM721	CTTGGAACTACCTACCTTGC
oM729	ATTGCTGCAGGTCGACTCTAGAGGATCCCCATGGACGTCCCAA AGAAGAAG
oM777	TGCAGGTCGACTCTAGAGATGGACA
oM778	ATTGTCTTTCCAGACTGCTTATCTC
oM801	TGTCCATCTCTAGAGTCGACCTGCAGAGGATATGCCAGGATTC CTCTTCC
oM802	AACGACGTAGTCGACAAGCTTGCATGCCGCCATAAAAGACAG AATAAGTC
oM905	GTAGTCGATCGACAAGCTTGCATGCCGCCATAAAAGACAGAA TAAGTCATC
oM906	CTCTTCTTCTTTGGGACGTCCATGGGAGGATATGCCAGGATTC CTCTTC
oM1002	GATATGTTATTCGGTTAATAGT
oM1003	TATTAACCGAATAACATATCTT
oM1004	ATAGAATAAGTCGAGAGTAGGT
oM1005	CTACTCTCGACTTATTCTATTT
oM1006	TTATGAGCTATATTAGTGATGT
oM1007	ATCACTAATATAGCTCATAATT
oM1008	GGAATTGTCAAAATAGGAGAGT
oM1009	TCTCCTATTTTGACAATTCCTT
oM1010	AGCACTCTTGACGGAGTTGA
oM1011	AGGGAGGGTTGAAATGTAGC

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