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Mini-Review

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Yeast Still a Beast: Diverse Applications of CRISPR/Cas Editing Technology in *S. cerevisiae*

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The recent discovery and use of CRISPR/Cas9 gene editing technology has provided new opportunities for scientific research in many fields of study including agriculture, genetic disorders, human disease, biotechnology, and basic biological research. The ability to precisely target DNA sequences and either remove, modify, or replace genetic sequences provides a new level of control in nearly all eukaryotic organisms, including budding yeast. Given the many discoveries made in *Saccharomyces cerevisiae* over the past decades spanning genetics, cell biology, and biochemistry, as well as the development of new technologies that have allowed high throughput screening, robotic automation, and a platform for synthetic genome engineering, the yeast community has also started to recognize the utility and complementary nature of CRISPR-based methodologies. Here we present and review a variety of recent uses of Cas9 in budding yeast—both nuclease dependent and independent applications spanning traditional gene editing and replacement, to transcriptional modulation, to novel uses including the development of living circuitry or robotic platforms for synthetic genome construction. Yeast continues to serve as a powerful model system, yet it can still benefit from use of CRISPR for basic research, industrial application, and innovation of new Cas9-based applications.

INTRODUCTION

Saccharomyces cerevisiae (budding yeast) is one of the most well studied, genetically tractable organisms. As a model eukaryote, it has provided critical insight into the basic biology of the cell cycle [1], endomembrane vesicular trafficking [2], autophagy [3], and many other cellular systems. Part of the success for the tractability of yeast in both industry and basic research stems from the ability to rapidly edit and manipulate genomes. This

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†Abbreviations: HR, homologous recombination; HDR, homology directed repair; PAM, protospacer adjacent motif; CRISPR, clustered regularly interspaced short palindromic repeats; SGA, synthetic genetic array; sgRNA, single guide RNA; DSB, double-stranded break; dCas9, nuclease inactive (dead) Cas9; PCR, polymerase chain reaction; RNAi, RNA interference; Mb, megabase, million base pairs; Kb, kilobase, thousand base pairs; TetO, tetracycline controlled transcriptional system; WT, wild type.

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has led to the development of genome-wide libraries [4-6], synthetic genetic array (SGA†) technology [7], and markerless integration methods [8], to name only a few. The recent interest and explosion of research into CRISPR/Cas9-based editing across many model systems has also finally reached the yeast community.

CRISPR (clustered regularly interspaced palindromic repeats) has evolved as a primitive immune system in prokaryotes with the ability to precisely target and edit any genome [9-12]. Briefly, the Cas9 endonuclease of the Class II CRISPR system (typically from *Streptococcus pyogenes*) is first bound to a single-stranded piece of RNA (sgRNA; single guide) containing a short stretch of nucleotides that bind and recruit the Cas9/RNA complex to the corresponding sequence within a target genome that is also marked with a "PAM" (protospacer adjacent motif) 5'-NGG-3' sequence [13]. The dual nuclease domains of Cas9 cause a double-stranded break (DSB) at the +3 position upstream of the PAM. Eukaryotes, including yeast, have evolved multiple DNA repair systems to handle the presence of DSBs, including repair by non-homologous end joining (NHEJ) [14] and homology directed repair (HDR) [15]. Introduction of DSBs at genomic position(s) by Cas9 has allowed for gene replacement, gene deletions, pathway construction, and single base editing in many eukaryotic organisms including humans [16-21]. This technology has obvious application to not only basic research, but industry, agriculture, biofuels, bioenergy, human pathogens, genetic disorders, and disease [22-25].

While the CRISPR system was first piloted in budding yeast in 2013 [26], a number of methodological and technical hurdles likely slowed its progression through the yeast community. First, we speculate that given the incredible efficiency by which *S. cerevisiae* *already* performs homologous recombination *in vivo* [27] sans any DSB, it seemed puzzling how the Cas9 nuclease might provide a significant advance from traditional molecular methodologies [5]. Second, and along these lines, a number of technical challenges including optimization of both expression and delivery of Cas9 and the sgRNA(s) had to first be overcome. However, recent efforts have provided a new suite of molecular tools using the CRISPR/Cas9 system that are being applied to a diverse array of methodologies in *S. cerevisiae* including multiplexed editing, markerless manipulation, chromosome splitting, transcriptional modulation, synthetic genome engineering, and gene drive technology.

YEAST GENOME MANIPULATION USING *S. PYOGENES* Cas9

As the CRISPR/Cas9 gene editing system was tested in model systems, editing was also successfully demonstrated in *S. cerevisiae* using an inducible promoter to drive Cas9 expression and a high copy plasmid-driven RNA polymerase III regulated promoter (*prSNR52*) to express the sgRNA cassette [26]. Activation of the Cas9/sgRNA complex resulted in double-stranded break formation in > 99 percent of yeast and subsequent cell death. However, introduction of donor DNA (as an oligonucleotide) greatly increased repair of the DSB and resulted in successful editing; this process is also extremely efficient when using amplified PCR fragments even with limited homology [28].

In contrast to other eukaryotes, NHEJ is extremely inefficient in yeast and double-stranded DNA breaks are repaired by HR. This may represent one of the challenges in adopting the new technology in *S. cerevisiae*. Indeed, a number of studies have provided insight into optimizing use and application of the CRISPR technology *in vivo*. Early efforts have examined the mechanism of delivery and expression of the Cas9 nuclease—moderate, rather than high-expression is preferred [29], as well as sequential delivery of Cas9 prior to the sgRNA cassette [30]. Moreover, various strategies have been developed for expression and delivery of the sgRNA component including (i) the original [26] Pol III promoter (*SNR52*) and terminator (*SUP4*) pair, (ii) the "natural" split two-component crRNA/tracrRNA [31], (iii) an amplified linear guide cassette (sans any circular plasmid backbone) [30], or (iv) a modular sgRNA design with use of a self-cleaving ribozyme fused to the 5' end of the guide sequence [29]. Finally, improvements to targeting multiple genomic loci (termed "multiplexing") in a single transformation event by cloning and delivery of unique sgRNAs [30,32] have greatly expanded the possibilities for yeast strain creation.

The ability to manipulate the genome at multiple loci in a single editing event [30,31,33-37] presents a serious upgrade from conventional cloning methods (such as homologous recombination (HR)-dependent integration or mating yeast followed by sporulation) for several reasons. First, as previously mentioned, since Cas9-induced DSBs are poorly tolerated in yeast, general survival following editing can be utilized as a powerful selection tool *without* the need for any selectable markers [28,29,32,35,38-41]. The ability to manipulate genomic loci sans auxotrophic or drug resistance cassettes provides a serious advantage for all research areas in budding yeast. This allows for (i) the use of more plasmid-borne constructs with classic selectable markers, (ii) the manipulation of yeast strains that are lacking a variety of auxotrophic marker(s), and (iii) the use of stably integrated mutations at their endogenous loci instead of plasmid-driven versions that require selection, and may provide yeast with an opportunity to vary the

plasmid copy number per cell. Second, this allows for introduction of precise genomic alterations including single point mutations [42] or editing of essential genes [28,36]. Third, DSB formation greatly aids in large-scale gene replacement, pathway integration, and modulation of existing (or new) biosynthetic pathways. Combining Cas9 editing with *in vivo* fragment assembly, Mans and colleagues reconstituted a six-gene pathway (pyruvate dehydrogenase complex) from *E. faecalis* at the *ACS2* locus in a single step (Figure 1A) [43]. Other groups have also demonstrated the great utility of engineering entire pathways *in vivo* for both basic research and potential industrial application [29,35,36,38,40,44-46].

The utility of Cas9 for DNA manipulation is continuing to expand beyond simple DSB formation and subsequent repair by HDR (Table 1). For instance, Sasano and colleagues have developed a modular toolkit for splitting and stable propagation of entire yeast chromosomes into two or more smaller chromosomes (Figure 1B). Following the DSB, HDR occurs on provided donor DNA modules that insert both a new centromere on the broken chromosome arm(s) as well as a telomere seed sequence repeat to the newly formatted chromosome ends [47]. Moreover, CRISPR has been used to delete large genomic fragments (> 30 Kb) [48] or to construct synthetic promoter elements *in vivo* [34].

NUCLEASE-DEAD Cas9 AS A TARGETING SCAFFOLD

Aside from its traditional role as an endonuclease, Cas9 has also been engineered to separate its DNA targeting function from that of its DNA cleaving enzymatic function [49]. Mutation of only two residues (D10A and H840A) inactivates both nuclease domains, yet does not disrupt the ability of Cas9 to bind the sgRNA nor target the intended genomic loci. Termed "dead" Cas9 (dCas9), this serves as a molecular recruitment tool to ultimately deliver a secondary protein of interest to target regulatory sequences. In 2017, Jensen and colleagues demonstrated the ability of dCas9 to modulate gene expression in yeast using both (direct) translational fusions and appended sgRNA-RNA-binding domain (indirect) tethers to transcriptional activators (VP64) or repressors (Mxi1). This group screened various sgRNA sequences for ideal positioning of dCas9 complex to the site of 14 yeast promoters with the intent of modulating flux through two biosynthetic pathways (Figure 1C) [50]. Similar studies have also focused on sgRNA identity (and dCas9 positioning) in order to perturb metabolic pathways [51]. Work in yeast has also illustrated the use and development of more complex RNA binding scaffolds to recruit multiple RNA-binding proteins (fused to transcriptional activators) [52]. However, the utility of such transcriptional modulation is not limited to endogenous transcriptional regulation. A recent study has adopted the dCas9-Mxi1 fusion to develop digital "logic circuits" in yeast [53] using guide RNA switches genetically wired together. An engineered cell-to-cell communication system was also developed in yeast using CRISPR transcription factors [54]. The utility of dCas9 extends far beyond that of the nuclease active protein since a variety of additional DNA/chromatin-modifying enzymes can be routinely fused or recruited to Cas9 and would provide a powerful platform for genome-wide screening in yeast not only restricted to transcriptional modulation. One example of this has been use of a Protein A fusion to dCas9 to isolate and identify by mass spectrometry the "epiproteome" of a yeast promoter [55]. There is enormous potential to utilizing dCas9 as a programmable physical scaffold onto which other DNA (or epigenome) modifying enzymes can be targeted. An explosion of Cas9 protein fusions has provided an ever-expanding suite of options for inducible, chemically regulated, or even split nuclease systems [56].

SYNTHETIC GENOME CONSTRUCTION

Work in budding yeast has been instrumental in the field of synthetic genome engineering. The *S. cerevisiae* 2.0 project (Sc2.0) aims to create the entire yeast genome *de novo* with a variety of designed modifications (removal of introns, grouping of tRNAs, introduction of loxP recombination sites, telomere modifications, etc.) [57]. While this project has relied mainly on traditional HR-directed integration of artificial chromosomal segments in place of the native sequence, CRISPR/Cas9 allowed for repair of mutations found during this construction process [58]. Moreover, entire 1 Mb bacterial genomes (*Mycoplasma* and *Escherichia*) have been edited *in vivo* in yeast cells using CRISPR [59,60] (Figure 1E). Recent work in *S. cerevisiae* has also demonstrated the ability to utilize a combination of high-throughput automation, genome-scale engineering, and Cas9-based gene editing [61]. This group created standardized fragments of native yeast genes (either overexpression or knockdown using either sense or antisense transcripts in an RNAi active yeast background) and these constructs were all integrated at repetitive DNA sequences in the yeast genome using Cas9. However, this system is (currently) unable to target native loci; the development of genome-wide collections of sgRNAs would provide the option to manipulate endogenous genomic sites.

At the interface of engineered yeast genomes and CRISPR-based editing, the first use of introduced *artificial* Cas9 target sites into the budding yeast genome was recently performed [28]. This application will likely have great utility as the use of synthetic genes (and

Table 1. Overview of recent applications of CRISPR/Cas9 gene editing technology in *S. cerevisiae*.

Category	Technology	Description	Reference	Additional Studies
Traditional Cas9-Based Gene Editing Methodologies	*Di-CRISPR* (Delta Integration CRISPR/Cas)	Multiplexing[1] of Cas9 (markerless, single-step integration of biochemical pathways) by targeting repeated delta sites[2] throughout yeast genome.	[38]	[30-32,37,41-45,70,71]
	mCRISTAR (Multiplexed CRISPR Transformation-Associated Recombination)	Use of Cas9 to target and replace endogenous promoter elements.	[34]	
	Large Chromosomal Fragment Deletion	Generation of chromosomal deletions up to 30 Kb.	[48]	
	Cas9 Nickase	Use of a Cas9 nickase[3] variant to edit bases distal (50+ bps) to the target site.	[72]	
Novel Cas9-Based Applications	*CRISPR-PCS* (CRISPR PCR-Mediated Chromosome Splitting)	Use of Cas9 to split and generate new chromosomes complete with centromeres and telomere seed regions.	[47]	[73,74]
	CRISPR-ChAP-MS (CRISPR-Based Chromatin Affinity Purification with Mass Spectrometry	Allows for dCas9-targeted purification of different chromatin regions coupled with protein and PTM identification via MS.	[55]	
Transcriptional Regulation (via Dead Cas9)	*CRISPRi* (Genome Scale CRISPR Interference)	Use of nuclease deficient ("dead") dCas9 for repression of gene expression of endogenous genes.	[49]	[52,53,75]
	dCas9-Mediated Transcriptional Reprogramming	Use of either direct or indirect dCas9 constructs to transcriptional activator (VPR) or repressor (Mxi1) to modulate gene expression.[4]	[50]	
	Graded Expression of Pathway Enzymes via dCas9 Positioning	Varied[5] sgRNA targeting of dCas9 for tuned expression of metabolic pathway genes.	[51]	
Synthetic Genome Engineering	Automated Multiplex Genome Engineering	High-throughput, robotic-based construction of overexpression or mutated alleles using Cas9 at repetitive genomic sequences.	[61]	
	In-Yeast engineering of a Bacterial Genome	Engineering of a deletion mutant of the *Mycoplasma* bacterial genome (1.2 Mb) using Cas9.	[59]	
	CasHRA (Cas9-Facilitated Homologous Recombination Assembly)	Construction of the Minimal *Escherichia coli* genome (1.03 Mb) using large circular DNAs that are subsequently cleaved via Cas9 and assembled into the genome.	[60]	
	Synthetic Yeast Genome (SynV) Construction	*De novo* synthesis of the yeast Chromosome V (0.54 Mb) and replacement of the endogenous sequence using Cas9 and HR.	[58]	

Table 1 cont'd.

	mCAL (Multiplexing of Cas9 at Artificial Loci)	Use of artificial[6] Cas9 target sequences (20+3 bp PAM) to multiplex Cas9 with a single sgRNA to different loci.	[28]
Gene Drive	Gene Drive Safeguarding	Development and testing of a yeast Cas9-based gene drives[7] to address safety concerns, positioning of the sgRNA (plasmid versus integrated), and fail safes to remove existing drives.	[67]

[1]Multiplexing: Targeting of Cas9 to multiple genomic targets. This can be accomplished by a single sgRNA (to a repeated genomic sequence—telomeres, delta elements, etc.—or "engineered" target sites [28] placed throughout the genome).
[2]Delta sites: Repeated Ty retrotransposon delta sites within the yeast genome.
[3]Cas9 Nickase: a mutated enzyme variant that has one of the nuclease cleavage domains mutated—this causes a single-stranded break (a nick) rather than a double-stranded break.
[4]Two versions of transcriptional activator/repressor tethers were used to dCas9: (i) direct translational fusions to VPR (VP64-p65-Rta transcriptional activator) or Mxi1 (repressor) or (ii) indirect recruitment of a MCP-VPR or PCP-Mxi1 (both RNA scaffold binding protein fusions) to the scaffold-extended sgRNA sequences.
[5]Positions between +30 to +750 bps upstream of the TATA box were analyzed by sgRNA targeted dCas9.
[6]Artificial Cas9 target sequences: 20 base paid target sequences and a 3 bp 5'-NGG-3' PAM sequence chosen to provide the maximum mismatch with the entire yeast genome were engineered and placed at several genomic loci.
[7]A Cas9 "gene drive" is defined as the placement of the Cas9 gene at a genetic locus (either deleting or modifying the native gene), accompanied by expression of an sgRNA that targets the Cas9 nuclease to the site of the WT endogenous gene on the opposite, homologous chromosome within a diploid cell. DSB formation causes the entire Cas9-containing drive to be copied to the second chromosome via homologous recombination.

genomes) becomes more widespread. Pre-loading a yeast strain with identical DNA target sites (23 bps) allows for only a single guide RNA construct to be expressed to multiplex Cas9 across the genome (Figure 1D). In this way, all genes within a signaling pathway, or within a macromolecular complex, or evolved gene paralogs could be simultaneously targeted in any combination desired. A major concern of the CRISPR field has been to reduce and eliminate off-target effects—recruitment and unintentional editing of other genomic positions [62-64]. Use of artificial programmable genomic site(s) and the corresponding sgRNAs could aid in reducing off-target Cas9 editing in yeast and other organisms.

GENE DRIVE TECHNOLOGY

One of the most intriguing and powerful arrangements of the CRISPR/Cas9 technology is in a "gene drive." Briefly, the endonuclease is integrated at the site of a target locus (typically replacing and deleting the endogenous gene) in addition to a guide RNA cassette (Figure 1F). The sgRNA targets Cas9 to a site present on the WT (wild type) copy of the gene on the homologous chromosome within a diploid cell. Double-stranded break formation and subsequent HR causes disruption of the WT allele on the homologous chromosome by the gene drive cassette. This mechanism bypasses the restrictions imposed by standard Mendelian genetics. This *super-Mendelian* arrangement can rapidly sweep through a population as nearly 100 percent of the progeny (from each generation) are homozygous diploid for the affected (or deleted) allele. Recent work has utilized this technology in flies and mosquitos with the intent of population control on a widespread level to restrict and eliminate the spread of insect-borne diseases [65,66]. Recent work in budding yeast has demonstrated the utility of this model organism for testing various gene drive arrangements with the intention of studying methods for (i) safely utilizing or (ii) halting active drives [67]. For example, the Church lab found that separation of the sgRNA-expressing cassette (on an unstable, high-copy plasmid) was a preferable safeguard to chromosomal integration adjacent to the Cas9 gene. Moreover, they piloted an experiment to illustrate the utility of a secondary gene drive-containing strain to actively target an original (theoretically "escaped") drive. Given the severity of accidental (or intentional) release of an engineered, active gene drive-containing organism (of any type) [68,69], yeast can serve as a safe and useful model system to assay various Cas9 drive arrangements for future implementation in insects or other eukaryotic systems.

FUTURE PERSPECTIVES

Given the profound contributions of the yeast community to many aspects of eukaryotic cell and

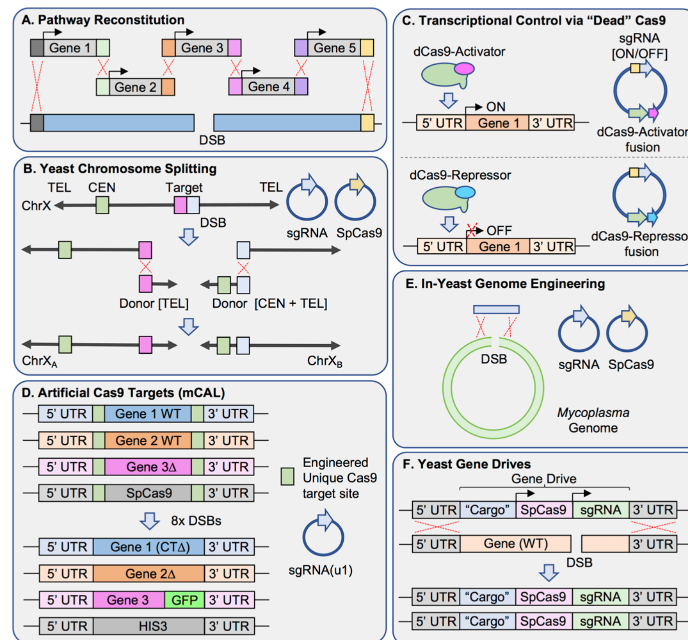

Figure 1. Diverse set of unique CRISPR/Cas9 methodologies employed in *S. cerevisiae* in recent years. (**A**) Traditional nuclease-based editing using Cas9 allows for the introduction of multiple non-native genes into the yeast genome in a single step. This study reconstituted the six genes (five illustrated) required for a pyruvate dehydrogenase complex (from *E. faecalis*) *in vivo* [43]. Each "Gene" represented contains both flanking UTR as well as unique 60-base pair segments (different colors used) to allow for homologous recombination. Following targeting of Cas9/sgRNA to the locus of interest, HR and subsequent integration of the entire six-gene cassette repairs the double-stranded break (DSB) and replaces the endogenous yeast gene. (**B**) Cas9 based method for chromosome splitting (CRISPR-PCS) [47]. A yeast chromosome (Arrow: telomere, Green box: centromere) is targeted for splitting into two or more smaller complete chromosomes by plasmid-expressed Cas9 and an sgRNA. Following introduction of the DSB, donor DNA is provided that will allow for repair of each fractured chromosome fragment. A homologous sequence (pink or blue) is included to link each unique donor DNA fragment to the appropriate chromosome segment. *Left*, the severed chromosome arm is lacking a telomere; the donor module includes a telomere seed sequence repeat. *Right*, the separated chromosome arm (now lacking a centromere) performs HR with the appropriate donor module to introduce both a capping telomere seed sequence and yeast centromere. This methodology allows for the generation of two (or more) functional chromosomes. (**C**) Transcriptional regulation of multiple yeast promoters using catalytic dead Cas9 (dCas9) fusions and an inducible sgRNA system [50]. The sgRNA cassette is under control of the TetO system (ON/OFF). Nuclease deficient Cas9 is fused to either a transcriptional activator (VPR; VP64-p65-RTA) or repressor (Mxi1). Expression of different sgRNAs recruits dCas9-A (activator) or dCas9-R (repressor) to the promoter element of interest (14 separate promoters tested with over 100 sgRNAs) to modulate transcription of the target gene(s). (**D**) Multiplexing using artificial Cas9 target sequences (mCAL) [28]. The introduction of non-native target sequences (20 bp target + 3 bp PAM) at multiple loci (illustrated as flanking three sample genes as well as an integrated copy of Cas9 at the *HIS3* locus) allows for a single sgRNA construct (u1; unique sequence 1) to target this identical sequence at every position in the genome. Introduction of donor DNA with appropriate flanking sequence allows for HR-based integration of any version of each gene (full deletion, repair, domain deletions, point mutations, or tagged versions) as well as simultaneous excision of the Cas9-expressing cassette. (**E**) In-yeast genome engineering of a bacterial genome [59]. The combination of active Cas9, a targeting sgRNA (both on plasmids) as well as the entire *Mycoplasma mycoides* bacterial genome (1.2 Mb) was transformed into yeast. CRISPR-based DSB induction and subsequent HR-based repair (with a synthetic oligonucleotide) allowed for the deletion of a particular *M. mycoides* gene. (**F**) The study of gene drives using *S. cerevisiae* [67]. The Cas9-based "gene drive" consists of the following: (i) the Cas9 gene, (ii) the sgRNA-expressing cassette, and (iii) an optional "cargo" for a new or modified gene. In yeast, the sgRNA can be expressed from a plasmid or be integrated as the site of the drive. The entire drive is integrated into the genome and replaces (full or partial deletion) an endogenous gene. Activation of the gene drive system causes targeting of Cas9 to the homologous WT gene copy on the opposite chromosome (in a diploid yeast cell). Creation of the DSB induces HR-based repair using the entire flanking chromosomal sequence as donor DNA. Therefore, the entirety of the gene drive is copied and replaces the endogenous WT gene target. Illustrations are adapted from various sources.

molecular biology and the many recent technologies (SGA, genome wide imaging, genetic screening, automation, and synthetic and directed evolutionary biology) that are possible, it is critical that further exploration and innovation be performed in budding yeast using CRISPR/Cas9 editing. Recent studies have already begun to illustrate the utility of the traditional Cas9-based techniques over conventional cloning and integrating methods. Moreover, because the basic components (nuclease, guide RNA, target DNA, etc.) of the CRISPR system are highly similar (if not identical) in practice across different organisms, many of the findings (*e.g.,* editing, Cas9 alterations, or guide specificity) can be directly applicable to the entire field and are not restricted to only *Saccharomyces* or fungi. The identification and use of dual Cas systems (Cas9 orthologs), genome-wide sgRNA collections, and new dCas9 translational fusions present exciting new platforms to merge with existing (or new) yeast technologies. Active research in budding yeast should continue to embrace the use of CRISPR/Cas9 to explore, innovate, and develop new molecular methodologies.

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