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**mCAL: a new approach for versatile multiplex action of Cas9
using one sgRNA and loci flanked by a programmed target sequence**

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Running title: Simplified Cas9-based genome manipulation

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Abbreviations: CRISPR, clustered regularly-interspaced short palindromic repeats; crRNA, 20-bp targeting sequence; dCas9, catalytically-dead (inactive nuclease) Cas9 variant; eGFP, enhanced GFP; 5-FOA, 5-fluoroorotic acid; HR, homologous recombination; LTR, long terminal repeat; NHEJ, non-homologous end joining; ORF, open reading frame; PAM, protospacer adjacent motif; sgRNA, single-guide RNA; tracrRNA, Cas9 nuclease-recruiting sequence; u1 and u2, “unique” exogenous Cas9-target sequences (23 nucleotides each) from two human genes; UTR, untranslated region; WT, wild-type.

ABSTRACT

Genome editing exploiting CRISPR/Cas9 has been adopted widely in academia and in the biotechnology industry to manipulate DNA sequences in diverse organisms. Molecular engineering of Cas9 itself and its guide RNA, and the strategies for using them, have increased efficiency, optimized specificity, reduced inappropriate off-target effects, and introduced modifications for performing other functions (transcriptional regulation, high-resolution imaging, protein recruitment, and high-throughput screening). Moreover, Cas9 has the ability to multiplex, *i.e.* to act at different genomic targets within the same nucleus. Currently, however, introducing concurrent changes at multiple loci involves: (i) identification of appropriate genomic sites, especially the availability of suitable PAM sequences; (ii) the design, construction, and expression of multiple sgRNA directed against those sites; (iii) potential difficulties in altering essential genes; and, (iv) lingering concerns about “off-target” effects. We have devised a new approach that circumvents these drawbacks, as we demonstrate here using the yeast *Saccharomyces cerevisiae*. First, any gene(s) of interest are flanked upstream and downstream with a single unique target sequence that does not normally exist in the genome. Thereafter, expression of one sgRNA and co-transformation with appropriate PCR fragments permits concomitant Cas9-mediated alteration of multiple genes (both essential and non-essential). The system we developed also allows for maintenance of the integrated, inducible Cas9-expression cassette or its simultaneous scarless excision. Our scheme—dubbed mCAL for “**M**ultiplexing of **C**as9 at **A**rtificial **L**oci”—can be applied to any organism in which the CRISPR/Cas9 methodology is currently being utilized. In principle, it can be applied to install synthetic sequences into the genome, to generate genomic libraries, and to program strains or cell lines so that they can be conveniently (and repeatedly) manipulated at multiple loci with extremely high efficiency.

INTRODUCTION

Discovery of CRISPR (Clustered Regularly-Interspaced Short Palindromic Repeats)-based RNA-mediated adaptive immunity in bacteria and archaea (SOREK *et al.* 2013; SHMAKOV *et al.* 2015), and especially the RNA-guided DNA endonuclease Cas9 from the Class II CRISPR system of *Streptococcus pyogenes* (JINEK *et al.* 2012; DOUDNA AND CHARPENTIER 2014), has provided a remarkably versatile tool for modifying genomes (HSU *et al.* 2014; WANG *et al.* 2016). Combining the normally separate DNA sequence-binding crRNA with the Cas9-stabilizing tracrRNA into a "single-guide" or "synthetic-guide" (sgRNA) streamlined target site recognition (JINEK *et al.* 2012; RAN *et al.* 2013). Changes to the stem-loop architecture of the tracrRNA portion of a sgRNA greatly strengthen its affinity for Cas9 (CHEN *et al.* 2013) and shortening of the crRNA portion of a sgRNA to just 20 nucleotides reduces off-target action while preserving efficiency (PATTANAYAK *et al.* 2013). The range of DNA/chromosome-based applications has been further extended by engineering of *S. pyogenes* Cas9 [or use of Cas9 orthologs from other bacterial species (JINEK *et al.* 2014)] to relax its requirement for initiating DNA sequence recognition at a so-called PAM ("protospacer adjacent motif") site (5'-NGG-3') (KLEINSTIVER *et al.* 2015), or to inactivate one or both of its two (McrA/HNH-like and RuvC/RNAaseH-like) catalytic sites to create a "nickase" (FU *et al.* 2014) or a catalytically "dead" (dCas9) version (GILBERT *et al.* 2013), or to insert new functionalities (OAKES *et al.* 2014). Cas9 and associated sgRNAs have been used in diverse organisms for genome editing, both gene knock-outs (GAJ *et al.* 2013) and gene fusions (WEI *et al.* 2013), as well as to force biased inheritance of a desired allele within entire populations ("gene drives") (DICARLO *et al.* 2015; DONG *et al.* 2015; GANTZ *et al.* 2015). Cas9-mediated genome alterations have been achieved in bacterial species (JIANG *et al.* 2013; TSARMPOPOULOS *et al.* 2016), various fungi (DICARLO *et al.* 2013; WAGNER AND ALPER 2015), zebrafish (HWANG *et al.* 2013), *C. elegans* (FRIEDLAND *et al.* 2013), *D. melanogaster* (GRATZ *et al.* 2013), plants (MAO *et al.* 2013), and human cells (CHO *et al.* 2013; JINEK *et al.* 2013; MALI *et al.* 2013; RAN *et al.* 2013), including clinical trials to explore Cas9-

mediated therapy in infectious and inherited disease (KAMINSKI *et al.* 2016; MENDELL AND RODINO-KLAPAC 2016; SU *et al.* 2016). Additional applications include sequence-specific repression or activation of gene expression (CHENG *et al.* 2013; GILBERT *et al.* 2013; LA RUSSA AND QI 2015), fluorescent labeling of chromosomal loci (CHEN *et al.* 2013; CHEN *et al.* 2016); and, RNA-scaffolded recruitment of proteins to a programmed chromosomal localization (ZALATAN *et al.* 2015).

For genome editing, the Cas9-sgRNA enzyme allows precise placement of a double-strand break (DSB) at any desired location(s) within a genome of interest. The DSB can be sealed in a highly error-prone manner via non-homologous end-joining (NHEJ) (RICHARDSON *et al.* 2016; VRIEND *et al.* 2016) or, more usefully, by homologous recombination (HR) (typically with PCR products provided in *trans*) to substitute a modification (deletion, insertion, allele replacement, fusion to a reporter sequence, etc.) (SHALEM *et al.* 2015; CHANDRASEGARAN AND CARROLL 2016; HU *et al.* 2016). Although accuracy and efficiency are generally high, an sgRNA-guided Cas9 can act at other sites in addition to the intended sequence (CHO *et al.* 2014; O'GEEN *et al.* 2015; ZHANG *et al.* 2015). To reduce such off-target action, specificity-enhancing alterations of Cas9 (KLEINSTIVER *et al.* 2016; SLAYMAKER *et al.* 2016) and sgRNA design (DANG *et al.* 2015; XU *et al.* 2015; DOENCH *et al.* 2016), and computational methods to search for optimal sgRNA-recognition sites (BOLUKBASI *et al.* 2015; NAITO *et al.* 2015), have been devised. By the same token, when provided with different sgRNAs concomitantly, Cas9 can effect simultaneous alterations at multiple locations within the genome in any given cell ("multiplex" genome engineering) (CONG *et al.* 2013), and this strategy has been successfully applied in *S. cerevisiae*, but almost exclusively to non-essential genes (RYAN AND CATE 2014; BAO *et al.* 2015; HORWITZ *et al.* 2015; JAKOCIUNAS *et al.* 2015; LAUGHERY *et al.* 2015; MANS *et al.* 2015; RONDA *et al.* 2015; TSAI *et al.* 2015). Here we describe a useful alternative strategy—introduction of unique, programmable, *artificial target sequences* into the genome, thereby permitting multiplex gene manipulation by Cas9 with a single sgRNA.

RESULTS

A new strategy for multiplex Cas9-mediated gene editing

When bound to an appropriate sgRNA, Cas9 is able to recognize repeated sequences within a genome, such as telomeres (CHEN *et al.* 2013) or the long terminal repeats (LTRs) (*delta* elements) of the yeast Ty1 retrotransposon (SHI *et al.* 2016). Given that fact, and that current limitations on genome editing by Cas9 include the necessity for an adjacent PAM sequence, the individuality of the desired target sequence itself (to avoid off-target effects), and unknown influences of local chromatin structure, we considered useful ways to circumvent these limitations.

In brief, we first integrate both upstream and downstream of any locus of interest, a unique 23-nucleotide sequence (a 20-bp target sequence plus a PAM) that has no detectable counterpart in the genome of interest. Second, we integrate at a safe harbor locus a cassette that expresses from an inducible Pol II promoter *S. pyogenes* Cas9 bearing a potent universal nuclear localization signal (NLS), which is also flanked by the same or a different unique 23-nucleotide sequence. Third, introduction by DNA-mediated transformation of a plasmid that expresses from a Pol III promoter a single sgRNA that matches the unique 23-bp target, along with PCR fragments to replace the excised loci by HR, completes the system.

As proof of principle, we chose genes encoding two members of the family of mitotically-expressed septins, *CDC11* and *SHS1*, to illustrate the utility of our method for exploiting the features of Cas9-mediated gene manipulation. *CDC11* is an essential gene, whereas cells lacking *SHS1*, although not normal, are viable (HARTWELL 1971; IWASE *et al.* 2007; GARCIA *et al.* 2011; MCMURRAY *et al.* 2011; FINNIGAN *et al.* 2015). At the genomic loci for both *CDC11* (Chromosome X) and an *shs1* Δ ::*Hyg*^R allele (Chromosome IV), we used standard techniques to insert (see Materials and Methods) both upstream and downstream of these two ORFs a human, 23-bp (or 24-bps, if necessary to maintain reading frame) PAM-containing sequence (designated "u1"), which does not match any other site in the *S. cerevisiae* genome by more

than a few nucleotides (Fig. 1A). To flank the genes of interest with the u1 (or u2) sequence, two successive rounds of *in vivo* homologous recombination-mediated plasmid assembly in yeast (FINNIGAN AND THORNER 2015) were used to separately introduce these target sites at each end of the desired genes. The resulting constructs were then PCR-amplified and used to transplace the endogenous chromosomal locus of interest by integrative recombination, as described in Materials and Methods. Of course, alternative methods could be used to insert the same (or other) Cas9 target sequences upstream and downstream of a gene of interest, including *in vitro* Gibson cloning (GIBSON 2011), inverse PCR with extended oligonucleotide tails (HARTL AND OCHMAN 1996), or artificial gene synthesis (STEMMER *et al.* 1995). Moreover, “traditional” Cas9-introduced double-strand breaks (JINEK *et al.* 2012) and appropriate PCR products for their repair could be used to introduce unique sites at desired locations in the genome. The directed placement of the u1 and/or u2 sites can be at any position flanking or within a gene (its UTR sequences, coding sequence, or introns). In our test cases, we inserted the u1 motifs as part of the coding sequence of one gene of interest (*CDC11*) and flanking a Hyg^R-marked deletion of another (*SHS1*). The former resulted in a 8-residue insertion at the N-terminal end and an 8-residue insertion at the C-terminal end of the Cdc11 polypeptide. Complementation tests revealed that, at least for Cdc11, such small N- and C-terminal extensions are tolerated *in vivo* (data not shown).

Such short “foreign” sequences are far below the length necessary for spontaneous loop-out from a yeast chromosome by HR, as observed, for example, with introduced *Salmonella hisG* repeats (1,100 bps) (ALANI *et al.* 1987) or the LTRs of retrotransposons (323-424 bps) (NEUVEGLISE *et al.* 2002). In the same strain (Table S1), a cassette expressing *S.p.*Cas9 bearing an SV40 NLS (DICARLO *et al.* 2013) under control of the inducible *GAL1/10* promoter was integrated at the *HIS3* locus marked by a Kan^R gene (Chromosome XV), flanked by u1 or by a different (u2) unique 23-bp PAM-containing sequence (Fig. 1B). To demonstrate how this method can be used to replace essential genes with a desired construct, the strain also

contained a “covering” plasmid carrying WT *CDC11* and *URA3* (a marker that can be counter-selected on 5-FOA medium) (BOEKE *et al.* 1984) (Fig. 1A). To initiate genome editing, a 2 μm DNA plasmid expressing sgRNA[u1] (Fig. 1C) and PCR fragments to integrate at each locus are introduced by transformation into cells in which Cas9 expression has been induced.

The rationale for flanking the target genes with identical sites for Cas9-catalyzed DSB formation is to demand repair of the resulting chromosomal lesions by HR with the PCR fragments provided, permit concurrent replacement of multiple loci using just a single sgRNA, allow for concomitant self-excision of the Cas9-expressing cassette, when desired, and avoid the spurious events that can occur upon standard multiplex Cas9 genome editing (see Fig. S1).

Multiplexing Cas9 to a programmed genomic target sequence using a single sgRNA

We confirmed, first, that Cas9 is expressed in a galactose-inducible manner and properly localized to the nucleus (Fig. S2A) and, second, that expression of neither Cas9 alone, nor sgRNA[u1] or sgRNA[u2] alone (Table S2), nor co-expression of Cas9 with either guide RNA, in otherwise WT cells (i.e. lacking u1 or u2 sequences) caused any detectable loss of viability or transformation efficiency (Fig. S2B). We constructed two tester strains, one (GFY-2002) for simultaneous manipulation of two loci (*CDC11* and *shs1Δ::Hyg^R* loci) (Fig. 2A, *left*) and one (GFY-2003) for simultaneous manipulation of three loci (*CDC11*, *shs1Δ::Hyg^R*, and *his3Δ::Cas9::Kan^R*) (Fig. 2A, *right*). After induction of Cas9, these strains were transformed with either empty vector or the same plasmid expressing sgRNA[u1] in the absence or presence of PCR fragments bearing homology to the genomic sequence upstream and downstream of each locus (Fig. 2B, *upper*). Additional control reactions were conducted in the absence of Cas9 expression or in the absence of sgRNA[u1] (Table S3). The PCR fragments used contained either 500 or 30 bps of flanking genomic sequence homology (Fig. 2B, *lower*). Control strains, in which Cas9 cleavage at the u1 sites produces DSBs that have no corresponding PCR fragment(s) for their repair, yielded very few viable colonies (Fig. 2B, *conditions B-D*; Fig. 2C), even though the intrinsic transformability of the cells was robust (Fig. 2B, *condition E*), because

Cas9-mediated DSB formation (with no subsequent repair of the locus) is lethal in yeast. By contrast, we observed a ≥ 300 -fold increase in the recovery of viable colonies when the PCR fragments present to mediate repair by HR had 500 bps of homology to the genomic sequence flanking each locus and a 20-40 increase in recovery of viable colonies even when the homology was only 30 bps (Fig. 2B, *condition A*; Fig. 2C). For the GFY-2002 strain (Fig. 2, *left*), the viable colonies recovered correspond to successful HR-mediated repair of two loci (*CDC11* and *SHS1*) loci, and for strain GFY-2003 (Fig. 2, *right*), the viable colonies recovered correspond to simultaneous successful repair at all three loci (*CDC11*, *SHS1*, and *HIS3*). Phenotypic characterization showed that, as expected, the vast majority ($\geq 97\%$) of ~ 200 randomly-chosen GFY-2002 survivors had become hygromycin-sensitive, and the vast majority ($\geq 87\%$) of ~ 200 randomly-chosen GFY-2003 survivors had become both hygromycin-sensitive and G418-sensitive (the status of *CDC11* had to be scored by other means; see below).

Unlike strain GFY-2003, where removal and replacement of the u1-flanked Cas9-expressing cassette occurs concomitantly with multiplex substitution at the other u1-flanked loci, strain GFY-2002 contains a Cas9 expression cassette flanked by u2, a different unique target site. This arrangement allows for additional Cas9-dependent integration (or deletion) events at other loci, if desired, but also allows for excision of the *his3 Δ ::Cas9::Kan^R* cassette upon introduction of a plasmid expressing sgRNA[u2]. To test the efficacy of this sequential scheme for removal of Cas9, an isolate of GFY-2002 in which direct DNA sequence analysis showed that sgRNA[u1]-driven genome editing had resulted in restoration of WT *CDC11* and *SHS1* at both loci (Fig. 2BC, *condition A*), was transformed with an empty *HIS3*-marked vector (pRS423) or a derivative expressing sgRNA[u2] cassette (Table S2). In this plasmid, the *HIS3* gene is flanked with significant lengths of genomic sequence (Fig. 2D, *right*); therefore, in theory, it serves both as the source of sgRNA[u2] to catalyze Cas9-mediated excision of the Cas9-expressing cassette and the source of the homologous DNA needed to repair the cleaved locus, without the necessity of co-transforming any PCR fragment or oligonucleotide. Indeed, reassuringly, nearly

all (99%) of ~200 His⁺ colonies obtained from cells exposed to sgRNA[u2] were G418-sensitive, indicating loss of the Cas9-expressing cassette, whereas all of ~200 His⁺ colonies exposed to the empty vector were Kan^R, as expected for retention of the Cas9-expressing cassette (Fig. 2D), *left*). The 2 μm DNA-based plasmids used to express sgRNA[u1] or sgRNA[u2] are themselves rapidly lost when not subjected to selection for the appropriate marker (Table S4).

Confirmation of successful multiplex gene replacement

Genomic DNA from ten randomly-chosen colonies from transformations with PCR fragments containing 500 bps of homology (Fig. 2, *condition A*) was analyzed by diagnostic PCR (Fig. 3) and direct nucleotide sequencing (data not shown) to examine each manipulated locus. Diagnostic PCR was also performed on colonies from transformations with PCR fragments containing only 30 bps of homology (Fig. 2, *condition A*) with very similar results (Fig. S3). For GFY-2002, PCR analysis showed that all ten isolates replaced the *shs1Δ::Hyg^R* allele with the WT *SHS1* gene (and, as expected, still harbored the Cas9-expression cassette) and seven of ten also properly replaced the u1-flanked *CDC11* locus with the WT *CDC11* gene, which was further confirmed by sequencing. Multiple PCRs tested for the presence or absence of the upstream and downstream u1 sites present at the *CDC11* locus; DNA of the covering plasmid expressing WT *CDC11* was not present since amplification of the parental strains (control lanes) only displayed single PCR bands corresponding to the chromosomal locus (Figs. 3 and S3).

For GFY-2003, all ten isolates replaced the *shs1Δ::Hyg^R* allele with the WT *SHS1* gene and also replaced the Cas9-expression cassette with the WT *HIS3*. For both, the PCR fragments used for gene replacement shared homology only with the genomic sequences flanking these two loci. In the same ten isolates, nine also properly replaced the u1-flanked *CDC11* locus with the WT *CDC11* gene and, in the remaining one, only the upstream u1 site was retained. In 36 total isolates tested from all experimental trials, 32 replaced both the upstream and downstream u1 sites with WT *CDC11* and only four retained just the upstream u1 site. The most likely explanation for these few exceptions arises from the fact that the *CDC11*-containing PCR

fragment we used for replacement shares homology across its entire coding region with the u1-flanked chromosomal *CDC11* locus and that the upstream u1 site lies just downstream and in-frame with the Met codon need to initiate Cdc11 translation. Thus, crossovers between the PCR fragment and the chromosome that occur within the *CDC11* ORF and in the 3'-UTR will heal a DSB at the downstream u1 site and yield a viable cell that can produce Cdc11, yet retain the upstream u1. These rare exceptions can be readily avoided by eliminating the internal homology by (i) starting with a genomic *cdc11* Δ null allele (covered by WT *CDC11* on a plasmid) or (ii) installing in the chromosome a synthetic ORF with codon alterations that minimize its nucleotide sequence identity to the authentic *CDC11* ORF on the PCR fragment.

Prior work has shown that repair of a DSB via HR in yeast is orders of magnitude more frequent than by NHEJ (STORICI *et al.* 2003), which is extremely inefficient (RATTRAY *et al.* 2001; DALEY *et al.* 2005; STORICI *et al.* 2006). Indeed, in sixteen of the very rare survivors obtained from the controls where the transformations lacked one or more PCR fragments to repair the DSBs (Fig. 2, *conditions B-D*), phenotypic analysis (Table S4) and diagnostic PCR (Fig. S5) showed that the majority did not have any replacements and likely escaped any Cas9-induced DSBs and a few isolates replaced one locus, but failed to cut and remove the u1 sites elsewhere. In only three (from Fig. 2, *condition B*), the *shs1* Δ ::*Hyg*^R allele was excised, but left a single intact u1 site, most consistent with repair of the Cas9-induced DSBs via HR between the u1 repeats rather than by NHEJ. We conclude from our data that correct replacement at all loci examined is nearly three orders of magnitude more frequent than any other event.

DISCUSSION

The crux of our method is first programming the desired Cas9 cleavage sites at will by installing a unique sequence of the investigator's own choosing, rather than relying on naturally-occurring genomic sequences (Fig. 4). By flanking any number of selected genes with the same "alien" sequence, and providing PCR fragments homologous to the loci of interest, expression of just a single sgRNA initiates multiplex Cas9-mediated removal and scarless replacement of these targeted genes. Here we achieved concurrent replacement of three ORFs on three different chromosomes, including one essential gene; however, the same approach can be used to excise or alter exons, introns, splice junctions, transcription factor-binding sites, locus control regions, etc. Also, our method eliminates the need for codon alterations to the integrated allele(s) [or any WT covering plasmid(s)] to prevent re-cutting of the newly substituted DNA by Cas9 (Fig. S1).

Other methods for modifying essential genes in yeast (Toh-e and Oguchi, 2000; Cross and Pecani, 2011; Horwitz et al., 2015) have been described. In our view, our approach provides an alternative that is, in the long run, substantially less cumbersome and markedly more efficient. For example, our strategy does not depend on the fortuitous presence of a unique restriction endonuclease site, as required by the "integration replacement/disruption" method of Toh-e and Oguchi (2000) to "loop-in" a mutagenized plasmid copy of the gene of interest. Although the HO endonuclease-based method of Cross and Pecani (2011) does not require selection, it does require the construction of strain backgrounds with inducible HO expression and demands the exclusive use of the pRS400 series of *ARS*-less and *CEN*-less integration vectors. In the use of Cas9 for editing of essential genes described by Horwitz et al. (2015), the endogenous target sequence used for DSB formation needs to lie as close as possible to the desired nucleotide change to prevent inappropriate HR downstream of the mutation resulting in repair of the DSB without incorporation of the desired allele, as we already pointed out (Fig. S1). Our approach circumvents all of the above issues, as well as increases the ease and efficiency by which

essential genes may be manipulated.

Our methodology is complementary to "traditional" Cas9-mediated multiplex gene editing that requires the design and expression of multiple sgRNAs. Our approach expands how Cas9-based genome editing technology can be deployed and, hence, enhances its utility. Although our strategy first requires the initial installation of a unique target site(s) within the genome to be manipulated, there are several long-term benefits of constructing strains with programmed Cas9 target sites that we feel outweigh the traditional Cas9 approach (Figs. 4 and S1). Our method is especially useful (i) when repeated targeting of a locus, or groups of genes (*e.g.*, paralogs or entire genetic pathways) is needed, (ii) for manipulation of essential genes, (iii) where minimizing off-target effects is critical, and (iv) in cases where an "alien" target sequence is required/desired, such as in the design of gene drives. Given the rapid movement toward programmable toolkits in synthetic biology, we envision that it would be worth investing the effort to flank every gene in a genome of interest with such synthetic Cas9 target sites. In fact, traditional Cas9 editing could be used to do so.

Moreover, as we demonstrated, Cas9 action at the artificially introduced sites can eliminate its own expression cassette without compromising its ability to mediate efficient gene editing elsewhere in the genome of the same cell. In addition, our method can be used to interrogate the effects of chromosome position and local chromatin structure on Cas9 action because the same 23-bp sequence can be installed at any location in a genome. In this way, apparent differences between species with regard to the efficiency with which Cas9 can access and cleave at sites within heterochromatin (YU *et al.* 2013; WU *et al.* 2014; KNIGHT *et al.* 2015; FENG *et al.* 2016) could be systematically explored. Finally, application of this approach should be extremely useful in generating strain libraries, constructing synthetic genomes, and introducing in a multiplex manner genomic changes to study multiple genes in a signaling pathway, the subunits of a multi-protein complex, paralogous gene sets, or any combination or collection of genes of interest.

MATERIALS AND METHODS

Yeast strains and plasmids

All budding yeast strains used in this study can be found in Table S1. Standard molecular biology methods were used in this study (SAMBROOK AND RUSSELL 2001). The introduction of the u1 and u2 Cas9 target sites was performed by first cloning vectors using *in vivo* ligation and homologous recombination harboring a single Cas9 site including the PAM sequence (FINNIGAN AND THORNER 2015). As an example, a vector (pGF-V130) containing the 5' UTR of *CDC11* was digested with a restriction enzyme (*NotI*) downstream of the promoter sequence and transformed with a PCR fragment of the *CDC11* coding region amplified with oligonucleotides containing overhanging "tails" to insert the Cas9 u1 target sequence in-frame. Two constructs, each with a single flanking u1 site placed upstream or downstream of *CDC11* were created separately and then combined by a second round of *in vivo* ligation to generate the final construct that contained both flanking u1 sites as well as flanking *CDC11* 5' and 3' UTR (330 bps of each). This process was repeated for the *shs1Δ::Hyg^R* cassette harboring flanking u1 sites and two Cas9-expressing cassettes containing either u1 or u2 sites at the *HIS3* locus (Table S1). The generated constructs were PCR amplified and integrated into the parent strain in successive yeast transformations. Diagnostic PCRs and Sanger sequencing (Univ. of California, Berkeley Barker Hall Sequencing Facility) of chromosomal DNA were performed to ensure proper integration of all manipulated loci.

Plasmids used in this study can be found in Table S2. Expression of the sgRNA cassettes was modeled after a previous study (DICARLO *et al.* 2013) using the snoRNA *SNR52* promoter and *SUP4* terminator sequences and were synthesized as custom genes with flanking *XhoI* and *BamHI* restriction sites (GenScript, Piscataway, NJ). The u1 and u2 sequences were chosen from two human genes, SEPT9 and MMP23A, respectively, using the DNA2.0 gRNA Design Tool (DNA2.0, Newark, CA). Putative guide sequences were then examined against the entire yeast genome using a nucleotide BLAST search (National Center for Biotechnology Information)

and sequences were considered for having the lowest possible number of matches to the 15 base pair sequence (PAM + upstream 12 bps) important for Cas9 “seeding” to minimize off-target effects (JINEK *et al.* 2012; JIANG *et al.* 2013). Additionally, the chosen u1 and u2 sequences were checked against the backbone vector sequences of the pRS316 covering vector, the high-copy sgRNA-expressing pRS425/pRS423 vectors, and both the Kan^R and Hyg^R cassettes (GOLDSTEIN AND MCCUSKER 1999) to ensure no highly similar matches existed in these exogenous non-yeast sequences.

Culture Conditions

Yeast were grown in rich YPD or YPGal medium (2% peptone, 1% yeast extract, 2% dextrose or 2% galactose), or in synthetic medium containing the necessary amino acids with either 2% dextrose or a 2% raffinose and 0.2% sucrose mixture. For transformation of yeast using the Cas9-mediated system, strains were grown overnight in synthetic medium with a raffinose/sucrose mixture lacking uracil (to select for the *CDC11*-expressing WT covering plasmid) to saturation, back-diluted into YPGal (to an OD₆₀₀ of approximately 0.25-0.35), and grown at 30°C for 4.5-5.0 hours. A modified lithium acetate transformation protocol (ECKERT-BOULET *et al.* 2012) was used to transform 10 OD₆₀₀ of yeast with combinations of purified plasmid DNA and/or PCR products. Yeast were heat shocked for 45-50 minutes at 42°C and recovered in fresh YPGal overnight at 30°C prior to plating onto selective media (selection for both plasmids and no selection for integrated knock-in alleles). An identical transformation protocol was used whether Cas9 was integrated at the *HIS3* locus or expressed on a CEN-plasmid.

The growth of single yeast colonies on various media (G418, Hygromycin, SD-HIS, etc.) were tested by first selecting isolated colonies, creating a small square “patch” (1 cm²) on an SD-URA plate, incubating overnight at 30°C, and then replica-plating to additional plates to be scored after 1 additional day of incubation. For yeast plates containing a significant number of colonies, the total colony count was estimated in several ways. First, several sectors (1/2, 1/4,

or 1/8, etc.) were selected on the agar plate and the total number of colonies in the sample sector was counted and extrapolated to the entire surface area. Second, subsequent repeated experiments plated various dilutions (1/10, 1/20, 1/50, etc.) of the final transformation product and the total colony counts were added, extrapolated, and averaged together. All experiments were performed in at least triplicate.

Fluorescence microscopy

For fluorescence microscopy, yeast were grown to saturation overnight in S+Raff/Suc-LEU, back-diluted into YPGal, grown for five hours at 30°C, harvested, washed with water, and prepared on a standard microscope slide with a coverslip. Samples were immediately imaged on an Olympus BH-2 upright fluorescence microscope (Olympus, Tokyo, Japan) with a 100x objective lens. A CoolSNAP MYO CCD camera (Photometrics, Tuscon, AZ), a SOLA light source (Lumencore, Beaverton, OR), Micro-Manager software (EDELSTEIN *et al.* 2010), and ImageJ software (National Institute of Health) were used to process fluorescent images. The cell periphery was determined using an over-exposed fluorescence image.

Polymerase Chain Reaction and DNA Sequencing

All PCR reactions were performed using either high fidelity KOD Hot Start DNA Polymerase (EMD Millipore, Billerica, MA) or PfuUltra II Fusion Hot Start DNA Polymerase (Agilent Technologies, Santa Clara, CA) according to the recommended manufacturer's conditions (KOD reactions all contained 3 mM Mg²⁺) on a PTC-200 Thermal Cycler (MJ Research, Bio-Rad). Oligonucleotides (Integrated DNA Technologies, Coralville, IA) used in this study can be found in Table S5. For PCR reactions used in the Cas9-mediated integrations, the template DNA was from either purified yeast chromosomal DNA or from bacterial-based plasmids (that cannot be propagated in yeast). Products for integration were confirmed to be the correct size on an agarose gel, but were not purified nor gel extracted; amplified DNA was directly added to the yeast transformation reaction. For diagnostic PCRs to confirm various manipulated loci, DNA agarose gels (1% or 2%) containing Ethidium Bromide were used to separate and image

(ChemiDoc System, Bio-Rad Laboratories, Hercules, CA) separated products. Sanger DNA sequencing was performed on all constructed vectors and plasmid intermediates. For sequencing of genomic loci, chromosomal DNA was isolated (AMBERG *et al.* 2006) and PCR amplified using a high-fidelity polymerase. The product sizes were confirmed on an agarose gel and the remaining DNA was purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA) and sequenced with overlapping coverage at each desired locus. For diagnostic PCRs, chromosomal DNA was first isolated from yeast strains, as follows. Two precautions were taken to avoid isolating DNA that contained the *URA3*-based covering vector expressing WT *CDC11*. First, cells were grown to saturation under non-selective conditions in rich (YPD) medium overnight (16 hrs) at 30°C. Second, DNA was isolated using a procedure that recovers only chromosomal DNA (AMBERG *et al.* 2006). Indeed, control F2/R2 PCR reactions carried out on DNA isolated using these approaches from the plasmid-containing parental strains GFY-2002 and GFY-2003 demonstrated that the preparations obtained generated PCR products diagnostic for the chromosomal *CDC11* locus and did not generate any PCR products diagnostic for the plasmid-borne DNA (Figs. 3, S3 and S5).

Statement on data and reagent availability. We will freely send all plasmids and strains and other research materials and procedures generated from this research to investigators at any and all non-profit institutions for research purposes upon request.

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FIGURE LEGENDS

FIGURE 1. Installation of programmed non-yeast Cas9 target sites at multiple loci.

(A) Haploid yeast strains were constructed in which the endogenous *CDC11* gene and a *shs1Δ::Hyg^R* allele were flanked by an identical 23-bp sequence containing a Cas9 target site (including a 5'-NGG-3' PAM sequence) from the human *SEPT9* gene, designated “unique Cas9 site 1”, u1. At *CDC11*, the upstream u1 site was placed in-frame with the initiator Met of the ORF and the downstream u1 was kept in-frame with the stop codon (via addition of an A to the 5'-end of each u1). Because *CDC11* is an essential gene, a *URA3*-marked *CEN* plasmid expressing WT *CDC11* (but with no 3'-UTR) was also present. *Red triangles*, site of Cas9-directed DSB (+3 upstream of the PAM). (B) A cassette for inducible *GAL1/10* promoter-driven expression of S.p.Cas9 bearing a C-terminal SV40 NLS and a *ADH1* transcriptional terminator was used to replace the ORF at the endogenous *HIS3* locus. In one variant (strain GFY-2002), this cassette was flanked by u2, a different 23-bp human sequence containing a Cas9 target from the human *MMP23A* locus. In another variant (strain GFY-2003), the cassette was flanked by u1. (C) The corresponding sgRNA[u1] and sgRNA[u2] sequences were expressed using the constitutive yeast pol III snoRNA *SNR52* promoter and yeast pol III tRNA *SUP4* terminator on high-copy (2 μm DNA) plasmids.

FIGURE 2. Multiplex Cas9-mediated scarless gene replacement (including an essential gene) and optional concurrent elimination of Cas9. (A) Otherwise isogenic yeast strains containing six programmed Cas9 target sites. In strain GFY-2002, the *CDC11* and *shs1Δ::Hyg^R* loci are flanked by u1, whereas the Cas9 expression cassette at the *HIS3* locus is flanked by u2. In strain GFY-2003, all three loci are flanked by u1. Both strains also carried a *URA3*-marked *CEN* plasmid harboring WT *CDC11*. (B) Cas9 expression was induced in strains GFY-2002 (*left*) or GFY-2003 (*right*) and then the cells were transformed with an empty *LEU2*-marked vector (pRS425) or with the same plasmid expressing sgRNA[u1] in the absence or presence of

various combinations of PCR fragments that span each of the genomic loci of interest, as indicated. The PCR fragments contained either 500 bps (*upper plates*) or just 30 bps (*lower plates*) of homology to the genomic sequence flanking each locus. *Asterisk*, for the *CDC11* PCR fragment, the flanking homology was 330 bps. After recovery in rich medium containing galactose (to support continued Cas9 expression), the cells were plated on SD-Ura-Leu medium. The plates were imaged and the number of colonies recovered were counted after incubation at 30°C for 3 days. Each independent trial was performed in triplicate. Representative plates are shown; *white numbers*, total colony count. The empty vector control confirmed that these conditions allowed for efficient transformation and selection for the *LEU2*- and *URA3*-marked plasmids. Individual colonies from Condition A, where all of the PCR fragments necessary to heal the Cas9-sgRNA[u1]-generated DSBs were provided, were tested for growth on various diagnostic media to ascertain whether successful gene replacement occurred (see Table S3). *Red values*, percentage of colonies scored that exhibited successful gene replacement at all loci tested. (C) The average colony count over all experimental trials for each condition (A, B, C and D), as indicated. *Error bars*, SEM. (D) An isolate of GFY-2002 from Condition A (B and C) in which both the u1-flanked *CDC11* locus and u1-flanked *shs1Δ::Hyg^R* allele were successfully replaced with WT *CDC11* (see Fig. 3) and WT *SHS1*, respectively, was grown in galactose to induce Cas9 expression and then transformed with either empty vector (pRS423) or the same plasmid expressing sgRNA[u2], plated on SD-Ura-His medium, and grown at 30°C for 3 days. The selectable marker in the sgRNA[u2]-expressing plasmid is the *S. cerevisiae HIS3* gene with 317 bps of 5'- and 201 bps of 3'-flanking genomic sequence. Therefore, this plasmid not only provides sgRNA[u2] to target Cas9 cleavage at the u2 sites flanking the *his3Δ::Cas9::Kan^R* cassette, but it also serves as a source of WT *HIS3* DNA to repair the cleaved locus. Representative plates are shown; *white numbers*, total colony count. To assess conversion of the u2-flanked *his3Δ::Cas9::Kan^R* cassette to WT *HIS3*, the His⁺ Ura⁺ colonies obtained were scored for loss of G418 resistance and complete elimination of the

entire cassette (Table. S4). *Red values*, percentage of colonies scored that exhibited successful elimination of the *his3Δ::Cas9::Kan^R* cassette.

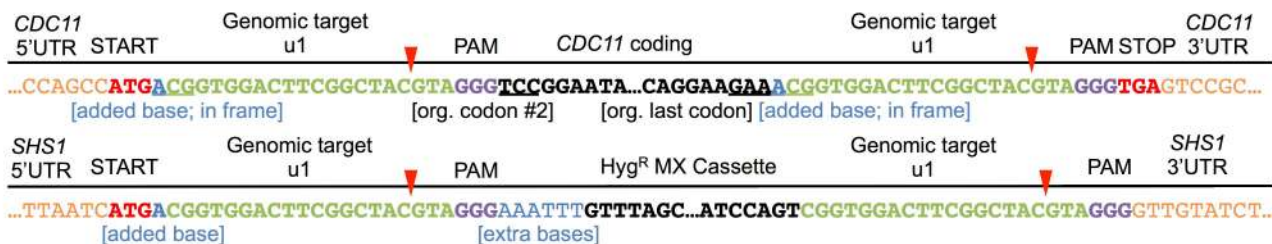
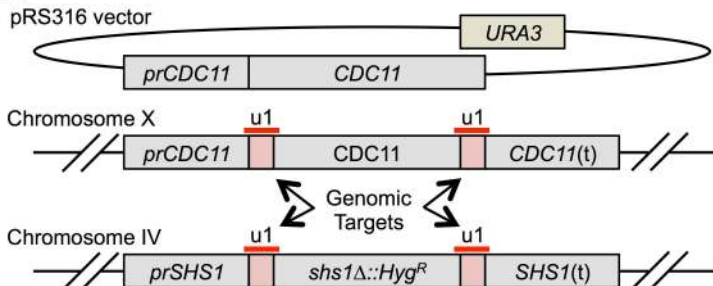
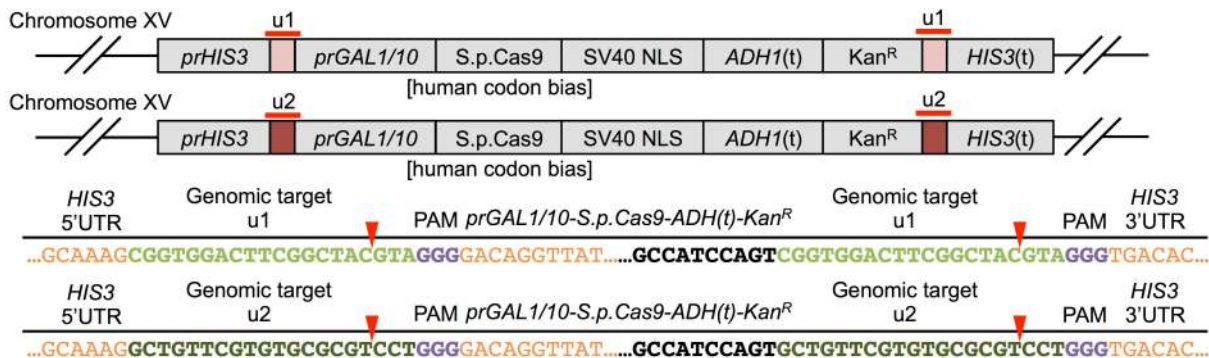
FIGURE 3. Diagnostic PCR confirms efficient multiplex gene replacement.

(A) Chromosomal DNA was purified (AMBERG *et al.* 2006) from ten, randomly chosen, clonal isolates from transformations of GFY-2002 in which PCR fragments with 500 bps of flanking genomic homology were provided to restore WT *CDC11* and WT *SHS1* loci, and which had lost the Hyg^R marker (see Fig. 2B), and tested by PCR with the indicated diagnostic primer sets. An identical analysis was performed on ten isolates in which PCR fragments with only 30 bps of flanking genomic homology were provided and which had lost the Hyg^R marker (see Fig. S3). The PCR products were resolved by agarose gel electrophoresis and visualized by staining with ethidium bromide. For *CDC11* (*top three gels*), the entire locus was amplified (primers F1/R1), as well as small fragments flanking the upstream (F2/R2) or downstream (F3/R3) u1 sites to determine whether or not the Cas9 target site was still present. For *SHS1* (*fourth and fifth gels*), PCR was performed using primers unique to either *SHS1* itself (F4/R4) or to the Hyg^R cassette (F4/R5). Finally, the *HIS3* locus (*bottom gel*) was testing using a unique primer internal to the Cas9 gene and to the Kan^R cassette (F5/R6). For optimal separation, 2% agarose was used for the second and third gels, 1% agarose was used for all of the others. *Left*, nearest DNA size marker (in kilobases) for each independent gel; *right*, expected PCR product sizes. (B) The same kind of analysis as in (A) was performed on chromosomal DNA purified from ten, randomly chosen, clonal isolates from transformations of GFY-2003, except that, in addition, PCR diagnostic for the *HIS3* locus was performed (F6/R7) to ascertain whether the u1-flanked *his3Δ::Cas9::Kan^R* cassette had been replaced by the WT *HIS3* gene. *Left red asterisks*, three representative isolates of GFY-2002 that diagnostic PCR indicated carried WT *CDC11* and WT *SHS1* loci, and retained the u2-flanked *his3Δ::Cas9::Kan^R* cassette, were confirmed as such by direct DNA sequencing (data not shown). *Right red asterisks*, three representative isolates of

GFY-2003 that diagnostic PCR indicated carried WT *CDC11*, WT *SHS1* and WT *HIS3* loci were also confirmed as such by direct DNA sequencing (data not shown). [For diagnostic PCR and sequencing of surviving colonies from controls (Fig. 2B, conditions B-D), see Fig. S5.]

FIGURE 4. Comparison of Cas9-mediated genome editing by multiplexing sgRNAs *versus* multiplexing loci with a unique target site. (A) Traditional targeting of Cas9 to multiple genomic loci (including one locus where Cas9 is integrated). Each of four loci is illustrated as requiring Cas9 action at two distinct sites. Hence, concurrent action of Cas9 at these four genes would require the selection of eight individual PAM-containing genomic sequences and the production of eight corresponding sgRNAs. In addition, it should be noted that, in this scenario, at least one target site lies within the coding sequence of each gene; therefore, PCR fragments used to replace Genes(1-3) would also require alterations of the coding sequence to avoid re-cutting by Cas9 (also see Fig. S1). Finally, for manipulation of any essential genes (e.g., Gene1), a counter-selectable plasmid expressing a WT copy will also need to be altered to not include the genomic target site(s), again to avoid its Cas9-mediated cleavage (Fig. S1). (B) The approach of multiplexing the target site(s) has a number of useful advantages. First, there is no need to restrict the target for Cas9 cleavage to sequences that exist within the genome of interest, which may be suboptimal (with regard to off-target effects) or may have a limited number or inopportune placement of available PAM sites. Second, the artificial target site chosen for insertion may be any stretch of 23 nucleotides (20 plus a 5'-NGG-3' PAM) taken from any known species (or designed *de novo*), as long as it has no counterpart in the genome of interest. In fact, such a programmed target site sequence should greatly reduce or eliminate off-target effects and also has the virtue that it can be inserted at a precise location (down to the base pair) to optimally facilitate recombination and precisely control the placement of the Cas9-mediated DSBs. The limiting step in this approach is, of course, introduction of these unique target site insertions into the parental genome at the desired locations. Once created, however,

such an engineered parental strain can be used repeatedly to install various different alterations at one or many loci using only a single sgRNA, allowing for rapid construction of multiple strain variants. Moreover, in this approach, the Cas9 expression cassette can be retained, targeted for simultaneous excision in parallel with the manipulations of other loci (*right*), or eliminated at a later time, if the Cas9 expression cassette is flanked with a separate unique target site (*left*). Finally, because the sequence of the target sites flanking each locus are distinct from any of the elements of the targeted genes themselves, no modifications to the sequence of the PCR fragments used for gene editing (or of a covering plasmid carrying the corresponding WT gene) are required to make them immune the further action of Cas9.

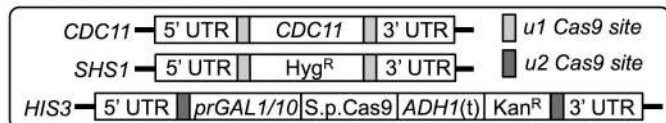
A**B****C**

sgRNA	Genomic Target	PAM
sgRNA[u1] ¹	CGGTGGACTTCGGCTACGTA	GGG
sgRNA[u2] ²	GCTGTTCGTGTGCGCGTCCT	GGG

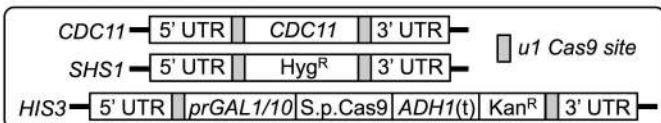
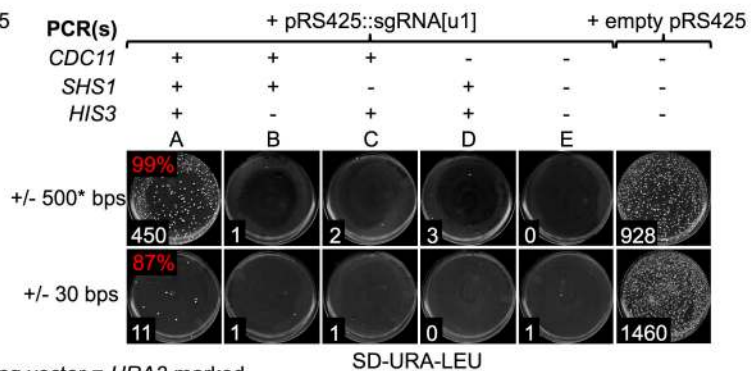
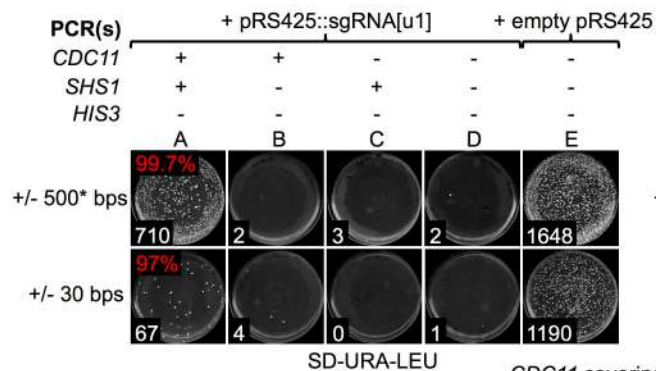
¹*Homo sapien* SEPT9; Chromosome 17, position 75,478,338

²*Homo sapien* MMP23A; Chromosome 1, position 1,632,617

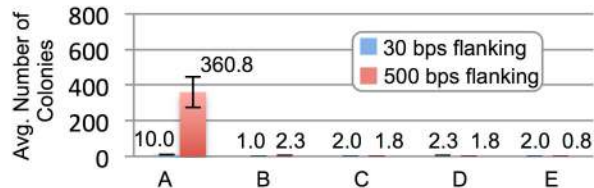
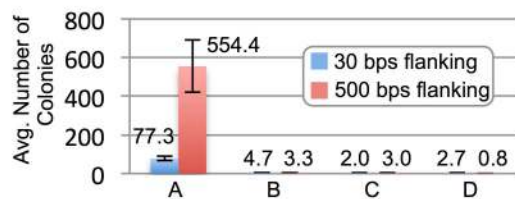
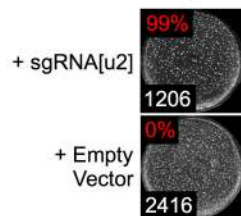
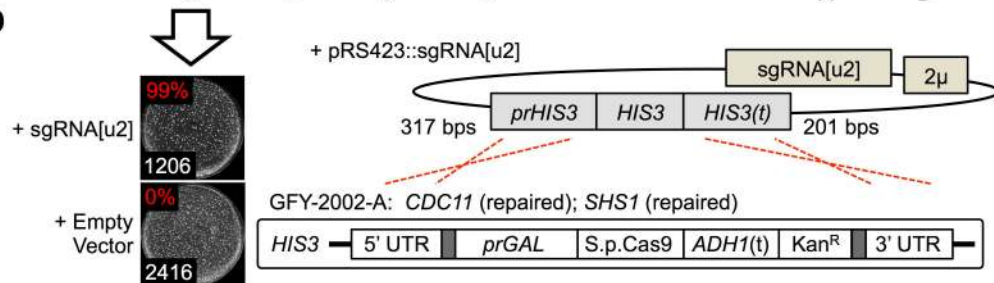
pRS425:	<i>prSNR52</i>	sgRNA[u1]	structural	<i>SUP4(t)</i>
pRS423:	<i>prSNR52</i>	sgRNA[u2]	structural	<i>SUP4(t)</i>

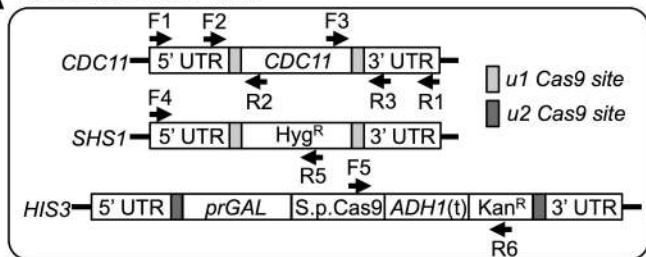
A Parental Strain GFY-2002


Parental Strain GFY-2003


B


CDC11 covering vector = URA3-marked
pRS425 = LEU2-marked

C

D


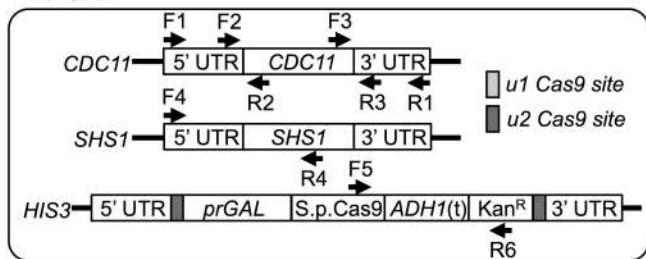
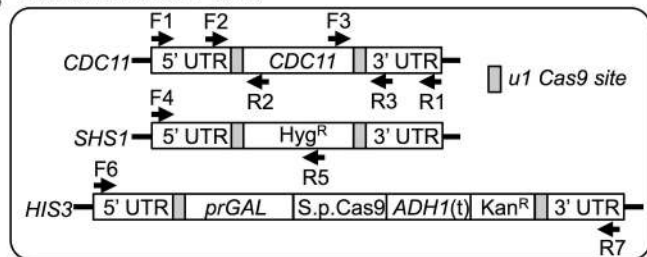
A Parental Strain GFY-2002


+ *CDC11* +/-330 bps [PCR]

 + *SHS1* +/-500 bps [PCR]

 + sgRNA[u1] [high-copy vector]

Final Strain


B Parental Strain GFY-2003


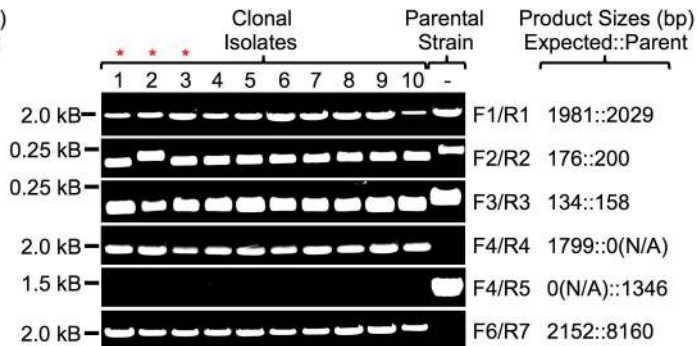
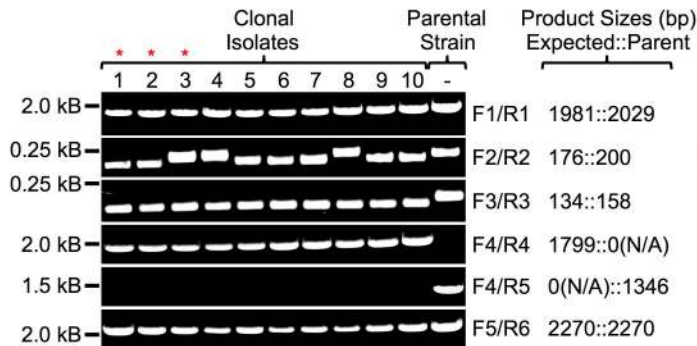
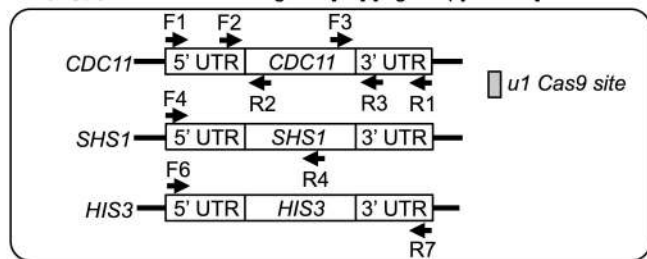
+ *CDC11* +/-330 bps [PCR]

 + *SHS1* +/-500 bps [PCR]

 + *HIS3* +/-500 bps [PCR]

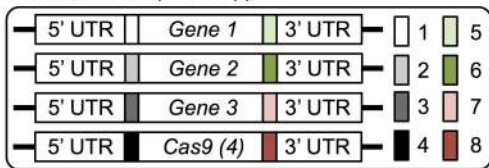
 + sgRNA[u1] [high-copy vector]

Final Strain



A Traditional method, multiplexing the guide RNA, various genomic targets

Parental Strain (one-step)



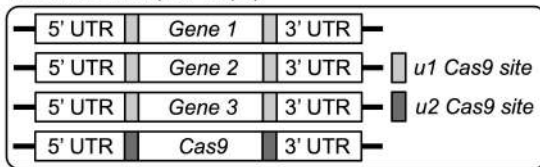
+ Altered Covering Vector (Gene1* WT)

Step 1:

sgRNA[1] + sgRNA[2] + sgRNA[3] + sgRNA[4] +
sgRNA[5] + sgRNA[6] + sgRNA[7] + sgRNA[8] +
PCR(Gene1*) + PCR(Gene2*) + PCR(Gene3*) +
PCR(Gene4)

B Novel method, multiplexing the target sites, not the guide RNA

Parental Strain (two-steps)

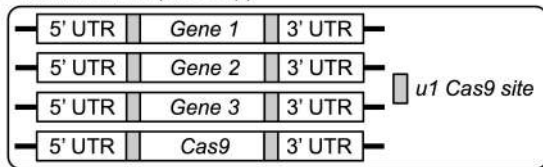


+ Standard Covering Vector (Gene1 WT)

Step 1: sgRNA[u1] + PCRs (native)

Step 2: sgRNA[u2] + PCRs (native)

Parental Strain (one-step)



+ Standard Covering Vector (Gene1 WT)

Step 1: sgRNA[u1] + PCRs (native)

SUPPORTING INFORMATION

for

**mCAL: a new approach for versatile multiplex action of Cas9
using one sgRNA and loci flanked by a programmed target sequence**

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Table S1. Yeast strains used in this study.

Strain	Genotype	Reference
BY4741	<i>MATa leu2Δ ura3Δ met15Δ his3Δ</i>	(BRACHMANN <i>et al.</i> 1998)
GFY-1517 ¹	<i>BY4741; NUP188::mCherry::ADH1(t)::S.p.HIS5</i>	This study
GFY-2002 ²	<i>BY4741; cdc11Δ::u1::CDC11::u1; shs1Δ::u1::Hyg^R::u1; his3Δ::u2::prGAL1/10³::S.p.Cas9::NLS::ADH(t)::Kan^R::u2 + pJT1520</i>	This study
GFY-2003 ⁴	<i>BY4741; cdc11Δ::u1::CDC11::u1; shs1Δ::u1::Hyg^R::u1; his3Δ::u1::prGAL1/10::S.p.Cas9::ADH(t)::Kan^R::u1 + pJT1520</i>	This study

¹Wild-type (WT) yeast was transformed with an amplified PCR product (from plasmid pJT2868) to tag the endogenous copy of *NUP188*, a nuclear envelope protein (AITCHISON *et al.* 1995; FABRE AND HURT 1997), with mCherry. The *S.p.HIS5* gene is from fission yeast *S. pombe* (and functions in place of *S. cerevisiae HIS3*).

²To construct GFY-2002, yeast strain GFY-153 (*cdc11Δ::Kan^R + pRS316::CDC11*) was transformed with the amplified *CDC11* product (from pGF-IVL972) containing both flanking u1 Cas9 sites in-frame with the ORF as well as 330 bps of 5'- and 3'-UTR and plated on synthetic complete medium containing 5-FOA (to select for the integration of the u1::CDC11::u1 copy and loss of the WT Cdc11-expressing *URA3*-based plasmid). The *CDC11*-expressing *URA3* plasmid (pJT1520) was subsequently transformed back into an isolate carrying the desired u1::CDC11::u1 integrant. Second, *SHS1* was deleted using a modified Hyg^R deletion cassette (GOLDSTEIN AND MCCUSKER 1999) containing flanking u1 sites (23 bps) upstream and downstream of the MX sequence (from pGF-IVL1026) with 500 bps of *SHS1* UTR. Third, the *HIS3* locus (*his3Δ0*) was repaired by amplifying a WT copy of *S.c.HIS3* with 500 bps of flanking UTR by PCR (template was genomic DNA from THS4213) and selected on SD-His medium. Fourth, the expression cassette for S.p.Cas9 (Cas9 from *S. pyogenes*; human codon bias; amplified from Addgene plasmid #43804) was integrated at the *HIS3* locus by PCR amplifying the following fragment in two, roughly equal-sized pieces (of approximately 5 kb each) that overlapped within the Cas9 gene, *prHIS3::u2::prGAL1/10::S.p.Cas9::ADH(t)::Kan^R::u2::HIS3-3'UTR* (from pGF-IVL975), and selected on rich medium containing G418. The Cas9 gene has the SV40 NLS signal (KALDERON *et al.* 1984) appended at its C-terminus. Following each chromosomal integration event, genomic DNA was purified, amplified by PCR, and confirmed by Sanger sequencing for all three manipulated loci (*CDC11*, *SHS1*, and *HIS3*) including their 5'-

and 3'-UTRs and the presence of each u1 and/or u2 Cas9 target sites.

³814 base pairs of the *prGAL1/10* promoter were used upstream of the initiator Met to overexpress Cas9.

⁴GFY-2003 was created similarly to GFY-2002, but used a Cas9-expressing cassette containing the flanking u1 sites rather than u2 (amplified from pGF-IVL1027), but is otherwise isogenic.

Table S2. Plasmids used in this study.

Plasmid	Description	Reference
pRS425	2 μ ; <i>LEU2 AMP</i>	(CHRISTIANSON <i>et al.</i> 1992)
pRS423	2 μ ; <i>HIS3 AMP</i>	(CHRISTIANSON <i>et al.</i> 1992)
pRS316	CEN; <i>URA3 AMP</i>	(SIKORSKI AND HIETER 1989)
pSB1/JT1520	CEN; <i>URA3 AMP</i> ; <i>prCDC11::CDC11</i> ¹	(VERSELE <i>et al.</i> 2004)
pGF-V796 ²	pRS425; sgRNA[u1]	This study
pGF-V798 ³	pRS423; sgRNA[u2]	This study
pGF-IVL977 ⁴	<i>prHIS3::u2::prGAL1/10::S.p.Cas9::NLS::Linker::eGFP::NL S::ADH(t)::Kan^R::u2::HIS3-3'UTR</i>	This study
pGF-V789 ⁵	pRS316; <i>prGAL1/10::S.p.Cas9::NLS::CDC10-3'UTR</i>	This study

¹There is no *CDC11* 3'-UTR present within this vector.

²The sgRNA[u1]-expressing cassette is under control of the snoRNA *SNR52* promoter and *SUP4* terminator sequences (DICARLO *et al.* 2013). The u1 target sequence is CGGTGGACTTCGGCTACGTA. The entire sgRNA-expressing cassette was synthesized (GenScript, Piscataway, NJ) with flanking *BamHI* and *XhoI* restriction sites in the vector pUC57 (GenScript Cat. No. SD1176; GenBank Y14837.1; A. Markauskas and G. Dreguniene, unpublished) and subcloned to pRS425 followed by sequence verification.

³The sgRNA[u2]-expressing cassette was mutated from the u1 sequence within pUC57 through successive rounds of PCR mutagenesis (ZHENG *et al.* 2004) to generate a u2 target sequence of GCTGTTCGTGTGCGCGTCCT followed by a final subcloning into vector pRS423.

⁴Plasmid pGF-IVL977 was constructed by first creating a parent vector by *in vivo* ligation and homologous recombination in yeast (FINNIGAN AND THORNER 2015) of the genotype: *prHIS3::u2::prGAL1/10::NotI restriction site::ADH1(t)::KanR::u2::HIS3-3'UTR* (pGF-IVL974). Next, a second round of *in vivo* ligation in yeast was used to insert the *S.p.Cas9::NLS::Linker::eGFP::NLS* sequence. The flexible linker has the sequence SGGGSG and the SV40 NLS sequence (KALDERON *et al.* 1984) is SRADPKKKRKV and is found after both the Cas9 and eGFP sequences. Under identical induction and growth conditions, a similar construct (pGF-IVL976) with only a single SV40 NLS present between Cas9 and a C-terminal eGFP tag did not yield as strong of a fluorescent signal within yeast cell nuclei compared to the

pGF-IVL977 construct containing two SV40 nuclear localization signals.

⁵This vector was constructed first by *in vivo* ligation in pRS315 to fuse the *prGAL1/10* promoter, Cas9 gene, *CDC10* 3'-UTR terminator (465 bps), and the Kan^R cassette with a unique *SpeI* site present between the terminator and drug cassette. A unique *NotI* site (upstream of the *prGAL1/10* sequence) was used to subclone the *prGAL1/10::Cas9::NLS::CDC10(t)* sequence to the same sites in pRS316 to yield pGF-V789.

Table S3. Transformation efficiency for controls lacking expression of either Cas9 or sgRNA.

Yeast Strain	Growth ¹	Plasmid transformed	Colonies ²	Trials
GFY-2002	Galactose	Empty pRS425	1614 ² +/- 427 ³	n=7
GFY-2002	Dextrose	pRS425::sgRNA[u1]	6025 +/- 4260	n=2
GFY-2003	Galactose	Empty pRS425	1310 +/- 186	n=7
GFY-2003	Dextrose	pRS425::sgRNA[u1]	5996 +/- 4239	n=2

¹Yeast cultures were grown overnight in S+Raf/Suc-URA, back diluted (to an $A_{600\text{ nm}}$ of roughly 0.3) in YP+Gal and incubated for 5 h at 30°C. The cells were transformed with equal amounts (1-2 μg) of empty pRS425 vector or pRS425::sgRNA[u1] using a modified lithium acetate protocol (ECKERT-BOULET *et al.* 2012) and recovered overnight in YP+Gal medium prior to plating onto SD-Ura-Leu plates. Colonies from each experimental trial were counted after a 3-day incubation at 30°C. Resulting isolates were grown in either galactose-containing medium to induce expression of Cas9, or in dextrose-containing medium to inhibit Cas9 expression.

²Colony number was estimated by plating several dilutions (1:10, 1:20, 1:100, etc.) to selective plates and the average total colony count was reported.

³Error is SEM.

Quantification of gene replacement from Figure 2B:

For conversion of *shs1* Δ ::*Hyg*^R to WT *SHS1*, loss of hygromycin resistance was scored (for both GFY-2002 and GFY-2003); and, for conversion of the *his3* Δ ::Cas9::Kan^R cassette to WT *HIS3*, loss of G418 resistance and gain of ability to grow on SD-His medium were also scored (for GFY-2003 only). Because the u1-flanked chromosomal *CDC11* locus carried no markers, its conversion could not be scored by such a phenotypic analysis.

GFY-2002-A (500 bps flanking): 5 independent trials, 334 total colonies tested.

GFY-2002-A (30 bps flanking): 3 independent trials, 213 total colonies tested.

GFY-2003-A (500 bps flanking): 5 independent trials, 364 total colonies tested.

GFY-2003-A (30 bps flanking): 3 independent trials, 30 total colonies tested.

Quantification of gene replacement from Figure 2D:

For conversion of the *his3* Δ ::Cas9::Kan^R cassette to WT *HIS3* from a confirmed (repaired) isolate from GFY-2002-A (WT *CDC11* and WT *SHS1*) (Fig. 2B), colonies were tested for the ability to grown on SD-His medium and were also scored for G418 resistance.

GFY-2002-A (*CDC11 SHS1*) (pRS423: sgRNA[u2]): 3 independent trials and 196 independent colonies tested.

GFY-2002-A (*CDC11 SHS1*) (empty pRS423): 3 independent trials and 200 independent colonies tested.

Table S4. Growth results for colonies from controls lacking at least one PCR product.

Parental Strain	PCR fragment(s)	YPD		YPD		Total Colonies
		SD-Ura	SD-His	+G418	+Hyg	
2002-B ¹	<i>CDC11</i>	+	-	+	-	7
		+	-	+	+	9
2002-C	<i>SHS1</i>	+	-	+	-	2
		+	-	+	+	7
2002-D	-	+	-	+	-	0
		+	-	+	+	3
2003-B ³	<i>CDC11</i> & <i>SHS1</i>	+	-	+	-	0
		+	-	+	+	6
2003-C	<i>CDC11</i> & <i>HIS3</i>	+	-	+	-	2
		+	-	+	+	7
2003-D	<i>SHS1</i> & <i>HIS3</i>	+	-	+	-	0
		+	-	+	+	6
2003-E	-	+	-	+	-	0
		+	-	+	+	6

Clonal isolates were pooled from multiple experimental trials, in which either 500 bps of flanking homology (Fig. 2) or 30 bps of flanking homology (Fig. S3) were used, and replica-plated onto various growth conditions (SD-Ura, SD-His, YPD+G418, and YPD+Hygromycin). A “+” score indicates growth/resistance whereas “-” designates no growth/sensitivity. For the initial genotypes indicated [strain and PCR fragment(s)], two growth patterns were observed and the number of colonies displaying each pattern were totaled (*far right column*). Yeast were also tested on SD-Leu medium for the presence or absence of the high-copy pRS425::sgRNA[u1] vector. After only two rounds of selection on SD-Ura (no selective pressure for the *LEU2* marker), 40/55 strains had lost the sgRNA-expressing plasmid. Representative isolates were taken from each category for further analysis by diagnostic PCR.

¹B, C, D or E designation is from the experiment in Fig. 2, in which no PCR fragments, one PCR fragment, or two PCR fragments were introduced by transformation, as indicated.

³For simplicity, only combinations of two PCR products (omitting a third) were tested for strain GFY-2003, rather than all possible combinations.

Table S5. Oligonucleotides used in this study.

Oligonucleotide	Description	Sequence (5' to 3')
F1	<i>CDC11</i> UTR ¹ +406 F	CATCTACAAAAGCAGGTTATAGCTCCG TTAAAC
F7	<i>CDC11</i> UTR +330 F	CACAACATGGAACATAACATTTAAACAT CGTTCTCAATC
R1	<i>CDC11</i> UTR -327 R	GCTAAGTGATGTTCTGGTCTTTCCAAAA TTCTC
F2	<i>CDC11</i> UTR +101 F	GATCGAAAAGTAAAATAGGTAGACACC ACGTATTG
R2	<i>CDC11</i> Int ² +75 R1	CACAGTGAAGGTTATACCTCTTTTCAAA TGCTTTC
F3	<i>CDC11</i> Int +1233 F	GAAGCCAGGTTGGAAAAAGAGGCGAAA ATC
R3	<i>CDC11</i> UTR -89 R	TAATATACCAGATGTATTTACTTATATAC AACACTCACTC
F4	<i>SHS1</i> UTR +800 F	CATTACTAGAGTTTGCTGGTGTTCAG ATCG
F8	<i>SHS1</i> UTR +500 F	GTTCTCCGGTTTAAACTGATCCATAGT GATAG
R4	<i>SHS1</i> Int +999 R	ACGGTAATTCTCGTAAAGCAAATTTTGC GTGG
R5	Int Hyg R	CTCCTACATCGAAGCTGAAAGCACGAG
F5	Cas9 Int +2903 F	CTGAAGTCTAAGCTGGTCTCAGATTTCA GAAA
F9	Cas9 Int +3477 F	GTGGAGAAAGGGAAGTCTAAAAAACTC AAAAGC
R6	Int Kan R	GAACACTGCCAGCGCATCAACAATATT TTC
F6	<i>HIS3</i> UTR +992 F	GGGTCAGTTATTTTCATCCAGATATAACC CG
F10	<i>HIS3</i> UTR +500 F	TTCTCGACGTGGGCCTTTTTCTTGCCAT AT

R7	<i>HIS3</i> UTR -497 R	GTAACCACCACGACGGTTGTTGCTAAAGAA
F11	<i>CDC11</i> UTR +30 F	CACAAGGCCTAAAGTTGCTAACCACCA GCCATGTCCGGAATAATTGACGCATCT TCTGCA
R8	<i>CDC11</i> UTR -30 R	AAGAAATAAGTGAGGAAGCCAAAAGCG GACTCATTCTTCCTGTTTGATTTTCGCC TCTTTTTCC
F12	<i>SHS1</i> UTR +30 F	TATAATTGCTAGAAAAATATATTATTAAT CATGAGCACTGCTTCAAC
R9	<i>SHS1</i> UTR -30 R	GCTCAGCTTTGGATTTTGTACAGATACA ACACTGGATGGCGGCGTTAGTATCGAA TC
F13	<i>HIS3</i> UTR +30 F	AATGAGCAGGCAAGATAAACGAAGGCA AAG
R10	<i>HIS3</i> UTR -30 R	GTATGCTGCAGCTTTAAATAATCGGTGT CA
F14	Int Kan F	CGTTGCATTCGATTCCTGTTTGTAATT GTCC
R11	<i>SHS1</i> UTR -469 R	CCACTAGAGCATTGGCAGAATTATATG GTAGT

¹UTR +/- designates sequence upstream (5') or downstream (3') of the specified gene.

²"Int" designates a position internal to the coding sequence of the specified ORF.

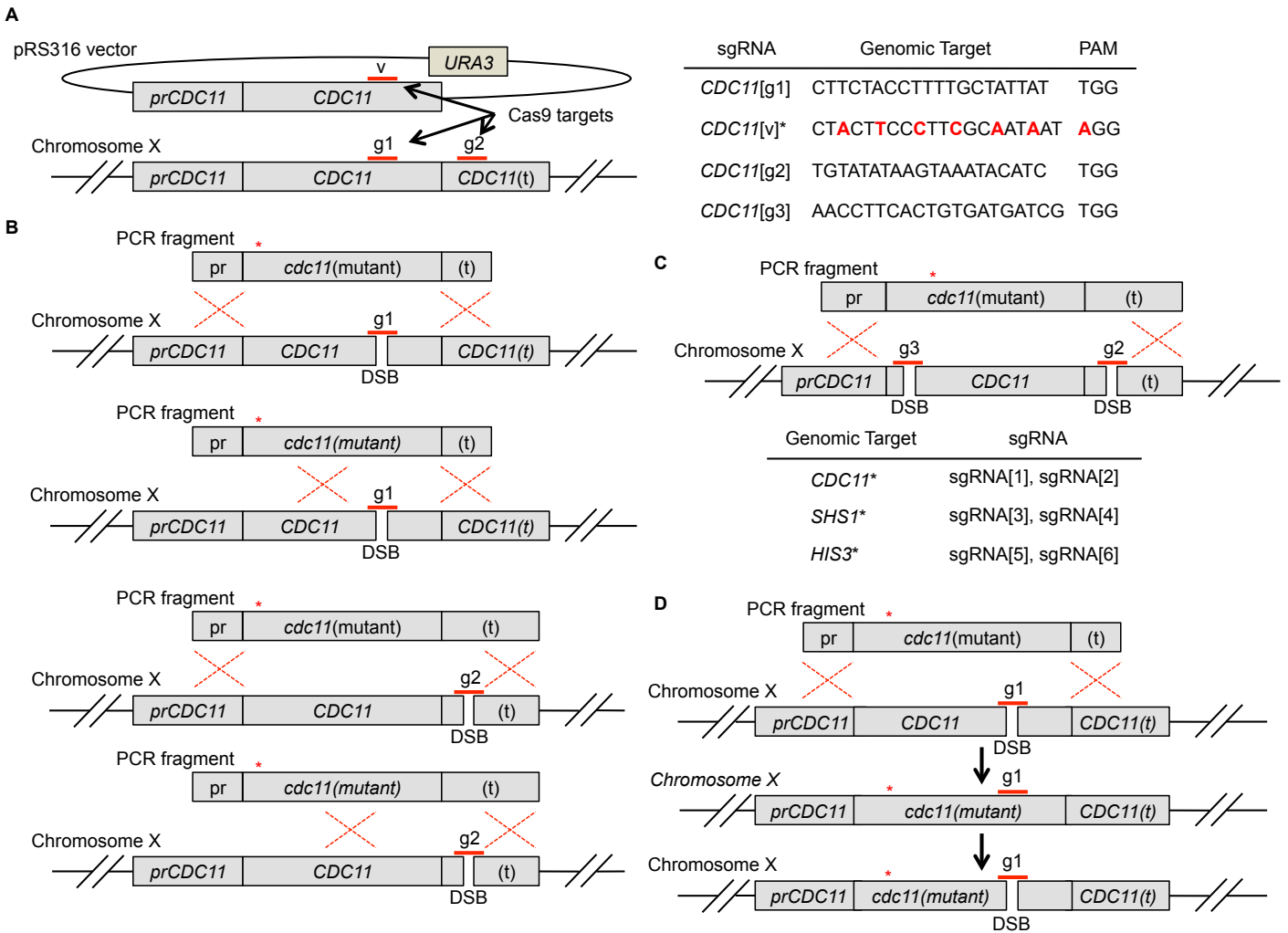


FIGURE S1. Limitations of “classical” Cas9-mediated manipulation of essential genes. (A) Targeting an essential gene *in vivo* requires the presence of a WT-expressing “covering” plasmid that can be counter-selected (harboring *URA3*) by growth on medium containing 5-FOA (BOEKE *et al.* 1984). Targeting the *CDC11* gene at two genomic positions, g1 (within the coding region) and g2 (within the flanking 3’ UTR) requires that both 23-nucleotide sequences are not present within the WT copy of the covering plasmid expressing *CDC11*. Example Cas9 target site sequences are shown based on the proposed collection of target sites within the yeast genome (DICARLO *et al.* 2013). If the g1 target sequence within *CDC11* were used, the corresponding sequence of the covering vector would have to be mutated to prevent inappropriate targeting by Cas9 to the vector sequence. Synonymous changes could be made (red text) to the covering vector, so as to not alter the amino acid sequence (target sequence v) with a priority to the nucleotides within the PAM motif and the 12 bps immediately upstream sequence, as these are the most significant for Cas9 target site “seeding” (JINEK *et al.* 2012; JIANG *et al.* 2013). Since the covering vector does not contain the native 3’-UTR of *CDC11*, no additional changes need to be made to allow for targeting at g2 position within the terminator. However, it should be noted that 3’-UTR sequences may pose difficult in finding suitable Cas9 target sites due to the AT-rich stretches, and the possibility for a TTTTTT sequence which can terminate RNA polymerase III transcripts (BRAGLIA *et al.* 2005; WANG AND WANG 2008) and lower GC content within the target sequence. (B) If a single genomic target is used, g1 or g2, to create a double-stranded break (DSB) within the *CDC11* locus, there are several scenarios that would allow for inappropriate crossover when integrating a mutant allele (red asterisk) in place of the WT endogenous copy. When using g1, which is downstream of the proposed mutant allele, the crossover event could occur either upstream of the mutation within the promoter

sequence (first panel) or downstream of the mutation within the coding sequence (second panel). The second event would repair the DSB, but fail to integrate the mutant allele. Similarly, use of g2 within the terminator sequence would also have the same issue with the possibility of the crossover event occurring downstream of the desired mutation. Thus, in order to optimize the integration efficiency of the mutant allele, one should (i) increase the 5'-homology (promoter) sequence and/or (ii) choose a Cas9 target site as close to the mutation as possible to prevent repair of the WT gene copy, as also suggested by others (HORWITZ *et al.* 2015). However, if the mutation is near the initiator Met within the coding sequence, or the coding sequence of the gene is long, then choosing a g2 terminator target (which is not present within the covering vector) encounters the same efficiency issues. Therefore, programming Cas9 to target a specific position close to the desired mutation g1 would increase integration efficiency, but would *also* require changing the same target site within the covering vector sequence *v*. Furthermore, if multiple mutant alleles spanning the entire length of the gene were to be integrated, this strategy would either require multiple Cas9 genomic targets, or varying efficiencies based on the position of the desired mutation(s). Additionally, for essential genes, the presence of the covering vector creates an additional step (to mutate the WT gene sequence) to prevent Cas9-dependent targeting of this protective vector. (C) One possible solution is to target multiple positions within the locus of interest and create two DSBs, one of which is upstream of the mutation of interest within the coding sequence g3. In this way, recombination can *only* occur upstream and downstream of the two DSBs, and would efficiently introduce the desired allele with virtually no inappropriate crossover events (assuming efficient targeting by Cas9). However, since g3 is still within the coding sequence, the *v* position within the covering vector would require alteration and rather than creating a single sgRNA construct, two constructs sgRNA[g2] and sgRNA[g3] would be required to manipulate this single locus. The amount of cloning required would be further increased if this strategy were extended to additional loci; targeting of three genes would require multiplexing 6 guide RNA cassettes *and* the covering vector sequences (see Fig. S1C, *lower*). (D) Finally, integration of an essential gene allele using a traditional Cas9 approach requires yet another cloning step. If any genomic target is chosen within the coding sequence, g1 or g3 for instance, then the coding sequence of the integrated allele itself at these same position(s) must *also* be altered so as to prevent Cas9-mediated targeting of the final integrated allele. Therefore, at minimum, a single guide g1 strategy requires (i) alteration of the covering vector, (ii) alteration of the mutant allele itself, and allows for (iii) inappropriate crossover between the mutant allele and g1 target site, and (iv), would likely have to be altered for various alleles or constructs spanning the length of the target gene. A two-guide strategy, targeting g2 and g3, which removes the possibility of inappropriate crossover, requires the same cloning alterations including the vector, the integrated allele itself, *and* design and construction of two sgRNA cassettes. These drawbacks make the traditional Cas9 strategy laborious, suboptimal, and require the separate construction of many components aside from Cas9 and the sgRNAs themselves.

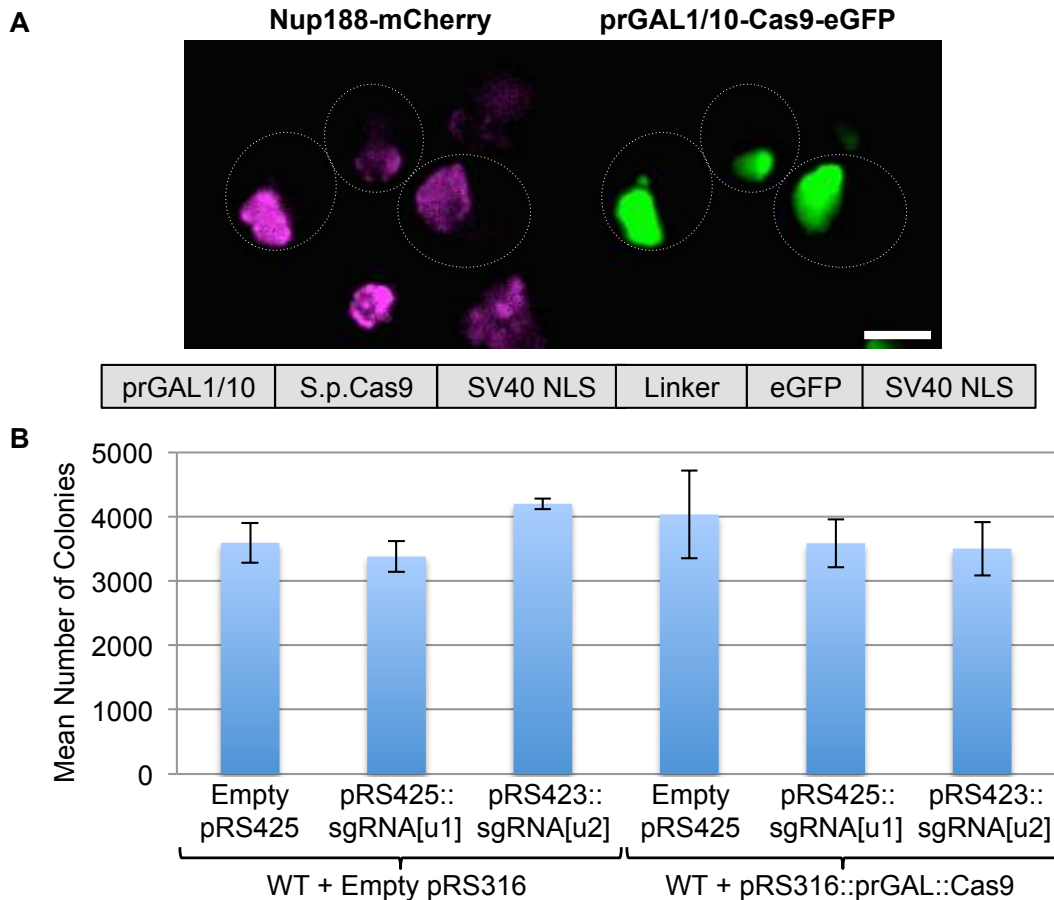


FIGURE S2. Expression of Cas9-eGFP and the benign effect of the programmable flanking site system *in vivo*. (A) Yeast (GFY-1517) expressing Nup188-mCherry (marker for the nuclear envelope) were transformed with a plasmid (pGF-IVL977) expressing under control of the *GAL1/10* promoter S.p.Cas9 containing an SV40 NLS sequence (SRADPKKKRKY), a flexible SGGGSG linker, eGFP, and a second SV40 NLS, as indicated, were grown overnight in S+Raf/Suc-Leu, back-diluted in YP+Gal, grown at 30°C for 3 h, and imaged by fluorescence microscopy. Representative cells are shown. White dotted lines, cell periphery; scale bar, 2 μ M. (B) Expression of Cas9 alone, or either sgRNA[u1] or sgRNA[u2] alone, or expression of Cas9 with either sgRNA[u1] or sgRNA[u2], did not result in any marked differences in transformation efficiency or viability of the resulting yeast colonies. WT cells (BY4741) were first transformed with either an empty pRS316 vector or a pRS316::prGAL1/10::Cas9 vector (pGF-V789). Next, the yeast were grown overnight in S+Raf/Suc-Ura medium to saturation, back-diluted into YP+Gal, grown at 30°C for 5 h, and then transformed with equal amounts (1-2 μ g) of empty pRS425, pRS425::sgRNA[u1], or pRS423::sgRNA[u2] vectors. After recovery overnight in YP+Gal, the cells were plated onto SD-Ura-Leu (for pRS425 vectors) or SD-Ura-His (for pRS423 vectors). Experiments were performed in triplicate; colonies were counted and the average colony number was plotted for each genotype (error bars, SEM). There was no statistically significant difference between WT yeast (*first strain*; unpaired t-test p-value = 0.59) and yeast over-expressing Cas9 (*fourth strain*). Similarly, there was no statistically significant difference between expression of sgRNA[u1] alone (*second strain*) compared to co-expression with Cas9 (*fifth strain*; p-value = 0.67). Similarly, there was no statistically significant difference with or without Cas9 for sgRNA[u2] (*third and sixth strains*; p-value = 0.18). These data suggest (i) that Cas9 expression *per se* is not toxic in yeast, as has been previously reported (DICARLO *et al.* 2013) and (ii) that neither sgRNA[u1] or sgRNA[u2] are toxic to yeast (*i.e.*, they do not mediate formation of any lethal DSB at off-target locations within the yeast genome).

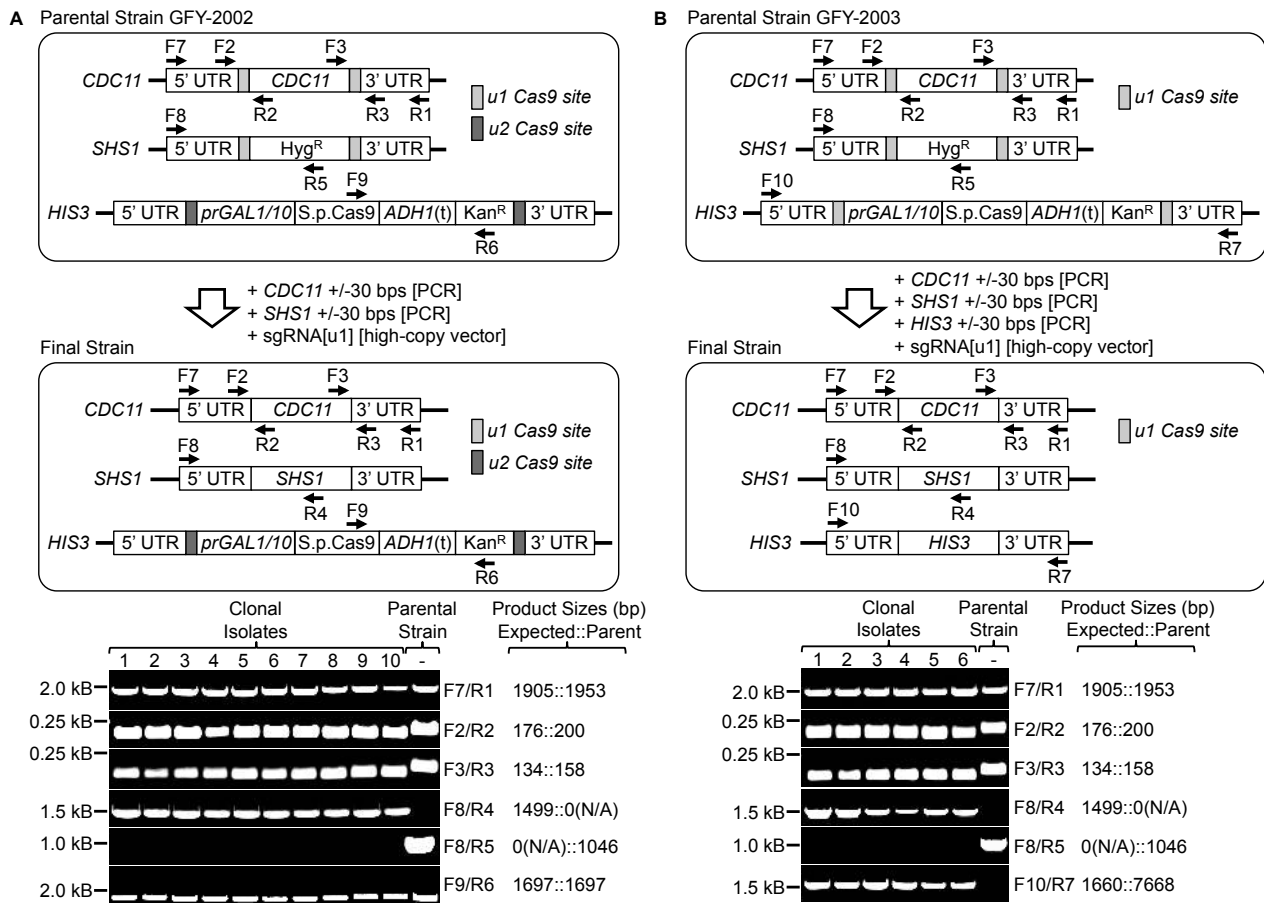


FIGURE S3. Concurrent integration at either of two (out of three) genomic loci with concomitant removal of Cas9 using the single sgRNA[u1] system and only 30 bps of flanking homology. (A) Strain GFY-2002 was induced for Cas9 expression and transformed with the sgRNA[u1] expression vector and PCR products for the *CDC11* and *SHS1* loci, as in Fig. 2. Each PCR fragment was flanked by 30 bps of homology to the genomic target. Chromosomal DNA was purified (AMBERG *et al.* 2006) from ten representative clonal isolates (chosen on the basis of their phenotype, assessed as in Table S4), and diagnostic PCRs were performed and the resulting DNA products analyzed by agarose gel electrophoresis. For *CDC11* (*top three gels*), the entire locus was amplified (oligos F7/R1) as well as small fragments flanking either the upstream u1 site (F2/R2) or the downstream u1 site (F3/R3) to determine if the 23-base pair Cas9 target site was still present or not. For *SHS1* (*fourth and fifth gels*), a PCR (F8/R4) was performed with a unique primer internal to the *SHS1* coding region that was not present within the parental strain, as well as a PCR containing a unique primer to the Hyg^R cassette that was present in the parental strain, but not in any putative integrant that repaired the *SHS1* gene. Finally, the *HIS3* locus was tested with a unique primer internal to the Cas9 gene and to the *HIS3* 3'-UTR (F9/R6) to illustrate this genomic site was unperturbed (it was programmed with flanking u2 sequences). For optimal separation on the second and third DNA gels, a 2% agarose mixture was used compared to the standard 1% concentration. DNA size markers, kB (kilobases). Finally, the expected fragment size for each diagnostic PCR is shown for each reaction. (B) An analysis identical to that in (A) was performed using parental strain GFY-2003 (which has u1 sites flanking the Cas9 cassette at the *HIS3* locus). Six independent clonal isolates were obtained and tested as in (A), except that one PCR fragment was included that contained the WT *HIS3* gene (also with 30 bps of flanking UTR) to eject and replace the endogenous Cas9 cassette. To monitor substitution of the Cas9 cassette with the repaired *HIS3* gene, PCR for the entire *HIS3* locus was performed (F10/R7). At least one of the two primers used for each diagnostic PCR corresponds to sequences that lie outside the region contained within the PCR fragments used for transformation.

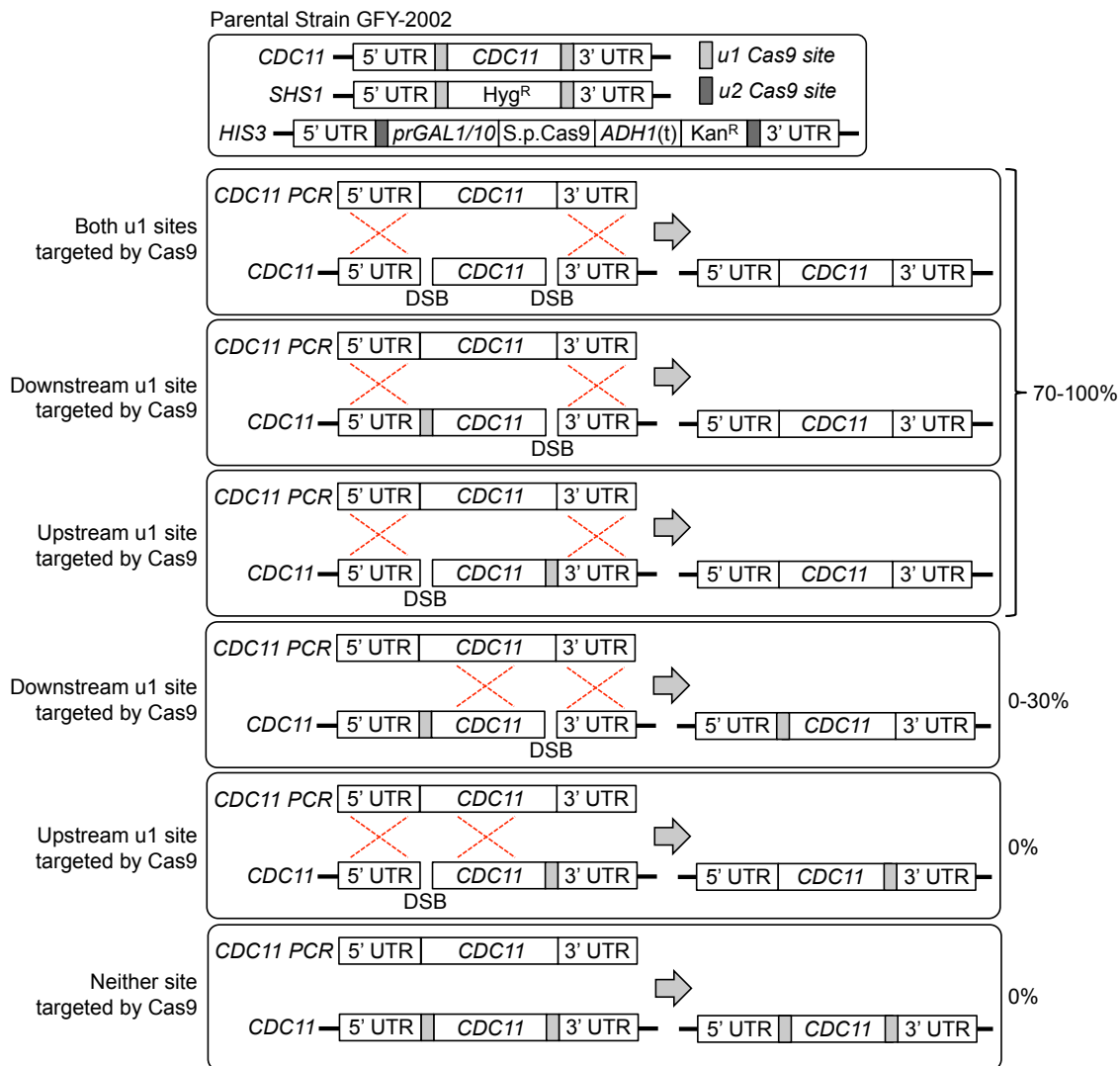


FIGURE S4. Possible outcomes of incomplete Cas9-targeting of u1 sites at the *CDC11* locus *in vivo*. Although the majority of the isolates tested displayed proper replacement of the u1-flanked *CDC11* locus with the WT *CDC11* gene carried on the PCR fragment, a small percentage of the isolates tested showed replacement of the downstream u1 site, but not the upstream u1 site (see Fig. 3). However, the *CDC11* locus replacement differed from that of *SHS1*, or *HIS3* (for parental strain GFY-2003 only), in that the genomic target had a sequence identical to that provided on the PCR fragment. For *SHS1*, the WT gene replaced the Hyg^R deletion, and for *HIS3*, the WT gene replaced the Cas9 cassette. If Cas9 targeted either or both of the u1 sites at the *CDC11* locus and the crossover events occurred outside of the coding sequence (*top three panels*), then the full WT *CDC11* gene would replace the parental u1-flanked cassette and neither Cas9 target site would remain, as we observed for 70-100% of the isolates screened in four independent experiments. If, by contrast, Cas9 failed to create any DSB at the *CDC11* locus, then both u1 sites would remain; reassuringly, none of the isolates tested displayed this pattern. If Cas9 only targeted one u1 genomic location at the *CDC11* locus, *and* the crossover occurred within the *CDC11* coding region, then the DSB would be repaired by PCR product provided, but one u1 site would remain intact. Out of 36 total isolates tested, 100% had the downstream u1 site removed, whereas just four retained the upstream site u1 site, which may reflect some difference in chromatin structure or occupancy by RNA polymerase or something else that affects their accessibility to Cas9.

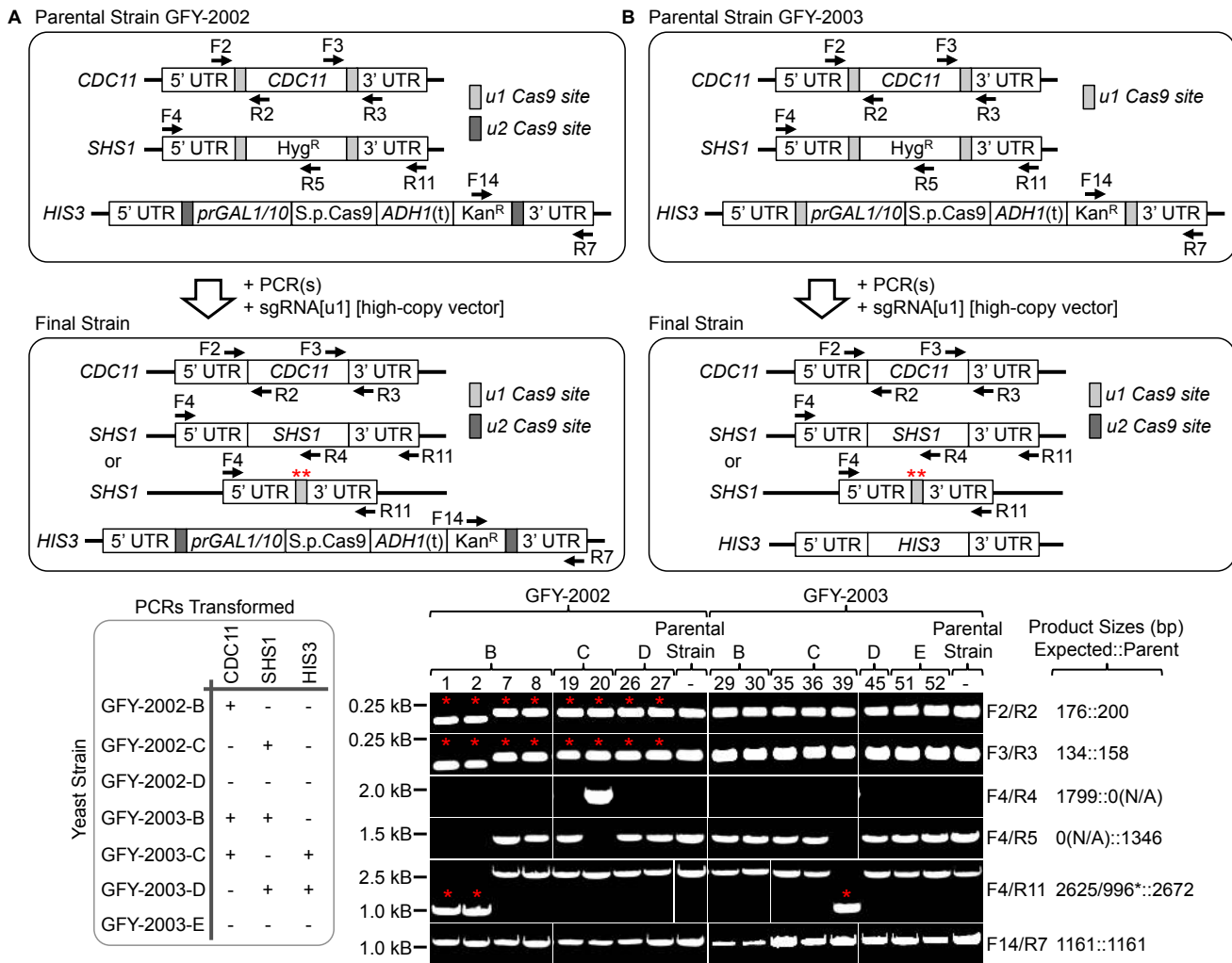


FIGURE S5. Analysis of very rare surviving isolates from controls where one or more PCR fragments for integration were not provided. (A and B) Clonal isolates from multiple experimental trials (Fig. 2B) were tested by both growth (all tested isolates; Table S4) and diagnostic PCR (1 to 4 isolates chosen from each growth category). A summary of the yeast strains used and the PCR fragment(s) added in each transformation are summarized (*lower left*). Primers flanking the *CDC11* u1 Cas9 sites (F2/R2 and F3/R3) were used for PCR to determine whether the WT *CDC11* allele has been integrated (*top two gels*). For the *SHS1* locus, PCR with a unique primer to the *SHS1* coding sequence (R4, *third gel*) or to the *Hyg^R* cassette (R5, *fourth gel*) were used. Because isolates 1, 2, and 39 appeared to have neither the WT *SHS1* gene nor the deletion cassette, primers flanking the *SHS1* locus (F4/R11) were used for amplification (*fifth gel*). If the full *SHS1* locus has been deleted, the diagnostic PCR product is expected to be 996 bps (*product size, black asterisk*). Finally, the *HIS3* locus was interrogated by PCR (F14/R7, *sixth gel*). Selected PCR products (*red asterisk*) were purified and sequenced. For isolates 1, 2, and 39, DNA sequencing revealed that the deletion present removed the *SHS1* locus, but left behind a single u1 site (*double red asterisk*), suggesting that after excision of the *SHS1* locus, the break was repaired by recombination between the two cut u1 sites. The diagnostic PCR products were analyzed on 2% agarose gels (*first and second gels*) or 1% (*gels three through six*); DNA size markers, kB (kilobases). For products resolved on different gels, the gel fragments are shown as separate images. All images were scaled identically and are marked with both DNA ladders and the predicted PCR product sizes from the parental strains GFY-2002 or GFY-2003, as indicated) using the same primer set.

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