Development of CRISPR-RNA Guided Recombinases for Genome Engineering

By

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ABSTRACT

Recombinases are powerful tools for genome engineering and synthetic biology, however recombinases are limited by a lack of user-programmability and often require complex directed-evolution experiments to retarget specificity. Conversely, CRISPR systems have extreme versatility yet can induce off-target mutations and karyotypic destabilization. To address these constraints we developed an RNA-guided recombinase protein by fusing a hyperactive mutant resolvase from transposon TN3 to catalytically inactive Cas9. We validated recombinase-Cas9 (rCas9) function in model eukaryote *Saccharomyces cerevisiae* using a chromosomally integrated fluorescent reporter. Moreover, we demonstrated cooperative targeting by CRISPR RNAs at spacings of 22 or 40bps is necessary for directing recombination. Using PCR and Sanger sequencing, we confirmed rCas9 targets DNA recombination. With further development we envision rCas9 becoming useful in the development of RNA-programmed genetic circuitry as well as high-specificity genome engineering.

DEDICATION

To all the *E.coli*, Yeast and HEK293T cells that heroically gave their life for this study.

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GLOSSARY OF ABBREVIATIONS

DNA: Deoxyribonucleic Acid

RNA: Ribonucleic Acid

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeat

sgRNA: single-guide RNA

PAM: Protospacer Adjacent Motif

Cas9: CRISPR-associated protein 9

dCas9 Catalytically InactiveCas9 (*i.e.* $Cas9^{D10A,H840A}$)

rCas9: Recombinase Fused Cas9

TE: Transposable Element

DBD: DNA-binding Domain

Bp: Base Pair

Kb: Kilobase Pair (1000 Bp)

GFP: Green Fluorescent Protein

mCherry: Red Fluorescent Protein

DSB: Double-stranded DNA Break

SSB: Single-stranded DNA Break

Indels: Insertion-Deletion Mutations

YPD Media: Yeast Extract Peptone Dextrose Media

Dropout Media: Media lacking defined amino acids, selects for prototrophic markers

INTRODUCTION

The development of new genome engineering technologies is crucial for the study of disease genetics and exploring the development of novel gene therapies[1,2]. Recombinases are a class of DNA manipulating enzymes. These are powerful tools for predictable and high specificity genetic manipulations. Serine recombinases are often employed for routine *in vivo* and *in vitro* DNA manipulations[3–5]. Recombinases have tremendous potential for therapeutic DNA manipulations. For instance, Karpinski and coworkers demonstrated directed evolution of Cre recombinase towards targeting conserved sequences of Human Immunodeficiency virus (HIV) long-terminal repeats (LTRs)[6]. This enabled removal of the HIV provirus from the human genome. However this required 150 rounds of directed evolution and counter-selection to ensure high specificity to generate the retargeted recombinase. Recombinase based DNA manipulations are tremendously hard to reprogram to new DNAs of interest.

DNA transposable elements (TEs) are capable of catalyzing movement and repair of DNA molecules. Some bacterial TEs contain sufficient enzymatic machinery to facilitate their requisite DNA manipulations[7–9]. Resolvases are a novel class of these proteins associated with TEs that facilitate highly targeted DNA repair have a well understood structure (**Fig. 2A**) and have been previously engineered target new DNAs of interest[10]. However, previous techniques rely on fusion of a resolvase with alternative DNA binding domains and are not easily targetable for new experiments and require coexpression of recombinase heterodimers[11].

Clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR associated proteins (*e.g.* Cas9) function as a bacterial immune system that enables recognition and cleavage of foreign nucleic acids (*ie.* DNA and RNA)[12]. The relative robustness and ease of implementing some engineered CRISPR systems in eukaryotic organisms has enhanced our ability to edit genetic material[13,14]. However, CRISPR based techniques may lead to off-target double stranded DNA breaks (DSBs). Off-target DNA damage may lead to mutations ranging from unwanted insertion and deletions (indels) to destabilizing the karyotype[15,16]. These unintended mutations impede application of CRISPR systems for manipulating organisms with large and highly complex genomes. The drive to avoid DSBs in genome editing lead to the development of Cas9-nickase base-editors[17]. However, it is still possible for the base-editors to induce off-target indels due to single-stranded DNA breaks[18]. Also these tools are relatively limited to single nucleotide changes preventing them from being used for targeted DNA deletion or integration. Since, recombinases are relatively unprogrammable, and conversely CRISPR systems may introduce off-target mutations, we reasoned we could fuse Cas9 with a hyperactive resolvase. This should result in an RNA-guided recombinase.

RESULTS

Identification of Genome Targeting Recombinases

To experimentally assess resolvase protein function, we constructed a reporter system in *Saccharomyces cerevisiae* (yeast) (**Fig. 1**). We choose yeast as it is a model eukaryote and can serve as a 'proxy-organism' for the development of genome engineering tools[19,20]. In the reporter assay, the green fluorescent protein (GFP) gene is

integrated into yeast chromosome 5 and is surrounded by resolvase recognition sites. When the resolvase protein is active, this results in deletion of GFP. At an adjacent position, mCherry is coexpressed as a constitutive marker. Flow cytometry allows for rapid and quantitative screening of resolvase function (**Fig. 1B**). We tested 3 resolvases described in the literature derived from transposon TN3 and bacteriophage mu (Gin). Using this technique we identified the mutant resolvase $TN3^{G79S,D102Y,E124Q}$ as functional in targeting eukaryotic genomic DNA (**Fig. 2B,C**). Henceforth, we refer to this recombinase as mTN3 and utilize this resolvase in genomic targeting experiments.

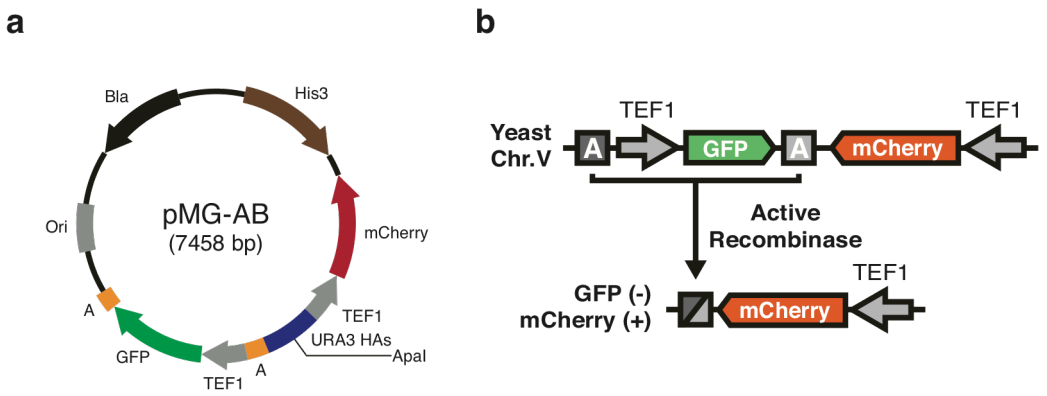

Figure 1: Genome Integrated Recombinase Assay. (**a**) To assess the functionality of recombinases' ability to target genomic DNA, we constructed a yeast integrative vector. This vector when digested with ApaI and transformed into yeast integrates into the URA3 locus on chromosome 5. The His3 cassette enables selection for integrants using hisitidine deficient plates. (**b**) GFP and mCherry are constitutively expressed from TEF1 promoters. When a recombinase mediates deletion between recognition sites (A) this results in GFP deletion, while mCherry expression remains intact.

Fusion of mTN3 Recombinase to dCas9

Following identification of an active resolvase in *S. cerevisiae*, we sought to fuse the catalytic domain of mTN3 resolvase with the programmable DNA binding of a catalytically inactive Cas9 protein (dCas9). We utilized molecular cloning techniques to

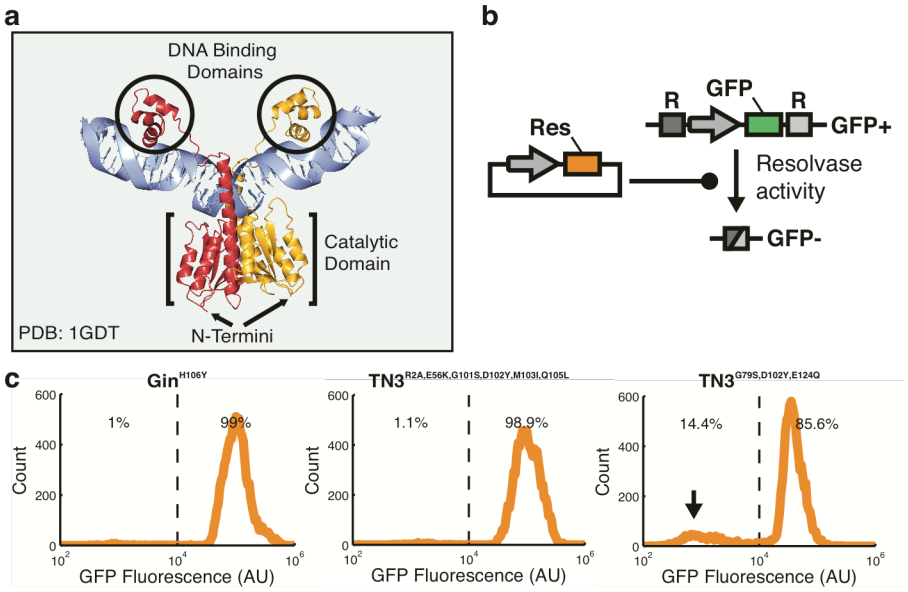

Figure 2: Identification of Active Recombinases. We utilized the assay described in figure 1 to determine the ability of different recombinase to effectively target deletion. (**a**) We focused on a series of recombinases homologous to γΔ (PDB 1GDT). Since these recombinases have relatively separated DNA binding and catalytic domains. (**b**) The resolvase was expressed from a yeast episomal vector (see methods) and targets GFP deletion when active. (**c**) Representative flow cytometry histograms of cells with various recombinase constructs. Left of the dashed line is GFP negative. $TN3^{G79S,D102Y,E124Q}$ was the sole recombinase substantially active in our deletion assay.

construct a resolvase-dCas9 fusion (rCas9) under control of a galactose responsive promoter (**Fig. 4A**). We cooexpressed rCas9 with multiple single-guide RNAs (sgRNAs) and an improved genome integrated fluorescent reporter (**Fig. 4B**). Given resolvases bind to substrate DNA as dimers (**Fig. 2A**), we reasoned sgRNAs would have to target around a resolvase "core" sequence (**Fig. 3**). Through initial flow cytometry screens we identified an rCas9-sgRNA design combinations with approximately one sixth the efficiency of the mutant resolvase (**Fig. 4C**). mTN3 with its native helix-turn-helix DNA binding domain (mTN3-DBD) resulted in 35% GFP deletion, while rCas9 resulted in 6-8% GFP deletion after 96 hours of galactose induction (**Fig. 4C**). We characterized

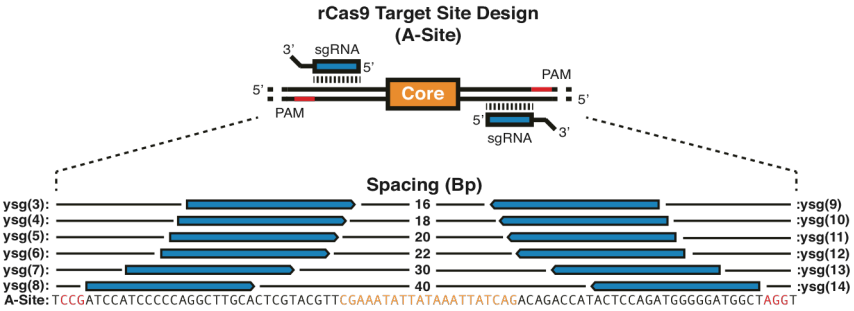

Figure 3: rCas9 Target Site Design. Since TN3 resolvase binds to its recognition site as a dimer we reasoned sgRNAs targeting the to the left and to the right of the core sequence should result in activity. TN3 core sequence is in orange, and PAMs are in red.

targeting of rCas9 by systematically testing sgRNA spacings. Interestingly, we found spacing of 22 and 40bp apart are ideal, however 30bp is relatively non-functional in symmetrical placement around the core sequence (**Fig. 4D**). However, we observed 31bp to be highly functional with asymmetric placement around the central core. This is consistent with the three dimensional structure of the Rosalind-Watson-Crick DNA double helix being 10-11bp per full rotation; combined with co-localization of resolvase catalytic domains on the same side of the DNA molecule. We further qualitatively characterized deletions using fluorescent microscopy (**Fig. 4F**). Expression of rCas9 results in cells without GFP, while mCherry expression remains. This suggests the recombinase is not prevalently resecting large parts of chromosome 5.

Analysis of Interdomain Linker Function

To analyze the functional effect of linker peptides between mTN3 and dCas9 we tested a series of linker domains in our deletion assay (**Fig. 5A**). These range from varying lengths of flexible glycine serine (GGS) linkers to the rigid XTEN, a previously described linker domain that has resulted in functional fusion proteins with Cas9[21]. We also tested a GGS-XTEN hybrid linker. We see a trend of longer linkers having slightly

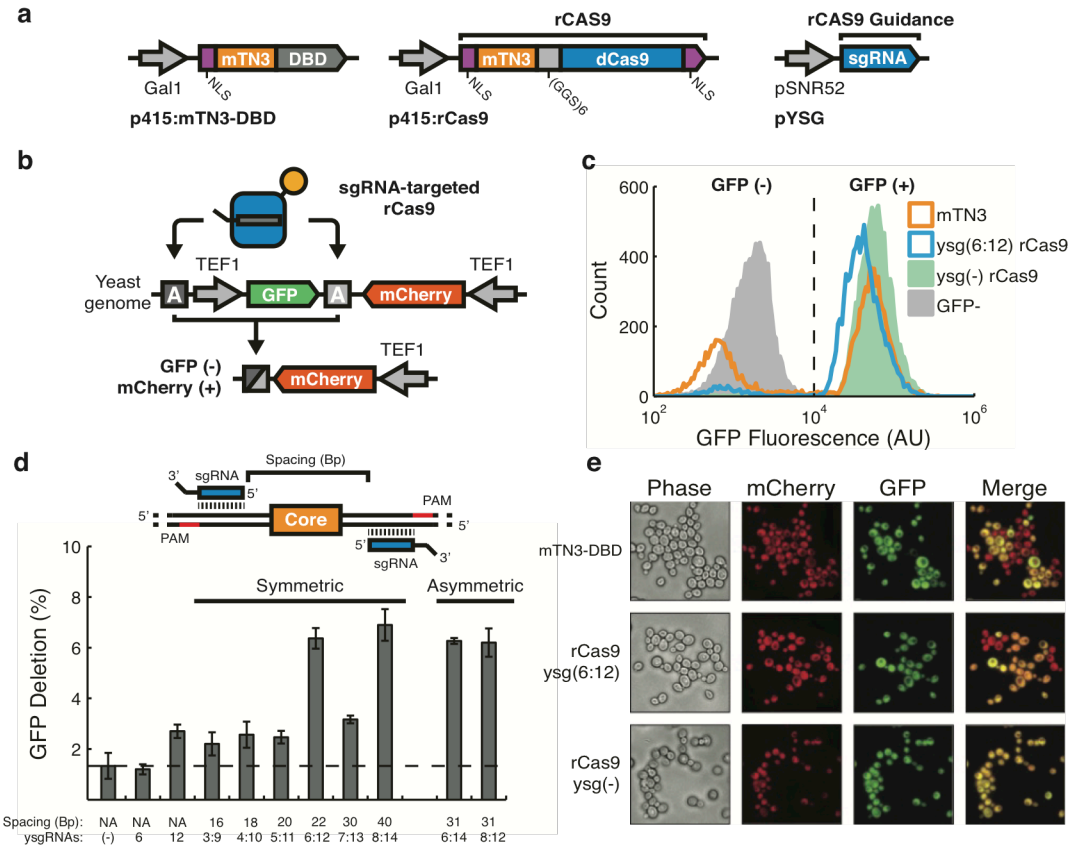

Figure 4: Demonstration of rCas9 Genomic Targeting. (a) representations of expression constructs for recombinase and rCas9 systems. mTN3 in organge represents the catalytic domain of the hyperactive mutant identified in figure 2. NLS indicates an SV40 nulcear localization sequence. Plasmids are described further in the methods. (**b**) rCas9 targeting of A-sites when active results in GFP deletion. (**c**) representative flow cytometry of deletion assay. mTN3 results in 35% GFP deletion, while rCas9 results in 6-8%. (**d**) We systematically tested sgRNA spacing and its effect on rCas9 function. (**e**) Fluorescent microscopy showing GFP deletions have intact mCherry expression. This results in red only cells when targeted.

higher efficiency compared to the shortest linker (*i.e.* linker-1), however we did not see a large (>10%) improvement of rCas9 efficiency (**Fig. 5B**). Perhaps, future investigation to improve rCas9 efficiency can focus on addition of larger linker sequences.

Confirmation rCas9 operates as a DNA recombinase

To confirm rCas9 operates as a DNA recombinase we utilized PCR with primers flanking the GFP expression cassette. The PCR product of the starting reporter is 5 Kb.

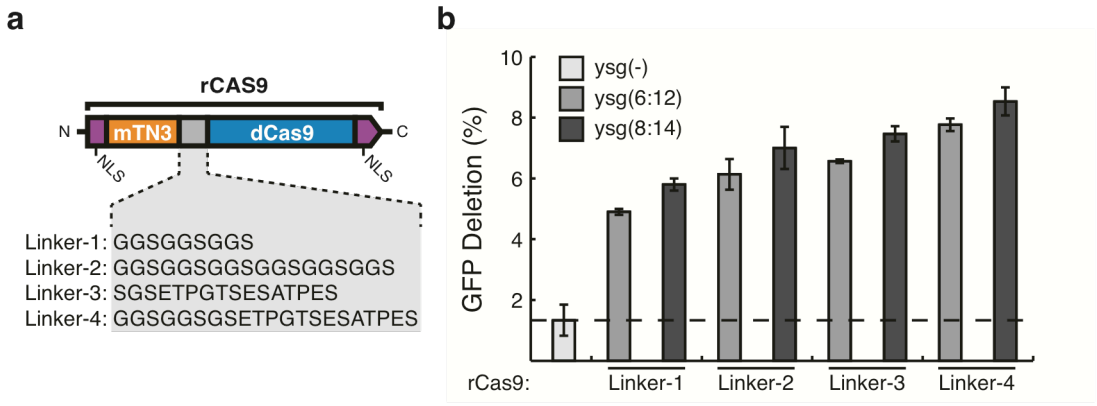

Figure 5: Analysis of Interdomain Linkers on rCas9 Function. (**a**) To asses the effect of interdomain linkers we constructed a series of interdomain linkers ranging from (GGS)3, linker-1, to longer amino acid sequences and hybrid flexible and rigid linkers. "N" indicates N-terminus and "C" C-terminus. (**b**) Deletion assay results for linker variant rCas9s. We focus on linker-2 in subsequence experiments.

Deletion of GFP results in a 4 Kb PCR product (**Fig. 6A**) . Expression of mTN3-DBD results in formation of the 4 Kb deletion product. When rCas9 is expressed with sg(-) (*i.e.* a guide not matching the reporter cassette) only the starting 5 Kb band is observed. When rCas9 binding is targeted to the reporter this results in the formation of the 4 Kb deletion product. These results indicated that rCas9 is resulting in DNA deletion. To further characterize the deletion product we gel-extracted and sub-cloned the deletion product. We sequenced 5 clones and found they consistently match the expected recombination product. We did not observe any insertion or deletion mutations (**Fig. 6B**). This indicates when CRISPR RNAs are cooperatively targeted to a recognition site rCas9 is capable of catalyzing DNA deletion.

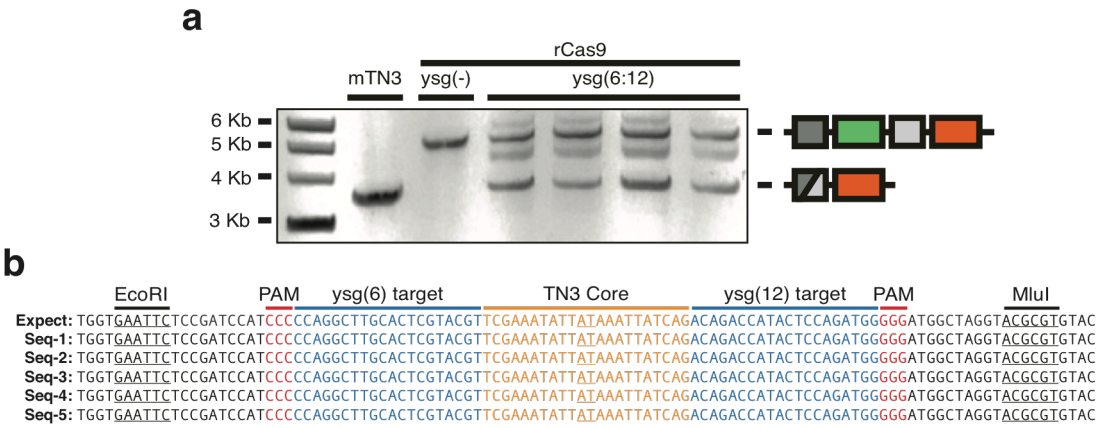

Figure 6: Confirmation rCas9 Targets DNA Deletion. (**a**) PCR of deletion reporter in cells expressing mTN3-DBD (mTN3 here), and rCas9. The 5 Kb band is the starting GFP-mCherry reporter (green and red icon). rCas9 conditionally results in deletion when ysg(6:12) guides it to its target site. This is illustrated by the occurrence of the 4 Kb deletion band (red icon). (**b**) sequencing of subcloned PCR products indicates formation of the expected deletion product. EcoRI and MluI sites function as landmarks that originally flanked the GFP expression cassette. We observed no indels within or near the target site.

CONCLUSION

Here we demonstrated the ability to fuse the catalytic domain of a hyperactive resolvase with the DNA binding functionality of catalytically inactive Cas9. This results in sgRNA targeting of recombinase functionality. This work focused on targeting the default Res1 core sequence. We reasoned that in proof of principle experiment we should assay the ability of rCas9 facilitate recombination against a substrate of known functionality. There have been conflicting reports regarding the versatility of resolvase catalytic activity. Some reports indicate directed evolution and catalytic reprogramming is necessary to target new DNAs, however other reports have demonstrated that homologous recombinases (*e.g.* Gin) can target many DNA sequences. Further experiments that focus on changing the core sequence would be necessary to know for certain what the versatility of rCas9 is. Similarly another current constraint of rCas9 is

it's relatively low efficiency. We saw minor improvements with varying interdomain linker sequences, however these improvements were relatively small compared to the activity of mTN3 with its native helix-turn-helix DNA binding domain. This may be in part due to Cas9's large structure, which may not present the mTN3 catalytic domains in close proximity to the target DNA. Nevertheless rCas9 future improvements in core sequences and target site design may lead to improvement of recombination efficiency.

rCas9 may be of broad use in genome engineering. Recombinases such as Cre and PhiC31 have been used for engineering eukaryotic genomes. These facilitate predictable high specificity DNA manipulations, however these lack any semblance of 'programmability' since they rely on complex protein-DNA interactions to target substrate binding. CRISPR RNAs target Cas9 binding with straightforward Watson-crick base-pairing rules. We sought here to synergize the binding of RNA-targeted binding of Cas9 with the catalytic activity of a hyperactive recombinase. This was successful. With further understanding of the versatility of rCas9 and its ability to target DNA manipulations beyond deletion (*e.g.* integration and inversion) it may prove helpful for high-specificity rewriting of genomes. This of course has application in the development of cell lines for basic science purposes or for development of gene therapies.

Likewise, rCas9 may be used in synthetic biology to implement recombinase based logic systems. Previously serine recombinases, such as Bxb1 and PhiC31, were employed to implement recombinase based logic gates[22,23]. This system requires coexpression of multiple recombinases. To add additional recombinases bioinformatics searches and functional screening for orthogonal recombinase proteins[24]. In the case of rCas9, sgRNAs can retarget DNA binding. sgRNAs placed under inducible promoters

should result in distinct recombination events in response to different experimental inputs. Perhaps more interestingly, since rCas9 can hypothetically target DNA integration, deletion products may be integrated at other chromosomal locations. This is an interesting opportunity for genome engineering and synthetic biology, in that it would impart the ability program DNA movement on the genome. This will likely require improved cooperative targeting for Cas9 integration and would have to be experimentally demonstrated, however it remains an interesting possibility.

In summary, we demonstrate the function of an RNA-guided recombinase in proof-of-principle experiments. We identified basic design principles for rCas9 protein design, such as functional sgRNA spacings and interdomain linkers. We determined rCas9 does indeed operate as a DNA recombinase using PCR and DNA sequencing. We envision this being the first step in developing RNA-guided recombinases that are amenable for use in human cells. This may ultimately be useful for ultra-specific DNA manipulations for basic science and translational gene therapies. rCas9 may be utilized for synthetic gene networks enabling RNA-programmed recombinase based logic, RNA-guided genetic 'recorders' and programmable genome 'SCRaMbLE' techniques[25–28].

METHODS

Bacterial Culture

Molecular cloning was conducted using *E.coli* NEB-10-Beta (New England Biolabs, NEB). LB Miller Medium (Sigma Aldrich, Sigma) was supplemented with appropriate antibiotics for plasmid maintenance: Ampicillin (100μg/ml) and/or Kanamycin (30μg/ml), or Chloramphenicol (30μg/ml). *E.coli* were cultured at 37˚C.

Yeast Culture

All yeast were cultured at 30˚C unless otherwise noted. *S. cerevisiae* YPH500 or FY834 (ATCC# 90845) (Ref. [29]) were cultured on YPD agar plates and in liquid medium containing glucose. Liquid cultures were shaken at 250-300 RPM. Yeast minimal dropout media contained either 2% glucose or 2% galactose with 1% raffinose and necessary amino acid dropout solutions (Clonetech). Yeast were made competent using the Zymo competent yeast kit and transformed using manufacturer protocol. Genomic Integrations and Plasmid transformations were selected for on yeast minimal dropout plates with amino acid combinations necessary for selection. Yeast were cultured in liquid yeast dropout media necessary for plasmid selection.

Molecular Cloning of Recombinases

Coding regions for $TN3^{G79S,D102Y,E124Q}$, $TN3^{R2A,E56K,G101S,D102Y,M103I,Q105L}$, Gin^{H106Y} (respective references [9,11,30]) containing native DNA-binding domains were synthesized as human codon optimized gBlocks from Integrated DNA Technologies (IDT). Coding regions were placed under control of a Gal1 promoter via cloning gBlock into the XbaI and XhoI sites of p415 Gal1-Cas9. This results in replacement of Cas9 with the recombinase coding sequences.

Construction of Yeast Genome Integration Vectors

The Yeast Genomic Integration Vector (pMG, Fig. 1B) was generated using vectors previously described (Ref. [31]). A modified Ura3 homology arm sequence, synthesized by IDT, was cloned into EcoRI and NotI sites. Gal promoters were replaced

with Tef1 promoters for constitutive expression of GFP and mCherry. To integrate into the yeast genome, 1-2µg of pMG was digested with ApaI in 50 µl reactions for ≥1 hour at 37˚C. 5µl of Restriction product was transformed into competent *S. cerevisiae* YPH500 or FY834 using protocol from Zymo Competent Yeast Kit (Zymo). The resulting pMG vector integrates into the URA3 locus of *S. cerevisiae* Chromosome V and confers histidine prototrophy.

To clone in recombinase recognition sequences into pMG, Res1, Gix and A-sites were synthesized as overlapping oligonucleotides. 5' phosphates were added to oligonucleotides by incubating 1ug of top/bottom oligonucleotides in 50 µl reactions containing 1X T4 DNA Ligase Buffer and 10 units of T4 Polynucleotide Kinase (T4 PNK) at 37°C overnight. Oligonucleotides were duplexed by heating the kinase reactions to 90°C on an aluminum heating block for 5 minutes followed by slowly returning the reaction to room temperature (25°C) over approximately 1 hour. Following duplexing, Res1, Gix or A-sites were ligated into EcorI and MluI sites surrounding GFP.

Molecular Cloning of rCas9

rCas9 was constructed using p415 Gal1-Cas9 (Addgene# 43804)(Ref. [32]) as a template for Cas9. The mTN3 catalytic domain along with D10A and H840A mutations to Cas9 were simultaneously generated using PCR primers containing SapI sites. Purified PCR products were digested with SapI and gel-extracted using the Sigma-Aldrich gel-extraction kit. rCas9 sub-fragments were ligated in equimolar amounts to p415 Gal1-Cas9 digested with XbaI and XhoI. The resulting p415 Gal1-rCas9 contains a Cen6 origin of replication and a leucine prototrophic marker.

Molecular Cloning of Interdomain Linkers

To clone new linker sequences, pUC19 containing a XbaI-SalI fragment of rCas9 was amplified outward with primers introducing SapI sites at the junctions of mTN3 and dCas9. Purified products were digested with SapI and ligated with oligo duplexes coding for various linker sequences. Following confirmation of correct ligation products, XbaI-SalI fragments were cloned to replace between XbaI and SalI sites in p415 Gal1-rCas9.

Cloning of sgRNAs

Yeast sgRNA expression cassettes, ysg(#)s, were contrstructed by cloning oligonucleotide duplexes into, pSB1C3 containing an SNR52 promoter with inverted SapI sites and an sgRNA hairpin recognized by *S. pyogenes* Cas9. ysgRNAs were then PCRed with primers adding EcoRI and SapI, or SpeI and SapI sites. Purified PCR product were then digested with respective restriction enzymes, heat inactivated and ligated into pRS424 (Ref. [33]) digested with EcoRI and SpeI. The resulting pYSG(#:#) contained pairs of sgRNA cassettes with a 2μ origin of replication and tryptophan prototrophic marker.

rCas9 Deletion Assay

To assay rCas9 function, YPH500 Ura3(MGab) with p415 Gal1-rCas9 and with various pYSG derivatives were cultured in 3ml YP –Leu, -Trp with 2% Glucose. After 24 hours, 5μl of the stationary phase culture was used to inoculate 3ml of YP –Leu, -Trp with 2% Galactose, 1% Raffinose. Cell were diluted down (5μl saturated culture in 3ml media) at 48 hour intervals. Cells were analyzed by flow cytometry and fluorescent

microscopy after 96 hours of galactose induction. Genomic DNA was also prepared after galactose induction. Recombinase screening (Fig. 2) was conducted as described above with 48 hours total of galactose induction.

Flow Cytometry

All flow cytometry was conducted on an Accuri C6 Flow Cytometer (BD Biosciences, CA). Samples were gated by consistent forward scatter (FSC) and side scatter (SSC) and 10,000 events within the FSC/SSC gate were collected. A 488 nm laser excitation and a 530±15 nm emission filter was used for GFP fluorescence determination. Flow cytometry files were analyzed using manufacture software and in MatLab (The MathWorks).

Fluorescent Microscopy

200µl of stationary phase cultures of yeast were spun down at 4000*g for 2 minutes and washed once in 1X PBS solution. Following washing, cells were concentrating by resuspending in 10-20µl of 1X PBS. 1-2 µl of cell solution was placed on glass microscope slides and visualized on a Nikon Ti-Eclipse inverted microscope with and LED-based Lumencor SOLA SE Light Engine with appropriate filter sets. GFP was visualized with an excitation at 472 nm and emission at 520/35 nm using a Semrock band pass filter. mCherry was visualized with excitation at 562 nm and emission at 641/75 nm.. Constant exposure times, LUT and image gain adjustments were applied to microscopy data.

Genomic DNA Isolation and PCR Analysis of Deletion

Yeast genomic DNA was prepared using the Zymo yeast genomic DNA preparation kit using the manufacturer's protocol with phenonl-chloroform steps included. To assay genomic deletion, PCR was conducted using Phusion DNA polymerase (New England Biolabs). Annealing temperatures and extension times were calculated using the manufacturer's protocol for High Fidelity Buffer. PCR products were visualized via 0.8% agarose gel eletcrophoresis.

Sequencing of Deletion Products

Following gel resolution of amplicons, deletion bands were gel-extracted using the Gen Elute gel extraction kit (Sigma-Aldrich) using the manufacturer's protocol. Following extraction, products with phosphorylated via incubation in 50µl reactions with T4 PNK and 1X T4 DNA ligase buffer. Reactions were heat inactivated and ligated in equimolar ratio to SmaI cleaved and dephosphorylated pUC19. Ligations were transformed into chemically competent NEB10B *E.coli* and plated on Ampicillin Plates supplemented with 40µl X-Gal solution (Promega). White colonies were picked and prepared using GeneElute Plasmid Preperation kit (Sigma-Aldrich). 300ng of plasmid DNA was sequenced via DNASU's Sanger Sequencing Core with primers flanking the A-site.

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