

# A Heritable Recombination System for Synthetic Darwinian Evolution in Yeast

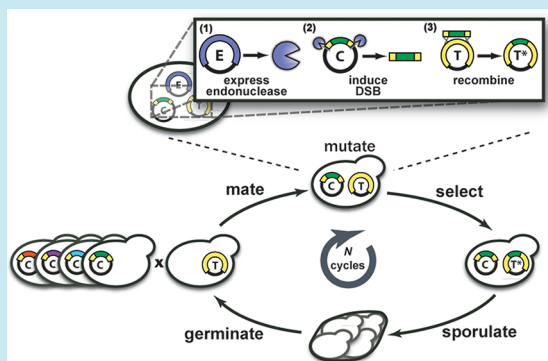
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## S Supporting Information

**ABSTRACT:** Genetic recombination is central to the generation of molecular diversity and enhancement of evolutionary fitness in living systems. Methods such as DNA shuffling that recapitulate this diversity mechanism *in vitro* are powerful tools for engineering biomolecules with useful new functions by directed evolution. Synthetic biology now brings demand for analogous technologies that enable the controlled recombination of beneficial mutations in living cells. Thus, here we create a Heritable Recombination system centered around a library cassette plasmid that enables inducible mutagenesis *via* homologous recombination and subsequent combination of beneficial mutations through sexual reproduction in *Saccharomyces cerevisiae*. Using repair of nonsense codons in auxotrophic markers as a model, Heritable Recombination was optimized to give mutagenesis efficiencies of up to 6% and to allow successive repair of different markers through two cycles of sexual reproduction and recombination. Finally, Heritable Recombination was employed to change the substrate specificity of a biosynthetic enzyme, with beneficial mutations in three different active site loops crossed over three continuous rounds of mutation and selection to cover a total sequence diversity of 10<sup>13</sup>. Heritable Recombination, while at an early stage of development, breaks the transformation barrier to library size and can be immediately applied to combinatorial crossing of beneficial mutations for cell engineering, adding important features to the growing arsenal of next generation molecular biology tools for synthetic biology.

**KEYWORDS:** homologous recombination, directed evolution, mutagenesis, libraries, sexual reproduction, yeast



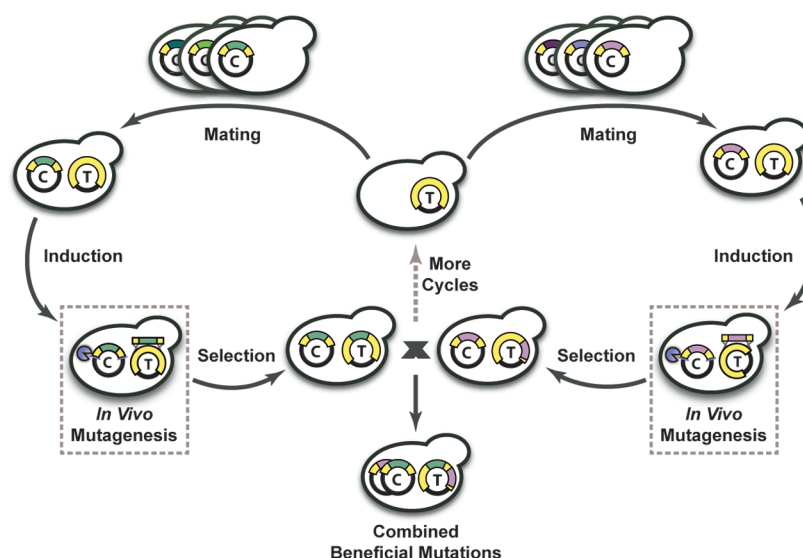
Progress in synthetic biology has spurred renewed interest in techniques for *in vivo* mutagenesis so that cellular components and pathways can be directly engineered in the intracellular environment in which they must perform.<sup>1–3</sup> To that end, recently improved *E. coli* mutator strains,<sup>4</sup> the co-opting of B cells for somatic hypermutation of exogenous genes,<sup>5</sup> recombination-mediated library mutagenesis by transformation,<sup>6</sup> a phage-based system for continuous mutagenesis,<sup>7</sup> and a Multiplex Automated Genome Engineering (MAGE) technology have all been reported.<sup>8</sup> None of these mutagenesis methods, however, also allows for crossing of beneficial mutations that arise in separate cells. We sought to design a system that would enable repeated rounds of mutagenesis and crossing of beneficial mutations *in vivo* with no intermediate *in vitro* manipulation of the DNA, built from classic genetic methods for recombination and mating and sporulation in the yeast *S. cerevisiae* (Figure 1).

Thus, we created Heritable Recombination, a system centered on library cassette plasmids that allow for inducible mutagenesis in a target gene via recombination and horizontal dissemination of beneficial mutations through sexual reproduction in yeast (Figure 2a). To provide specificity for mutagenesis of the target gene(s), the cassette is designed to utilize homologous recombination (HR) as the mechanism for mutagenesis. The high efficiency of HR in *S. cerevisiae* is well

documented and widely exploited in both classical genetics and genetic engineering.<sup>9–11</sup> Additionally, the cassette is designed such that the mutagenesis can be initiated by an inducible endonuclease that introduces a double strand break (DSB) in the cassette plasmid.<sup>12,13</sup> Experiments using endonucleases to create double strand breaks *in vivo* for the study of HR and for chromosome mutation suggested that induction of the DSB would be rapid and efficient, but also that intact cassette plasmids would be regenerated and passed on to daughter cells, thereby allowing for repeated cycles of evolution and straightforward tracking of beneficial mutations.<sup>14</sup> A significant difference between Heritable Recombination and previous HR technologies such as Delitto Perfetto<sup>11</sup> is that the DSB is engineered in the mutagenic cassette rather than in the target gene. As elaborated here, there are likely numerous advantages to the flexibility provided by making the DSB in the cassette. Because the cassettes are encoded on plasmids, cell populations carrying two different cassette libraries can be subjected to selective pressure and the winners then crossed with one another by mating and sporulation to produce daughter cells with a beneficial mutation from one library in the target plasmid and a beneficial mutation from the other library in the cassette

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**Figure 1.** Heritable Recombination: a system for mutating DNA and crossing beneficial mutations directly in living cells. DNA mutagenesis is effected by homologous recombination between a mutagenic cassette (plasmid “C”) and a target gene (plasmid “T”). Because the mutagenic cassette is introduced by mating, successive rounds of mutagenesis and selection can be carried out with no intermediate isolation of the target gene. Significantly, evolving populations can be crossed with one another to allow consolidation of beneficial mutations in an unplanned evolutionary walk through sequence space, exploiting cycles of mating and sporulation in yeast.

plasmid. Thus, Heritable Recombination breaks the transformation barrier to library size by enabling combinatorial exploration of extremely large libraries by crossing multiple different cassettes. Alternatively, or in addition, it can enable virtual searches of extremely large sequence space along different pathways by varying the content and ordering of cassette library introduction (Figure 1).

## RESULTS AND DISCUSSION

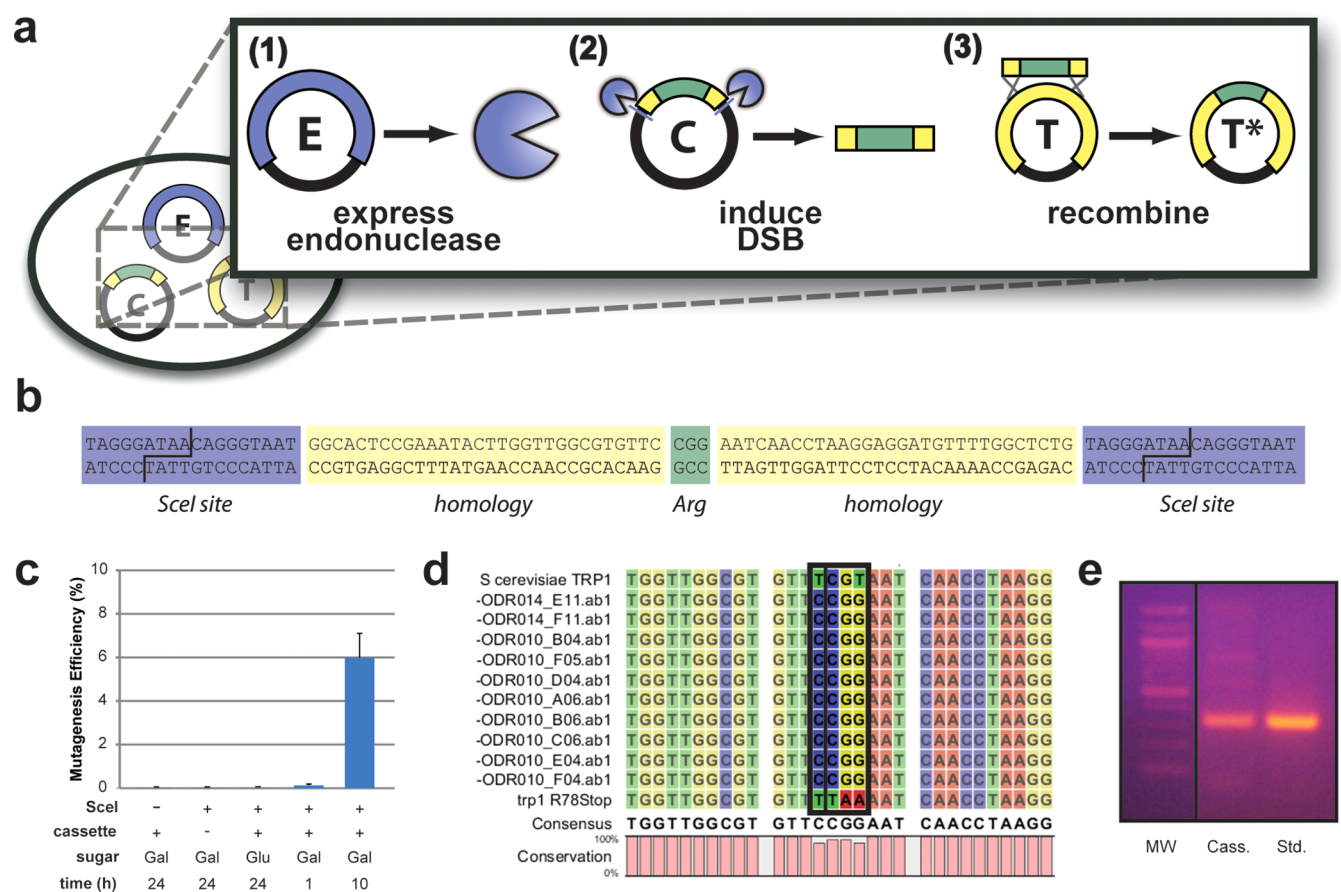
The first step in building the Heritable Recombination system was to effect endonuclease-inducible recombination so that mutagenesis could be initiated by a double-stranded break (DSB) to the cassette plasmid rather than by transformation. While designed to be modular and to enable library mutagenesis, the system was validated on the basis of repair of a nonsense codon in the *trp1-R78<sup>TAA</sup>* gene. The *TRP1* gene is one of the most commonly used auxotrophic markers in yeast and was the focus of our initial studies on oligonucleotide mutagenesis.<sup>6</sup> For the catalytic endonuclease, we chose the meganuclease I-SceI (SceI), an established tool for genetic engineering in yeast;<sup>15,16</sup> SceI was placed under control of the tightly repressed, yet strongly inducible, *GAL1* promoter. Finally, the mutagenic cassette plasmid was designed with 30 bp of homologous sequence on either side of the wild-type Arg78 codon and flanked on both sides by the 18-bp recognition sequence for SceI (Figure 2b). Previous studies have shown that 30 bp is the minimum amount of homology needed to obtain high efficiency recombination in yeast,<sup>17</sup> yet at the same time, this length of DNA corresponds to 10 amino acids and thus should allow mutagenesis of multiple regions within a single gene. As depicted in Figure 2a, in this initial inception, the target gene *trp1-R78<sup>TAA</sup>*, the galactose-inducible SceI endonuclease, and the mutagenic Arg78 cassette are each encoded on their own plasmid.

Satisfyingly, we were able to readily optimize this initial design to obtain the high recombination efficiencies essential for library mutagenesis. Our definition of recombination efficiency is the ratio of the number of colonies that grow in

the absence, versus the presence, of tryptophan, indicating successful repair of the *trp1* nonsense codon by the mutagenic Arg78 cassette plasmid. Our initial experiments yielded growth in only 0.12% of cells, but protocol optimization led to an average maximum efficiency of  $6 \pm 1\%$  after 10 h of induction with galactose (Figure 2c). Numerous control experiments confirmed the requirement for all three components of our system: no mutagenesis above background levels was observed in strains without the endonuclease plasmid, without the mutagenic cassette plasmid, or with a cassette plasmid that lacked endonuclease recognition sites. Sequencing of individual clones confirmed that the expected mutations had in fact occurred without any other detected changes in the *TRP1* gene in 24/25 of the samples analyzed (Figure 2d).

Furthermore, we were able to confirm that intact cassette plasmids were successfully passed on to daughter cells following induction. Plasmids were harvested from cells that displayed the  $\text{Trp}^+$  phenotype, and PCR amplification of the cassette region produced products of the expected size (Figure 2e). Sequencing of the PCR products showed not only that the cassettes retained all of their essential parts but also that they were not mutagenized, even after undergoing prolonged DSB induction and successful mutagenesis of the target gene. Together, these results establish that the high-copy cassette plasmid is competent for targeted mutagenesis and further dissemination to daughter cells, providing a simple way to track and cross the accumulated mutations in an evolving population of cells.<sup>18</sup>

Addition of the sexual reproduction step was executed in a model system in which stop codons in two different biosynthetic genes were successively repaired with no intermediate *in vitro* manipulation of the DNA. Specifically, we first repaired a nonsense mutation in the *hisA* gene from *Thermotoga maritima* to complement a *his6<sup>-</sup>* genotype and then repeated the process with the *trp1* gene to complement *trp1<sup>-</sup>*, exploiting mating and sporulation in *S. cerevisiae* as the mechanism of sexual reproduction (Figure 3a).<sup>19</sup> Two cassette plasmids were used to achieve this sequential mutagenesis: the

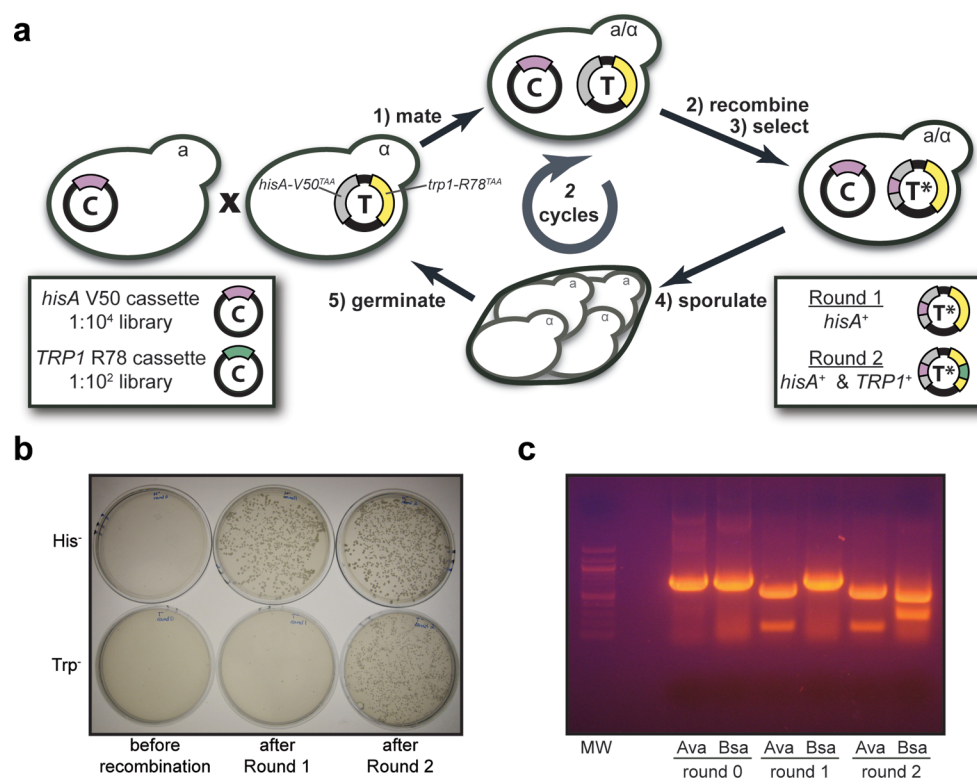


**Figure 2.** Endonuclease-inducible mutagenesis via recombination between a cassette plasmid (C) and a target plasmid (T) in *S. cerevisiae*. (a) Schematic depiction of the endonuclease-inducible recombination step of Heritable Recombination. (1) An endonuclease (E) is inducibly expressed. (2) The endonuclease makes double-stranded breaks (DSBs) in the cassette plasmid (C), which encodes the mutation(s) (green) flanked by homology regions (gold). (3) Induction of the DSB initiates homologous recombination between the cassette plasmid (C) and the target plasmid (T), effectively mutating the target gene. (b) Sequence of the cassette plasmid in the vicinity of the mutation(s). The mutation(s), here the GCC codon for the wild-type Arg78 residue in the *S. cerevisiae TRP1* target gene, is shown in green in the center. The mutation(s) is flanked on either side by 30 bp of homology (gold) to the target gene. The homology regions are both followed by the 18-bp meganuclease I-SceI recognition site (blue); the cleavage sites are indicated by black lines. (c) Mutagenesis efficiencies based on repair of a nonsense codon in *S. cerevisiae trp1-R78<sup>TAA</sup>*. *S. cerevisiae* bearing the appropriate endonuclease plasmid, cassette plasmid, and target plasmid were preinduced in lactate media for 3 h before transfer to galactose induction (Gal) or glucose control (Glu) media. Cells were incubated in the respective media for the time indicated and then transferred to pairs of plates differing only by the presence or absence of tryptophan. Efficiency is calculated as the percentage of colonies growing on Trp<sup>-</sup> plates relative to the number of colonies growing on Trp<sup>+</sup> plates. Experiments were carried out in triplicate, and error bars represent one standard deviation from the mean. (d) Sequence alignment of wild-type *TRP1*, R78<sup>TAA</sup> nonsense mutant, and 10 isolates after recombination-mediated mutagenesis. Only the mutations encoded in the cassette are observed. (e) Analysis of cassette integrity following DSB induction. The *trp1-R78<sup>TAA</sup>* repair strain was subjected to continuous DSB formation by growth in galactose induction media for 21 h and plated on Trp<sup>-</sup> plates. Total plasmids were isolated from the resulting colonies, and the cassette region was PCR amplified using primers surrounding the multiple cloning site. The major PCR product (Cass.) is of equal size to the product obtained using a control plasmid as template (Std.).

first encoded a wild-type Val50 residue for repair of the corresponding nonsense mutation in an engineered *hisA-V50<sup>TAA</sup>* gene; the second cassette encoded Arg78, as above, to repair *trp1-R78<sup>TAA</sup>*. Importantly, while the cassette plasmids were initially introduced into the haploid  $\alpha$ -type donor cells by transformation, in this experiment mutation of the *hisA* gene was executed simply by mating haploid  $\alpha$ -type cassette cells with haploid  $a$ -type target cells containing the target gene and inducible endonuclease. Sporulation followed by lyticase digestion of any unsporulated diploids was used to regenerate haploid cells between rounds of selection for mating with additional cassette cells (see Supporting Information, Section S.3). Cells were selected for histidine prototrophy after the first round of mutagenesis and for tryptophan prototrophy after the second round. To confirm the link between the observed

phenotypes and the desired recombination events, individual plasmids were isolated and retransformed into the background strain, yielding cells that exhibited the expected growth profile (Figure 3b). Restriction mapping and sequencing of both pooled DNA and DNA from individual colonies showed that both encoded mutations had been successfully incorporated into the *hisA* and *TRP1* genes with no other detected changes to their sequences (Figure 3c).

This two-stage experiment was also executed as a mock selection to confirm the capability of the system to effect mutagenesis from a library. For these experiments, cassette strains carrying mutagenic plasmids with the appropriate repair codon were mixed with an excess of empty cassette cells in which the plasmid contained no homology to the target gene and a single SceI site. The resulting mock libraries were mated



**Figure 3.** Multiple, successive rounds of mutation in living cells. (a) Schematic depiction of the two-stage mutagenesis experiment. First, a Target Strain (T) carrying the target genes, *hisA-V50<sup>TAA</sup>* from *T. maritima* (depicted in gray) and *trp1-R78<sup>TAA</sup>* from *S. cerevisiae* (gold), is mated (1) with a mock library of cassette strains (C) harboring the cassette to repair the nonsense *V50<sup>TAA</sup>* mutation (purple) or a blank cassette with one *SceI* site but no homology to *hisA* or *TRP1*. After recombination-based mutagenesis (2) and selection on histidine dropout media (3), diploids are sporulated (4) and germinated (5) to produce haploid daughter cells with the repaired *hisA* gene. A second round of mating with a new cassette strain library introduces either a new cassette (green) designed to repair the *R78<sup>TAA</sup>* mutation in *trp1* or the blank cassette. Cells that receive the repair cassette can properly repair the *trp1-R78<sup>TAA</sup>* gene and are selected on media lacking tryptophan. Plasmids isolated after this round contain the mutations encoded in both cassettes. (b) Transformations of plasmids recovered following each round of the two-stage mutagenesis experiment. The initial plasmid, carrying the *hisA-V50<sup>TAA</sup>* and *trp1-R78<sup>TAA</sup>* genes, does not support growth on either histidine or tryptophan dropout media. Plasmids isolated after the first round complement histidine auxotrophy in the *his6<sup>-</sup>* genetic background. Plasmids from the second round complement both *his6<sup>-</sup>* and *trp1<sup>-</sup>*, consistent with repair of both genes. (c) Restriction digest of the *hisA* and *trp1* genes at each stage of the experiment. Genes were isolated after each round and PCR amplified, and restriction enzymes were added directly to the completed PCR reaction. After one round of mutagenesis, the *hisA* gene contains a new *AvaII* (Ava) site purposely encoded in the mutagenic cassette immediately after the Val50 repair codon. During the second round, a *BsaWI* (Bsa) site is introduced into *TRP1* adjacent to the repaired Arg78 codon. Genes isolated after each round are cleaved by the appropriate restriction enzyme(s).

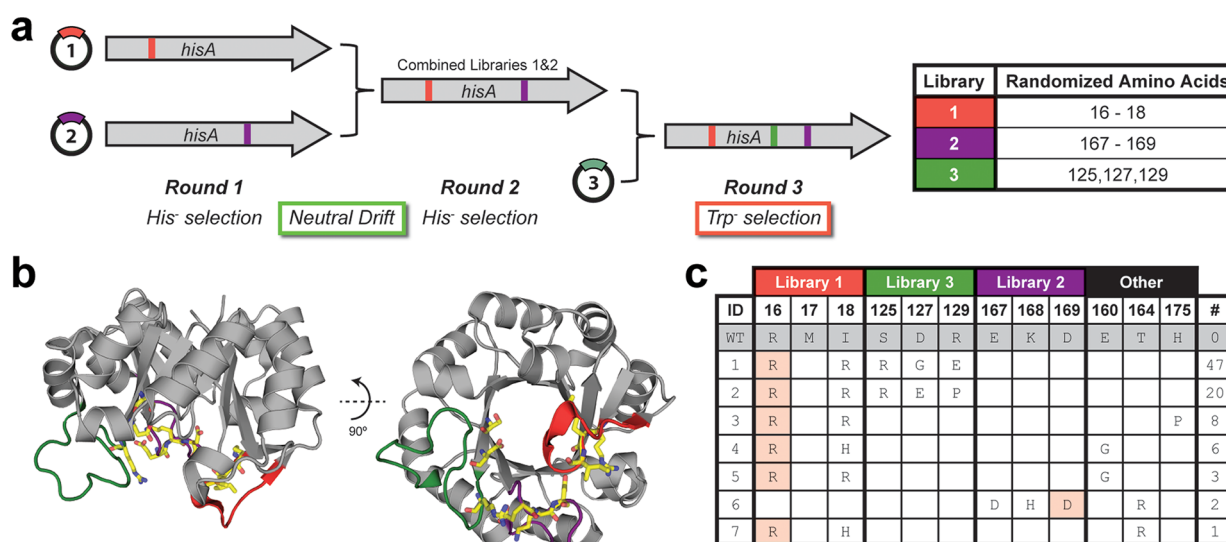
to the target strain at the start of each round of Heritable Recombination, and cells carrying both repaired genes were successfully isolated from a 10<sup>4</sup> excess of empty cassette plasmids in the first round and a 10<sup>2</sup> excess in the second round. Thus, while validated here with just two cassettes, Heritable Recombination allows multiple rounds of library evolution to be carried out simply through repeated cycles of sporulation followed by mating with prepackaged cassette cells under conditions of selective pressure without any intermediate steps of DNA isolation and manipulation.

Finally, we challenged the utility of crossing beneficial mutations in the course of selection with Heritable Recombination by rapidly evolving the *hisA* biosynthetic enzyme to accept a new substrate. Wild-type *hisA* codes for *N*'-[(*S*'-phosphoribosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide (ProFAR) isomerase, a ( $\beta\alpha$ )<sub>8</sub>-barrel enzyme that catalyzes an Amadori rearrangement as the fourth step in the *de novo* biosynthesis of histidine. Previous work by the Sterner group has shown that directed evolution followed by rational combination of beneficial mutations can be used to convert ProFAR into highly active variants of the evolutionarily

related tryptophan biosynthetic enzyme phosphoribosylanthranilate isomerase (PRAI).<sup>20,21</sup> Thus, we reasoned that Heritable Recombination should allow us to evolve *hisA* variants with combinations of beneficial mutations in a rapid and straightforward manner.

Rewordingly, three rounds of mutagenesis and selection via Heritable Recombination yielded *hisA* variants that could complement a *trp1<sup>-</sup>* auxotrophy in yeast in under a month (Figure 4a). Three separate libraries of mutagenic cassettes were designed targeting distinct loops at the C-terminal end of the *hisA* ( $\beta\alpha$ )<sub>8</sub>-barrel (Figure 4b). Loops at this face of the protein contribute residues for substrate recognition and catalysis in nearly all known examples of ( $\beta\alpha$ )<sub>8</sub>-barrel enzymes.<sup>22</sup> Each cassette randomized three residues using degenerate NNS codons (N = A,T,C,G; S = G,C) to provide a complexity of 3.4 × 10<sup>4</sup> variants per cassette, or 3.5 × 10<sup>13</sup> total variants with crossing of all three cassettes.

In the first round, cells containing a target plasmid with the *hisA* gene were separately mated to two populations of cells carrying libraries 1 and 2. Recombination-mediated mutagenesis was followed by selection against the *his6<sup>-</sup>* auxotrophy



**Figure 4.** Facile directed evolution of new substrate specificity in *hisA* using Heritable Recombination to cross beneficial mutations from individual lineages. (a) Diagram of the directed evolution experiment. The *hisA* gene is represented by a gray arrow, and the mutagenic cassette libraries are represented by color-coded numbered plasmids. In Round 1, libraries 1 and 2 were individually mated to a target strain carrying the *hisA* gene. Mutations were introduced using homologous recombination, and variants were subjected to neutral drift by growth in His<sup>-</sup> media. The winners from this round were sporulated and mated to each other to merge libraries 1 and 2. After a second round of neutral drift with this combined lineage, library 3 was introduced through mating, and the cells were then grown in media lacking tryptophan to select for variants that could complement a *trp1*<sup>-</sup> genotype. (b) Rendering of the *hisA* enzyme based on the X-ray crystal structure (PDB id: 1QO2), showing the location of the randomized libraries at the C-terminal face of the ( $\beta\alpha$ )<sub>8</sub>-barrel. The three major catalytic loops are color-coordinated with the corresponding mutagenic cassette libraries. Residues that were subjected to saturation mutagenesis are shown as yellow sticks. (c) Table of mutations found in Trp<sup>+</sup> variants of *hisA*. Seven different sequences were found from 87 clones; the frequency of each is denoted in the column marked “#”. Amino acid positions are listed in bold across the top with the wild-type residues in the gray row below. Blank boxes indicate wild-type codons, filled boxes denote the new amino acid at that position, and filled boxes with red backgrounds list sites where a new DNA sequence encoded wild-type residues. Together these experiments show the power of Heritable Recombination to readily cross beneficial mutations and produce synergistic solutions directly within living yeast cells.

to generate a pool of variants that retained *hisA* activity. This “neutral drift” step allowed the identification of mutations that still led to folded and functional proteins, providing a privileged pool of starting points for discovery of *hisA* mutants that could complement *trp1*<sup>-</sup> auxotrophy in yeast.<sup>23,24</sup>

Surviving cells from these initial selections were induced to sporulate and then mated to each other to combine the cassette plasmids from these first two libraries. A second round of mutagenesis and neutral drift selection was then carried out to merge the winning mutations from the first round before mating the surviving cells with a population carrying library 3.

Following a third round of mutagenesis, the cells were placed in media lacking tryptophan to select for enzymes with the desired new substrate specificity. Mutated target plasmids were isolated after the final round of selection, retransformed into yeast to confirm the Trp<sup>+</sup> phenotype, and sequenced. Significantly, by using an intermediate selection to hone the libraries so that they contained only functional mutations, we were able to combine three libraries of  $3.4 \times 10^4$  variants to virtually explore a sequence space equal to the product of the three libraries, or approximately  $3.5 \times 10^{13}$  sequences, several orders of magnitude greater than the current transformation limit to library size in yeast. In theory the process of mutation followed by sexual reproduction could be continued with more cassette libraries to explore vast amounts of sequence diversity.

This efficient search of sequence space readily produced a variety of *hisA* mutants with different cassette combinations that could fully complement a *trp1*<sup>-</sup> genotype in yeast (Figure 4c). Sequencing of these mutants showed that Heritable Recombination was working properly: several cassettes were

reused in multiple different solutions, indicating combinatorial sharing of beneficial mutations among members of the population. Despite cassettes encoding full saturation at each targeted locus, several residues were unchanged from the wild type in all variants that were sequenced (see Supporting Information, Section S.2). Several of the sequenced genes contained additional single-base mutations that were not encoded in the initial degenerate libraries but rather were located in the homology region for library 2; we are currently engaged in studies to understand the origin of these mutations as well as the overall mechanism of mutagenesis in this system.

While the design of our libraries benefited from insight into this enzyme class from published work,<sup>20,21</sup> the ability to cross beneficial mutations *in vivo* using sexual reproduction allowed us to readily identify several different cassette combinations that produced enzymes that strongly complemented the *trp1*<sup>-</sup> genotype. The ease with which we isolated these combinations stands in contrast to previous efforts that required rational engineering to combine beneficial mutations arising from error-prone PCR to achieve highly active enzymes.<sup>21</sup> We discovered seven distinct sequence solutions, and most of the mutations we found were at sites other than those previously reported. Interestingly, all of our results contained at least one new basic amino acid, supporting the hypothesis that electrostatic effects play a dominant role in recognizing the negatively charged substrate phosphoribosylanthranilate.<sup>20,25</sup> The preliminary neutral drift steps show that nondeleterious mutations can potentially provide increased evolvability under changing selective pressure. We are currently exploring ways to test the

evolutionary significance of this concept using Heritable Recombination.

The success of this directed evolution experiment showcases all three parts of our technology working in concert to efficiently search sequence space and distinguishes it from previously reported *in vivo* mutagenesis techniques. Homology-directed library cassettes target the mutations to the regions of interest in a vast excess of chromosomal DNA, thus avoiding the undesired genomic instability often observed with mutator strains that prevents continuous rounds of mutation and selection.<sup>26</sup> The recently reported PACE technology uses continuous phage infection of *E. coli* to surmount the genomic instability problem but does not enable targeted mutations or addition or deletion of DNA and does not address recombination of beneficial mutations.<sup>7</sup> By using an endonuclease-induced double-strand break in a heritable cassette plasmid inside the cell to initiate mutagenesis, our method is distinct from previous efforts, both long-standing work in yeast<sup>27–30</sup> and the more recent MAGE technology in *E. coli*<sup>8</sup> and work from our laboratory in yeast,<sup>6</sup> to generate molecular diversity that rely on transformation of linear DNA fragments to achieve recombination. A technique called “gene gorging” uses DSBs generated by *SceI* to initiate lambda Red-mediated recombination in *E. coli*, but this process has not been explored for library creation and cannot be iterated over multiple rounds.<sup>31</sup> Several methods using *in vivo* recombination in *E. coli* to generate antibody libraries have been reported,<sup>32,33</sup> but these methods are often limited to antibody-derived genes and, unlike Heritable Recombination, have no mechanism for repeated diversification through sexual reproduction. DNA shuffling can mimic sexual reproduction but relies on PCR and therefore lacks the other benefits of our entirely *in vivo* process.<sup>34</sup> Thus, Heritable Recombination uniquely breaks the transformation barrier to library size and, by analogy to computational algorithms such as dead-end elimination, enables virtual searches of extremely large libraries.<sup>35</sup> Most important, our strategy allows heritable cassette plasmids to be exchanged among cells *via* mating and sporulation and so provides a simple and efficient way to track and combine beneficial mutations. In contrast to CAGE, which relies on bacterial conjugation to combine large contiguous regions of *E. coli* chromosomes, mutations can be crossed organically with Heritable Recombination over many rounds of the mutation and sexual reproduction cycle.<sup>36</sup>

Heritable Recombination is immediately compatible with any screen or selection available in yeast. While we have demonstrated it here using classic yeast auxotrophic growth selections, we anticipate that it will be equally useful under a variety of existing assays, including analytical screens and affinity-based selections. Currently, we are applying Heritable Recombination in conjunction with yeast cell surface display for the evolution of antibodies<sup>37</sup> and enzymes.<sup>38</sup> Our long-term goal is to couple Heritable Recombination with an expanded chemical complementation selection to enable the routine evolution of new chemistry directly in living cells.<sup>39</sup>

We foresee this technology becoming an efficient production platform for the directed evolution of biomolecules. For example, a privileged scaffold, such as the  $(\beta\alpha)_8$ -barrel or an immunoglobulin fold, could be used as a common starting point, and libraries specific to the scaffold would be stored in yeast as frozen glycerol stocks. While demonstrated here with diversification of active-site loops in a single gene, we also plan to apply Heritable Recombination to several genes at once,

ultimately targeting multiple loci in the yeast genome simultaneously. Regardless of the desired target(s), the appropriate libraries could be combined and searched through mating and *in vivo* mutagenesis to identify useful sequences in a matter of days. Further maturation of the activity of the target(s) could be executed simply by additional rounds of mating with pre-existing cassette cell libraries.

In a broader context, the methods presented here represent an advance in synthetic biology. The manipulation of nucleic acid sequences *in vivo* is a rapidly developing field with implications ranging from conditional gene expression to completely synthetic genomes. We foresee techniques based on homologous recombination, in yeast, bacteria, and mammalian cells, gaining even greater relevance as the mechanism of these processes becomes further elucidated.<sup>40–42</sup> We have already begun related projects using Heritable Recombination for the evolution of enzymes and binding proteins, metabolic engineering, and pathway construction.<sup>43</sup>

## METHODS

**General.** Standard molecular biology protocols were used for plasmid and strain construction. For specific details regarding strains and plasmids, individual experiments, and mock selections, please refer to the Supporting Information.

***In Vivo* Mutagenesis Protocol.** Overnight cultures of mutagenesis strains were grown in synthetic complete media with 2% (w/v) glucose (SC-Glu) lacking the necessary nutrients for plasmid maintenance. Cells were centrifuged, the supernatant was removed, and the cells were resuspended in preinduction media (SC with 2% lactate). Preinduction cultures were shaken at 30 °C for 3 h and then split evenly into two tubes and centrifuged. Supernatants were removed, and one cell pellet was resuspended in SC with 2% galactose while the other was resuspended in SC-Glu. The cultures were shaken at 30 °C for the desired induction time, typically 24 h, and then serial dilutions were plated on selective and non-selective SC-Glu plates. Colonies were counted after incubation at 30 °C for 48 h.

**Sporulation and Mating.** Diploid cells were placed in 10 mL of presporulation media (YP + 10% glucose) at an initial cell density of  $OD_{600} = 0.1$ . The cultures were shaken at 30 °C until they reached a density of 0.5–0.7, typically 7–9 h. Cells were then collected by centrifugation and resuspended in an equal volume of sporulation media (0.5% potassium acetate) and shaken at 30 °C for 48 h. The spores were harvested by centrifugation and resuspended in 1 mL of water. Zymolyase was added (10 units), and the spores were placed in a 37 °C bath for 2 h and then sonicated for 35 min. Cells were germinated by overnight growth in SC media. Freshly germinated cells were mixed in a 1:3 ratio with cells containing the new cassette ( $\sim 10^7$  total cells), and this cell mixture was plated on YPD. The mating plate was incubated at 30 °C for 8 h, and then the cells were harvested with water and diluted into SC-Glu media to select for diploids carrying all plasmids.

## ASSOCIATED CONTENT

### Supporting Information

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## Author Contributions

D.W.R., P.P.-Y., V.M., and V.W.C. conceived and designed the research. D.W.R., P.P.-Y., and V.M. conducted the experiments. D.W.R. and V.W.C. analyzed the data and wrote the paper.

## Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

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