Supporting Information

Wingler and Cornish 10.1073/pnas.1100507108

SI Materials and Methods

General Methods. Standard methods for molecular biology in *Saccharomyces cerevisiae* and *Escherichia coli* were used (1, 2). *S. cerevisiae* strains were grown at 30 °C on media containing 2% glucose unless otherwise noted. Restriction enzymes, Vent DNA polymerase, and DNA ladders were purchased from New England Biolabs. The dNTPs used for PCR were purchased from GE Healthcare Life Sciences. Oligonucleotides were purchased from Integrated DNA Technologies. For yeast colony PCR, cells were prepared by vortexing colonies resuspended in a 0.2% sodium dodecyl sulfate solution and then heating to 95 ° Celsius for five minutes, and amplifications were performed with GoTaq DNA polymerase (Promega). Plasmid DNA was purified using QIAprep miniprep kits (Qiagen), and PCR products were purified with agarose gel electrophoresis and QIAquick spin columns purchased from Qiagen. Yeast genomic DNA was purified with a Yeastar Genomic DNA kit (Zymo Research). Five-fluoroorotic acid was obtained from Oakwood Products. For the overlay assays, 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt (X-Gluc) was obtained from Gold Biotechnology and 5-bromo-6-chloro-3-indolyl-β-D-galactopyranoside (Magenta-Gal) from Biosynth International. DNA concentrations were determined by absorption at 260 nm. Absorbance measurements were taken on a Molecular Devices SpectraMax Plus 384 or a Tecan Infinite M200 instrument. All aqueous solutions were made with distilled water prepared from a Milli-Q Water System. For PCR, a MJ Research PTC-200 Pellier Thermal Cycler was employed. Transformation of *E. coli* was carried out by electroporation using a BioRad *E. coli* Pulser. Yeast transformation was carried out by electroporation using a BioRad Gene Pulser Xcell and a previously reported protocol (3). DNA sequencing was performed by Genewiz. Plasmid maps were made using BVTech Plasmid (BioVisualTech).

Reiterative Recombination Plasmids. All plasmids were constructed from pRS416 (ATCC 87521) using a combination of standard subcloning techniques and plasmid gap repair via yeast transformation. Key regions were verified by sequencing. Maps for the essential set of Reiterative Recombination plasmids are provided in Fig. S5, and details of their construction are available upon request. The universal odd and even donor plasmids pLW2592 and pLW2593 each contain unique restriction sites between the *CYC1* terminator of the endonuclease gene and the endonuclease cleavage sites in order to linearize the plasmids in preparation for plasmid gap repair. In addition to the universal donor plasmids, we made donor plasmid pLW2594 containing the *HO(L)*-KanMX fragment from plasmid *HO*-poly-KanMX4-*HO* (4) to use during round 1. The *HO(L)* region provides homology to facilitate integration of the first subfragment. The KanMX region can be cut out by restriction digest before gap repair to make the desired linearized round 1 donor plasmid. Uncut pLW2594 can be used as a positive control during the first round of Reiterative Recombination, as cured recombinants acquire resistance to G418 due to integration of the KanMX marker.

Reiterative Recombination Parental Strain Construction. The HO cleavage site in the *MATa* locus of BY4733 (ATCC 200895, *MATa his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0*) was replaced with the noncleavable *MATa-inc* 4–28 allele (5) using "pop-in/pop-out" gene replacement (6), and the genotype of the resulting strain LW2589Y was confirmed by PCR and restriction analysis of the genomic DNA. An integration fragment (Fig. S5) containing the acceptor module p*PYK*-GFP-*HIS3*-(HO cleavage site) between the *HO(L)* and *HO(R)* regions from plasmid *HO*-poly-KanMX4-*HO* (4) was created by digestion of plasmid pLW2590 and transformed into LW2589Y. Integrants were selected on SC(-Histidine) media, and correct integration of the acceptor module into the HO locus of LW2591Y was confirmed by PCR and restriction analysis of the strain's genomic DNA. This strain was used as the parental acceptor strain for all Reiterative Recombination experiments described here.

Fragment, Subfragment, and Primer Design. Primers were designed to provide homology regions as described in *Materials and Methods* and shown in Fig. S4. As an illustrative example, we show the design of the reporter gene proof-of-principle experiment. The primers used to amplify fragments and subfragments are shown in Table S2. The assembled construct is shown schematically in Fig. S2, and its sequence is shown in the *Appendix*. In Table S2, promoters are in plain font, genes are in bold underlined font, and terminators are in italics. Lower-case black letters in the primers indicate homology to the donor plasmids. We arbitrarily designate the *HO(L)* as the 5′ end and the acceptor module as the 3′ end of the assembled construct.

In Table S2, the numbers (1) and (2) indicate the primers used for the first and second rounds of amplification of the subfragments. We used two rounds of PCR (Vent DNA polymerase) so that we could create universal outer primers that added homology to the donor plasmids. This decreased the cost of primers and kept them short enough to require only standard desalting purification. Primers LMW374 and LMW367 would be used as the outer primers for all subsequent odd rounds of Reiterative Recombination, and primers LMW374 and LMW375 serve as the outer primers for all even rounds of Reiterative Recombination. From 5′ to 3′, inner primers contain an annealing region for the outer primers (if necessary), 20 bases of homology to the adjacent fragment or subfragment, and a priming region for the subfragment being amplified. Typically we could use the unpurified PCR from first reaction as a template for the second round.

For the round 1 subfragment, we added homology to *HO(L)* and used the round 1 donor plasmid pLW2594 rather than the universal odd donor plasmid pLW2592.

Induction Controls. Donor plasmids pLW2595 and pLW2596, identical to pLW2592 and pLW2593, respectively, but containing only p*GAL*-t*CYC* rather than p*GAL*-*HO*-t*CYC* or p*GAL*-*SceI*-t*CYC*, were constructed to use for (−)-endonuclease negative controls (Fig. 2*C*). Uncut donor plasmids, with or without the endonuclease, were used as the (−)-homology negative controls. Inductions in 2% glucose media were carried out in parallel to the galactose inductions and generated marker switching rates comparable to the galactose negative controls.

Reporter Proof-of-Principle Assays. Cured recombinants from the reporter proof-of-principle experiment were phrogged onto SC(-Histidine), SC(-Leucine), SC(-Histidine, -Methionine), and SC(-Leucine, -Methionine) plates for phenotypic analysis. Expression of the *lacZ* and *gusA* reporters was assayed using a previously described agarose overlay assay (9).

Lycopene Library Construction and Screening. Based on phylogenetic analyses of the *crtB* and *crtI* genes (7, 8), we selected two adjacent, fully conserved amino acid residues in each gene to replace with stop codons. Using plasmid gap repair, plasmids were created

containing *crtB* alleles with the following sequences at nucleotide positions 514 through 531 (mutated residues in lower-case):

crtB-stop: AAC ATt GCG taa tag ATT (stop codons at amino acid residues 175 and 176 and a new BsrDI restriction site, underlined).

crtB-silent: AAC ATC GCG AGg GAT ATc (only silent mutations and a new EcoRV restriction site, underlined).

and *crtI* alleles with the following sequences at nucleotide positions 52 through 66:

crtI-stop: ATA taa tag CAA GCT (stop codons at amino acid residues 19 and 20 and a new Cac8I restriction site, underlined).

crtI-silent: ATt cgt ctc CAA GCT (only silent mutations and a new BsmBI restriction site, underlined).

These alleles were used as PCR templates for amplification of the *crtB* and *crtI* genes for the lycopene libraries, and purified PCR products were mixed in the desired ratios (0:1, 10:1, or 100:1 stop/silent) during the round 2 and round 3 transformations. "Libraries" containing only the stop and only the silent alleles at each position were constructed in parallel, and the resulting cured colonies are shown in Fig. 3 *B–E*. Following the third round of Reiterative Recombination, libraries were plated on SC(-Leucine, -Tryptophan, 0.1% FOA) media for curing. Plates were grown at 30 °C for 3 d rather than 2 d to allow full development of the orange color. The expected percentage of orange colonies was, for example, $100 \times (1/101) \times (1/11) = 0.90\%$ for the 100:1 *crtB* stop/silent × 10:1 *crtI* stop/silent library. Single colonies from the libraries were analyzed by colony PCR and restriction analysis, following streak purification if necessary.

SI Results

Analysis of Colonies with Inactive Reporters. To determine the source of the recombinants with inactive *lacZ* and *gusA* reporters in the reporter proof-of-principle system, we purified the genomic DNA of three of the white round 1 colonies and the single white round 2 colony shown in Fig. 2*D*. PCR and restriction analysis of the white round 2 colony indicated correct construction of the pathway. Sequencing of the integrated *gusA* gene showed that it had two mutations in the amino acid sequence, D436G and F551V. For the white round 1 colonies, we could amplify the 5′ end of the expected construct (*HO*(*L*) → p*ADH*) but not the 3′ end (t*ADH* → *LEU*2), indicating that the complete fragment had not integrated as expected. We have not observed this result for any of the other constructs tested. Analysis of the original *lacZ* PCR product transformed during round 1 revealed that there was a truncated DNA fragment that could not be removed by gel purification. We subsequently repeated round 1, amplifying *lacZ* as two shorter, overlapping subfragments rather than as a single subfragment (as described in Table S2). As shown in Fig. S1*D*, a higher percentage of colonies (42/48, or 87.5%) tested positive for *lacZ* using this modified protocol, and PCR analysis of the white colonies indicated that all contained the complete fragment.

Analysis of *HIS LEU* Mutants. The pool of cured recombinants was lifted from the curing plate for the rounds of Reiterative Recombination shown in Table S1. Serial dilutions of the resuspended cells were plated on SC(-Leucine) and SC(-Histidine) media. After 2 d of growth at 30 °C, the number of colonies on each type of media was counted. Colonies that grew on the "wrong" media [SC(-Leucine) for even rounds, and SC(-Histidine) for odd rounds] were assayed for growth on the "correct" media [SC (-Histidine) for even rounds, and SC(-Leucine) for odd rounds] to confirm that they were prototrophic for both amino acids, and all tested colonies grew on both types of media. The percentages of colonies with this *HIS LEU* phenotype observed in four different Reiterative Recombination trials are shown in Table S1.

Appendix

Sequence of the completed reporter gene proof-of-principle assembly, which is also shown schematically in Fig. S2. To show the context of the sequence in the chromosome, the last 30 bp of the *HO(L)* region on the 5′ end and the first 30 bp of the acceptor module (containing the SceI cleavage site and the *LEU2* terminator) on the 3′ end are shown.

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1..30 HO(L)
31..4145 Fragment 1 (pADH-lacZ-tADH)
4146..5957 Subfragment 2a (gusA, reverse complement)
5958..6452 Subfragment 2b (pCYC, reverse complement)
6453..8086 Subfragment 3a (tMET-MET15, reverse complement)
8087..8498 Subfragment 3b (pTEF, reverse complement)
8499..8528 Acceptor module
1 AAAATTGTGC CTTTGGACTT AAAATGGCGT CAACTTCTTT TCTTTTTTTT
51 TCTTTTCTCT CTCCCCCGTT GTTGTCTCAC CATATCCGCA ATGACAAAAA
101 AATGATGGAA GACACTAAAG GAAAAAATTA ACGACAAAGA CAGCACCAAC
151 AGATGTCGCT GTTCCAGAGC TGATGAGGGG TATCTCGAAG CACACGAAAC
201 TTTTTCCTTC CTTCATTCAC GCACGCTACT CTCTAATGAG CAACGGTATA
251 CGGCCTTCCT TCCAGTTACT TGAATTTGAA ATAAAAAAAA GTTTGCTGTC
301 TTGCTATCAA GTATAAATAG ACCTGCAATT ATTAATCTTT TGTTTCCTCG
351 TCATTGTTCT CGTTCCCTTT CTTCCTTGTT TCTTTTTCTG CACAATATTT
401 CAAGCTATAC CAAGCATACA ATCAACTCCA AGCTTGAATT AATACCGGGC
451 GGAATGACTA AATCTCATTC AGAAGAAGTG ATTGTACCTG AGTTCAATTC
501 TAGCGCAAAG GAATTACCAA GACCATTGGC CGAAAAGTGC GGAATTCCAA
551 GCTTGGCCAA GCCCGGATCC GGAGCTTGGC TGTTGCCCGT CTCACTGGTG
601 AAAAGAAAAA CCACCCTGGC GCCCAATACG CAAACCGCCT CTCCCCGCGC
651 GTTGGCCGAT TCATTAATGC AGCTGGCACG ACAGGTTTCC CGACTTAATC
701 GCCTTGCAGC ACATCCCCCT TTCGCCAGCT GGCGTAATAG CGAAGAGGCC
751 CGCACCGATC GCCCTTCCCA ACAGTTGCGC AGCCTGAATG GCGAATGGCG
801 CTTTGCCTGG TTTCCGGCAC CAGAAGCGGT GCCGGAAAGC TGGCTGGAGT
851 GCGATCTTCC TGAGGCCGAT ACTGTCGTCG TCCCCTCAAA CTGGCAGATG
901 CACGGTTACG ATGCGCCCAT CTACACCAAC GTAACCTATC CCATTACGGT
951 CAATCCGCCG TTTGTTCCCA CGGAGAATCC GACGGGTTGT TACTCGCTCA
1001 CATTTAATGT TGATGAAAGC TGGCTACAGG AAGGCCAGAC GCGAATTATT
1051 TTTGATGGCG TTAACTCGGC GTTTCATCTG TGGTGCAACG GGCGCTGGGT
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1101 CGGTTACGGC CAGGACAGTC GTTTGCCGTC TGAATTTGAC CTGAGCGCAT
1151 TTTTACGCGC CGGAGAAAAC CGCCTCGCGG TGATGGTGCT GCGTTGGAGT
1201 GACGGCAGTT ATCTGGAAGA TCAGGATATG TGGCGGATGA GCGGCATTTT
1251 CCGTGACGTC TCGTTGCTGC ATAAACCGAC TACACAAATC AGCGATTTCC
1301 ATGTTGCCAC TCGCTTTAAT GATGATTTCA GCCGCGCTGT ACTGGAGGCT
1351 GAAGTTCAGA TGTGCGGCGA GTTGCGTGAC TACCTACGGG TAACAGTTTC
1401 TTTATGGCAG GGTGAAACGC AGGTCGCCAG CGGCACCGCG CCTTTCGGCG
1451 GTGAAATTAT CGATGAGCGT GGTGGTTATG CCGATCGCGT CACACTACGT
1501 CTGAACGTCG AAAACCCGAA ACTGTGGAGC GCCGAAATCC CGAATCTCTA
1551 TCGTGCGGTG GTTGAACTGC ACACCGCCGA CGGCACGCTG ATTGAAGCAG
1601 AAGCCTGCGA TGTCGGTTTC CGCGAGGTGC GGATTGAAAA TGGTCTGCTG
1651 CTGCTGAACG GCAAGCCGTT GCTGATTCGA GGCGTTAACC GTCACGAGCA
1701 TCATCCTCTG CATGGTCAGG TCATGGATGA GCAGACGATG GTGCAGGATA
1751 TCCTGCTGAT GAAGCAGAAC AACTTTAACG CCGTGCGCTG TTCGCATTAT
1801 CCGAACCATC CGCTGTGGTA CACGCTGTGC GACCGCTACG GCCTGTATGT
1851 GGTGGATGAA GCCAATATTG AAACCCACGG CATGGTGCCA ATGAATCGTC
1901 TGACCGATGA TCCGCGCTGG CTACCGGCGA TGAGCGAACG CGTAACGCGA
1951 ATGGTGCAGC GCGATCGTAA TCACCCGAGT GTGATCATCT GGTCGCTGGG
2001 GAATGAATCA GGCCACGGCG CTAATCACGA CGCGCTGTAT CGCTGGATCA
2051 AATCTGTCGA TCCTTCCCGC CCGGTGCAGT ATGAAGGCGG CGGAGCCGAC
2101 ACCACGGCCA CCGATATTAT TTGCCCGATG TACGCGCGCG TGGATGAAGA
2151 CCAGCCCTTC CCGGCTGTGC CGAAATGGTC CATCAAAAAA TGGCTTTCGC
2201 TACCTGGAGA GACGCGCCCG CTGATCCTTT GCGAATACGC CCACGCGATG
2251 GGTAACAGTC TTGGCGGTTT CGCTAAATAC TGGCAGGCGT TTCGTCAGTA
2301 TCCCCGTTTA CAGGGCGGCT TCGTCTGGGA CTGGGTGGAT CAGTCGCTGA
2351 TTAAATATGA TGAAAACGGC AACCCGTGGT CGGCTTACGG CGGTGATTTT
2401 GGCGATACGC CGAACGATCG CCAGTTCTGT ATGAACGGTC TGGTCTTTGC
2451 CGACCGCACG CCGCATCCAG CGCTGACGGA AGCAAAACAC CAGCAGCAGT
2501 TTTTCCAGTT CCGTTTATCC GGGCAAACCA TCGAAGTGAC CAGCGAATAC
2551 CTGTTCCGTC ATAGCGATAA CGAGCTCCTG CACTGGATGG TGGCGCTGGA
2601 TGGTAAGCCG CTGGCAAGCG GTGAAGTGCC TCTGGATGTC GCTCCACAAG
2651 GTAAACAGTT GATTGAACTG CCTGAACTAC CGCAGCCGGA GAGCGCCGGG
2701 CAACTCTGGC TCACAGTACG CGTAGTGCAA CCGAACGCGA CCGCATGGTC
2751 AGAAGCCGGG CACATCAGCG CCTGGCAGCA GTGGCGTCTG GCGGAAAACC
2801 TCAGTGTGAC GCTCCCCGCC GCGTCCCACG CCATCCCGCA TCTGACCACC
2851 AGCGAAATGG ATTTTTGCAT CGAGCTGGGT AATAAGCGTT GGCAATTTAA
2901 CCGCCAGTCA GGCTTTCTTT CACAGATGTG GATTGGCGAT AAAAAACAAC
2951 TGCTGACGCC GCTGCGCGAT CAGTTCACCC GTGCACCGCT GGATAACGAC
3001 ATTGGCGTAA GTGAAGCGAC CCGCATTGAC CCTAACGCCT GGGTCGAACG
3051 CTGGAAGGCG GCGGGCCATT ACCAGGCCGA AGCAGCGTTG TTGCAGTGCA
3101 CGGCAGATAC ACTTGCTGAT GCGGTGCTGA TTACGACCGC TCACGCGTGG
3151 CAGCATCAGG GGAAAACCTT ATTTATCAGC CGGAAAACCT ACCGGATTGA
3201 TGGTAGTGGT CAAATGGCGA TTACCGTTGA TGTTGAAGTG GCGAGCGATA
3251 CACCGCATCC GGCGCGGATT GGCCTGAACT GCCAGCTGGC GCAGGTAGCA
3301 GAGCGGGTAA ACTGGCTCGG ATTAGGGCCG CAAGAAAACT ATCCCGACCG
3351 CCTTACTGCC GCCTGTTTTG ACCGCTGGGA TCTGCCATTG TCAGACATGT
3401 ATACCCCGTA CGTCTTCCCG AGCGAAAACG GTCTGCGCTG CGGGACGCGC
3451 GAATTGAATT ATGGCCCACA CCAGTGGCGC GGCGACTTCC AGTTCAACAT
3501 CAGCCGCTAC AGTCAACAGC AACTGATGGA AACCAGCCAT CGCCATCTGC
3551 TGCACGCGGA AGAAGGCACA TGGCTGAATA TCGACGGTTT CCATATGGGG
3601 ATTGGTGGCG ACGACTCCTG GAGCCCGTCA GTATCGGCGG AATTCCAGCT
3651 GAGCGCCGGT CGCTACCATT ACCAGTTGGT CTGGTGTCAA AAATAATTAC
3701 AACAGGTGTT GTCCTCTGAG GACATAAAAT ACACACCGAG ATTCATCAAC
3751 TCATTGCTGG AGTTAGCATA TCTACAATTG GGTGAAATGG GGAGCGATTT
3801 GCAGGCATTT GCTCGGCATG CCGGTAGAGG TGTGGTCAAT AAGAGCGACC
3851 TCATGCTATA CCTGAGAAAG CAACCTGACC TACAGGAAAG AGTTACTCAA
3901 GAACAAGAAT TTTCGTTTTA AAACCTAAGA GTCACTTTAA AATTTGTATA
3951 CACTTATTTT TTTTATAACT TATTTAATAA TAAAAATCAT AAATCATAAG
4001 AAATTCGCTT ATTTAGAAGT GTCAACAACG TATCTACCAA CGATTTGACC
4051 CTTTTCCATC TTTTCGTAAA TTTCTGGCAA GGTAGACAAG CCGACAACCT
4101 TGATTGGAGA CTTGACCAAA CCTCTGGCGA AGAAGTCCAA AGCTTTCATT
4151 GTTTGCCTCC CTGCTGCGGT TTTTCACCGA AGTTCATGCC AGTCCAGCGT
4201 TTTTGCAGCA GAAAAGCCGC CGACTTCGGT TTGCGGTCGC GAGTGAAGAT
4251 CCCTTTCTTG TTACCGCCAA CGCGCAATAT GCCTTGCGAG GTCGCAAAAT
4301 CGGCGAAATT CCATACCTGT TCACCGACGA CGGCGCTGAC GCGATCAAAG
4351 ACGCGGTGAT ACATATCCAG CCATGCACAC TGATACTCTT CACTCCACAT
4401 GTCGGTGTAC ATTGAGTGCA GCCCGGCTAA CGTATCCACG CCGTATTCGG
4451 TGATGATAAT CGGCTGATGC AGTTTCTCCT GCCAGGCCAG AAGTTCTTTT
4501 TCCAGTACCT TCTCTGCCGT TTCCAAATCG CCGCTTTGGA CATACCATCC
4551 GTAATAACGG TTCAGGCACA GCACATCAAA GAGATCGCTG ATGGTATCGG
4601 TGTGAGCGTC GCAGAACATT ACATTGACGC AGGTGATCGG ACGCGTCGGG
4651 TCGAGTTTAC GCGTTGCTTC CGCCAGTGGC GCGAAATATT CCCGTGCACC
4701 TTGCGGACGG GTATCCGGTT CGTTGGCAAT ACTCCACATC ACCACGCTTG
4751 GGTGGTTTTT GTCACGCGCT ATCAGCTCTT TAATCGCCTG TAAGTGCGCT

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4801 TGCTGAGTTT CCCCGTTGAC TGCCTCTTCG CTGTACAGTT CTTTCGGCTT
4851 GTTGCCCGCT TCGAAACCAA TGCCTAAAGA GAGGTTAAAG CCGACAGCAG
4901 CAGTTTCATC AATCACCACG ATGCCATGTT CATCTGCCCA GTCGAGCATC
4951 TCTTCAGCGT AAGGGTAATG CGAGGTACGG TAGGAGTTGG CCCCAATCCA
5001 GTCCATTAAT GCGTGGTCGT GCACCATCAG CACGTTATCG AATCCTTTGC
5051 CACGTAAGTC CGCATCTTCA TGACGACCAA AGCCAGTAAA GTAGAACGGT
5101 TTGTGGTTAA TCAGGAACTG TTCGCCCTTC ACTGCCACTG ACCGGATGCC
5151 GACGCGAAGC GGGTAGATAT CACACTCTGT CTGGCTTTTG GCTGTGACGC
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5251 ACTTGCAAAG TCCCGCTAGT GCCTTGTCCA GTTGCAACCA CCTGTTGATC
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5401 TCGTCCACCC AGGTGTTCGG CGTGGTGTAG AGCATTACGC TGCGATGGAT
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5651 CCTGATTATT GACCCACACT TTGCCGTAAT GAGTGACCGC ATCGAAACGC
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6651 GAAAAAAAGG ATATTCATTT CAATAAAGTT CGTTTTATAA AAGTATAGTA
6701 CTTGTGAGAG AAAGTAGGTT TATACATAAT TTTACAACTC ATTACGCACA
6751 CTCATGGTTT TTGGCCAGCG AAAACAGTTT CAAAAGATTG CTGGAAGTCT
6801 GCAATAATGT CATCAATAAA TTCGATACCA ACAGAGACAC GAATTAAGTC
6851 CTTGGTAACA CCAGATGCCA ACTTTTCTTT GTCATTTAAT TGTTTGTGGG
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6951 AAGTTAGAGG CAAGCTTTAA ATTGTCAACA ACTTGAGCAC CAGAAAGTTT
7001 GAATGGGTCA GTTTCCTTGT CGGCATTTGG TAAGTCTTTT ACACCGAAAG
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7551 GGAATACCGT GTTTGTGAGC AATTGCAACA ATTTTTTCAA AATCCGGAAC
7601 ATTGTACTTT GGATTACCAA TGGTTTCCAA ATAAACAGCC TTGGTTCTTT
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8301 TTTCGACGAA GAAAAAGAAA CGAGGCGGTC TCTTTTTTCT TTTCCAAACC
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8401 AGTATGCTGT GCTTGGGTGT TTTGAAGTGG TACGGCGATG CGCGGAGTCC
8451 GAGAAAATCT GGAAGAGTAA AAAAGGAGTA GAAACATTTT GAAGCTATAT
8501 TACCCTGTTA TCCCTAagat tgtactga
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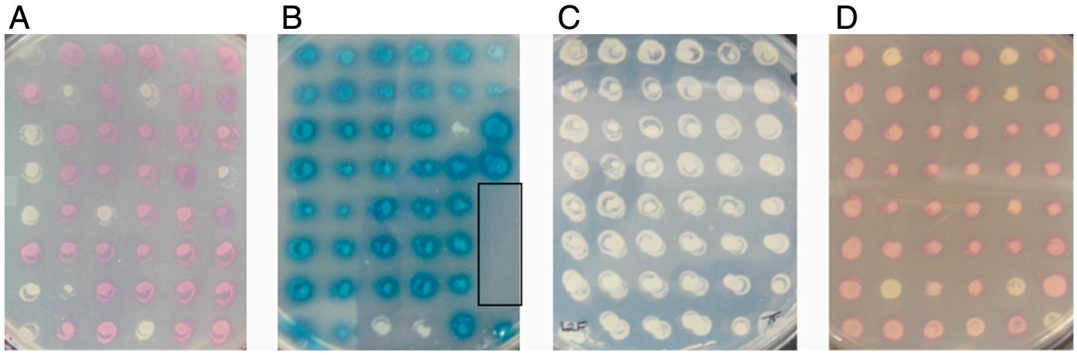
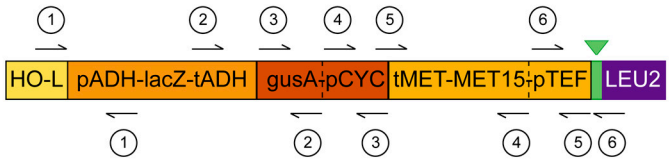


Fig. S1. Phenotypic analysis of phrogged cured recombinants from (*A*) round 1, (*B*) round 2, and (*C*) round 3 of the reporter gene proof-of-principle system assayed with Magenta-Gal, X-Gluc, and SC(-Methionine) media, respectively, as in Fig. 2*D*. No colonies were phrogged in the boxed area of (*B*). (*D*) Phenotypic analysis with Magenta-Gal of cured round 1 recombinants resulting when *lacZ* was amplified as two overlapping subfragments, as described in SI Results.



	Amplicon	Total length (bp)	Enzyme	Expected fragments (bp)
1	HO-L - pADH	455	BfuAI	320,135
2	tADH - gusA	1932	BsaWI	823,682,427
			BsrGI	1132,426,374
			BsrGI/HindIII	1132,426,266,108
3	gusA - pCYC	2336	BsaWI	1163,569,427,177
4	pCYC - MET	2179	BsaWI	1289,377,277,236
5	MET - pTEF	2114	BsmAI	1505,290,195,124
6	pTEF - LEU	472	BsmAI	277,195

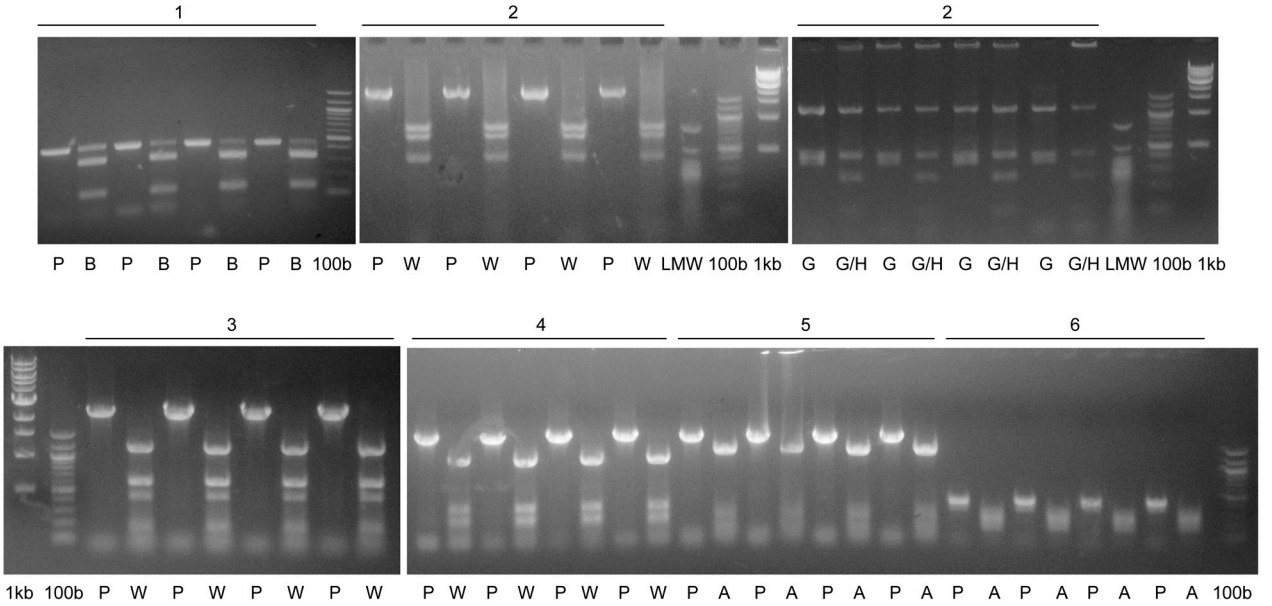


Fig. S2. PCR and restriction analysis of genomic DNA from four unique colonies from round 3 of the reporter gene proof-of-principle system. In the diagram of the integrated construct, solid lines between regions of different color represent new junctions that were created during endonuclease-stimulated integration; dashed lines indicate new junctions that were created between PCR fragments by plasmid gap repair. (P = undigested PCR product, B = BfuAI digest, W = BsaWI digest, G = BsrGI digest, G/H = BsrGI/HindIII digest, A = BsmAI digest, LMW = low molecular weight ladder, 100 b = 100 bp ladder, 1 kb = 1 kb ladder)

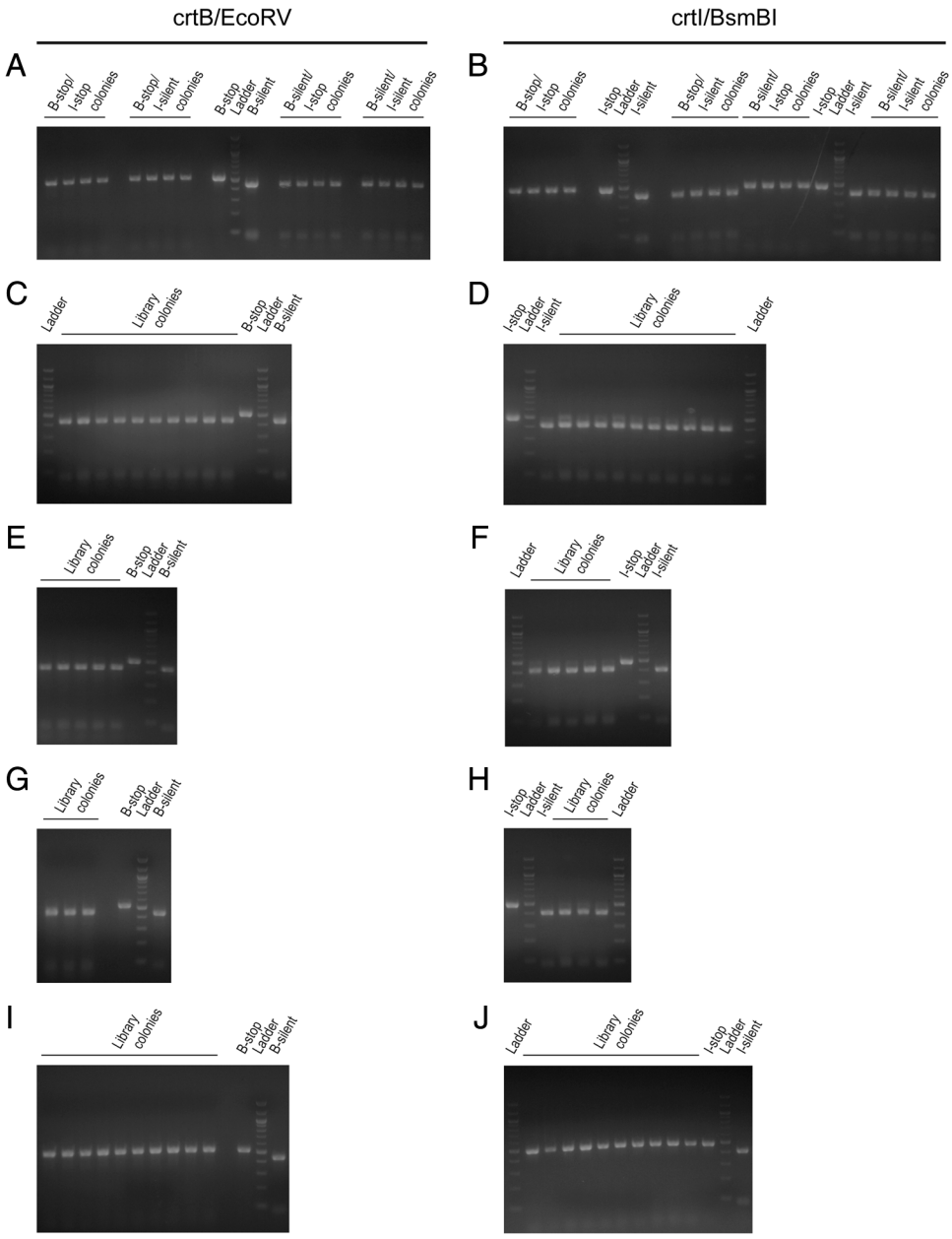


Fig. S3. Restriction analysis of cured recombinants from the lycopene library screen. Regions of the *crtB* (*A*, *C*, *E*, *G*, and *I*) and *crtI* (*B*, *D*, *F*, *H*, and *J*) alleles containing the diagnostic mutations were amplified by colony PCR and digested with EcoRV and BsmBI, respectively. Only alleles containing the silent mutations are cut by these enzymes. The plasmids with the *B*-stop, *B*-silent, *I*-stop, and *I*-silent alleles that served as PCR templates for the subfragments were PCR amplified and digested in parallel as controls. "Ladder" is a 100 bp ladder. (*A*, *B*) From left to right, four colonies each from the *crtB*-stop + *crtI*-stop, *crtB*-stop + *crtI*-silent, *crtB*-silent + *crtI*-stop, and *crtB*-silent + *crtI*-silent "libraries" from Fig. 3 *B*–*E*. (*C*, *D*) Ten orange colonies from the 10:1 *crtB* stop:silent + 0:1 *crtI* stop:silent library. (*E*, *F*) The five orange colonies from the 100:1 *crtB* stop:silent + 0:1 *crtI* stop:silent library. (*G*, *H*) The three orange colonies from the 100:1 *crtB* stop:silent + 10:1 *crtI* stop:silent library. (*I*, *J*) Ten white colonies from the 100:1 *crtB* stop:silent + 100:1 *crtI* stop:silent library.

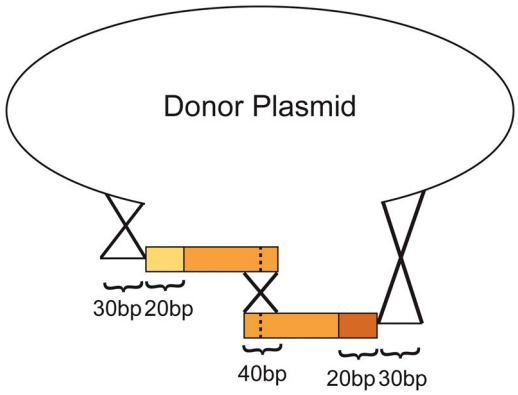


Fig. S4. General design of subfragment homology regions for plasmid gap repair and Reiterative Recombination. The recombination events that create a construct-specific donor plasmid from a universal donor plasmid and subfragment PCR products are shown.

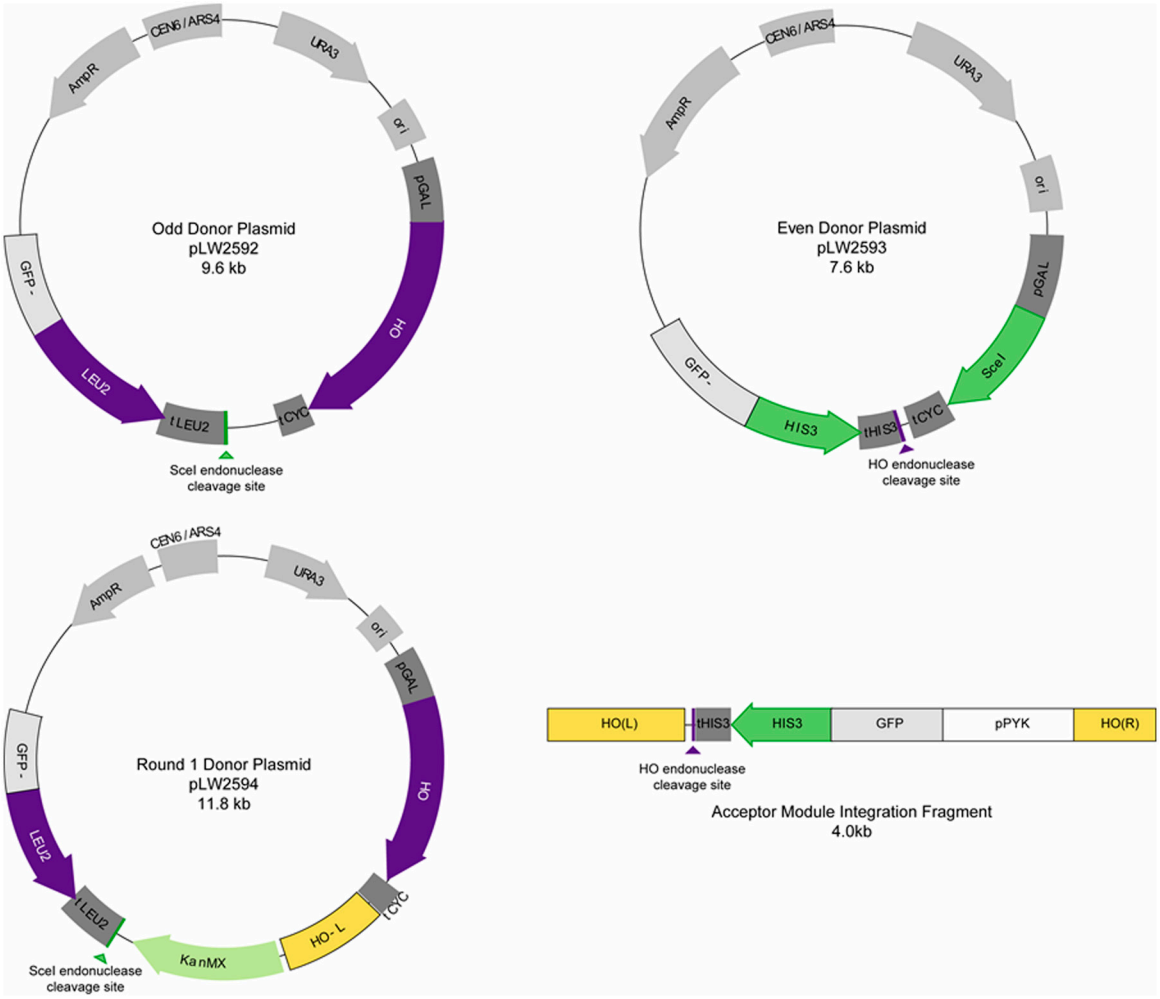


Fig. S5. Maps of donor plasmids and the acceptor module integration fragment for Reiterative Recombination.

Table S1. Percentage of cells with the *HIS LEU* phenotype in cured recombinant pools from various rounds of Reiterative Recombination

Round	Percentage of recombinants with *HIS LEU* phenotype
Reporter proof-of-principle	
Round 1	0.2%
Round 2	0.00007%
Lycopene library round 2	
10:1 *crtB* stop:silent	0.006%
100:1 *crtB* stop:silent	0.01%

Table S2. Primers for amplification of subfragments in the reporter proof-of-principle experiment

Subfragment	Amplicon	Primers
1	pADH-lacZ-*tADH*	1) 5′ primer LMW419 AAAATTGTGCCTTTGGACTTAAAATGGCGTCAACTTCTTTTCTTTTTTTTTCT 3′ primer LMW420 cttagggataacagggtaat**AGCAGGGAGGCAAACAATGA***AAGCTTTGGACTTCTTCGC* 2) 5′ primer LMW419 aaaattgtgcctttggacttaaaatggcgtCAACTTCTTTTCTTTTTTTTTCT 3′ primer LMW367 tcagtacaatcttagggataacagggtaat
2a	gusA	1) 5′ primer LMW421 ggacgctcgaaggctttGGCGAAGAAGTCCAAAGCTT**TCATTGTTTGCCTCCCTGCTG** 3′ primer LMW422 ATACACACACTAAATTAATA**ATGTTACGTCCTGTAGAAAC** 2) 5′ primer LMW374 tgagaaggttttgggacgctcgaaggcttt 3′ primer LMW422 ATACACACACTAAATTAATA**ATGTTACGTCCTGTAGAAAC**
2b	pCYC	1) 5′ primer LMW423 **GTTTCTACAGGACGTAACAT**TATTAATTTAGTGTGTGTATTTG 3′ primer LMW424 ctgttgcggaaagctgaaa*AAGAGGATGATGGAGGTTTC*TTTGGAAAACCAAGAAATGAA 2) 5′ primer LMW423 **GTTTCTACAGGACGTAACAT**TATTAATTTAGTGTGTGTATTTG 3′ primer LMW375 gcacagttatactgttgcggaaagctgaaa
3a	*tMET15*-MET15	1) 5′ primer LMW425 ggacgctcgaaggctttTCATTTCTTGGTTTTCCAAA*GAAACCTCCATCATCCTC* 3′ primer LMW426 ATCTAAGTTTTAATTACAAA**ATGCCATCTCATTTCGATAC** 2) 5′ primer LMW374 tgagaaggttttgggacgctcgaaggcttt 3′ primer LMW426 ATCTAAGTTTTAATTACAAA**ATGCCATCTCATTTCGATAC**
3b	pTEF	1) 5′ primer LMW427 **GTATCGAAATGAGATGGCAT**TTTGTAATTAAAACTTAGATTAGA 3′ primer LMW428 cttagggataacagggtaatATAGCTTCAAAATGTTTCTACT 2) 5′ primer LMW427 **GTATCGAAATGAGATGGCAT**TTTGTAATTAAAACTTAGATTAGA 3′ primer LMW367 tcagtacaatcttagggataacagggtaat