File S1

Supplement

gRNA constructs

Plasmid pRB1017, containing a *C. elegans* U6 promoter from *R07E5.16*, was used to drive expression of all gRNAs. pRB1017 was made by cloning a gBlock (sequence:
GGGAAGCTTCAAAAAAAACTAGCAATAAAGGAATAAAAAACTGTACACCTTAAAGGCGCACACTCTGTTTTGCAAATTTTATTTTT
AGTTGTGAATTTTCTGCTGAGACtTGAAAATAGCAACTTTAGTACTACTATAATTTGTCAACCTTTTCAAAAAAAGCATGCAATTTTT
GAGAAACTCTTATAAAAGCTATTATTAAAAAAACACCTTTTTTCCAAAATTATTCCACAAAAAATATGTTATGAAATGCCTACACCCT
CTCACACACACTCTTTATACTACTCTGTCAAACTCACGAGATGTCTGCCGCCTCTTGTGTtgCCCCTATATAAACACCTCCTATTGCGA
GATGTCTTggagaccggtaaccatggctcgagaaaccggtactcggtctctGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTT
ATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTGTGAAATTTGCTAGCGG, BsaI-excised insert for gRNA cloning in lower case, see below) between the NheI and HindIII sites of pDR274 (HWANG *et al.* 2013).

gRNA plasmids were designed and cloned as follows (Figure S3): pRB1017 contains two opposing non-palindromic BsaI sites, i.e. 5'-…GTCTTGGAGACC N_{29} GGTCTCTGTTTT…-3', BsaI sites underlined. As BsaI cuts outside of its recognition site and leaves a four nucleotide 5'overhang, BsaI digestion of pRB1017 leaves the vector backbone without BsaI sites, but with 3'-AGAA-5' and 5'-GTTT-3' overhangs. For a given target mutation, a nearby PAM site (NGG) was located, ideally so that the gRNA overlapped the position of the mutation of interest (site is N_{19}NGG). Forward and reverse oligonucleotides bearing the gRNA site and complimentary overhangs were ordered (forward: 5'-TCTTGN_{19}-3' and reverse 5'-AAACN*$_{19}$C-3', where N*$_{19}$ denotes the reverse complement of N_{19}). The additional G is required for transcription from the U6 promoter, and in our experiments a mismatch with the target site at this position does not confer adverse effects on gRNA functionality. Forward and reverse oligonucleotides were annealed, and then ligated into the cut vector. All plasmids were confirmed by restriction digest as well as sequenced with the M13 forward primer. We noted a tendency for single gRNA vectors to concatamerize in *E. coli* (even in *recA1* strains) and avoided gRNA plasmid preps where this was observed to be the case.

Microinjection

Plasmid DNA was prepared using Cetyl Trimethyl Ammonium Bromide (CTAB) precipitation (MELLO and FIRE 1995), and quantified using the Qubit Broad Range assay (Life Technologies). In all injections, the Cas9 plasmid (pDD162 (DICKINSON *et al.* 2013)) was present at 50ng/ul, and unless otherwise indicated, donor DNA was present at 20ng/ul (~600nM for ~100nt ssDNA). DNA mixtures were made up in injection buffer (20mM potassium phosphate, 3mM potassium citrate, 2% PEG, pH 7.5) and spun for >10' at >13,000 rcf. Attempts were made to microinject the distal arms of both gonads of young adult animals. Injected animals were rehydrated in recovery buffer (1mg/ml salmon sperm DNA, 4% glucose, 2.4mM KCl, 66mM NaCl, 3mM $MgCl_2$, 3mM $CaCl_2$, 3mM HEPES pH 7.2) prior to being placed on a small NGM plate seeded with OP50.

Throughout the paper, the number of injected animals includes animals that died yielding few progeny. We estimate on average ~20-40% of injected animals died before giving rise to sufficient progeny to accurately screen activity of the CRISPR/Cas9 system.

Screening

The F1 was screened for Rol, Dpy, and/or Unc phenotypes ~3-4 days after injection. Because some of the Unc phenotypes cause a developmental delay, and the Rol phenotypes do not manifest until ≥L3 stage, we found it helpful to move the injected parent to a new plate every ~12 hours. We found the majority of HR animals were born between 12 and 48 hours after injection.

Where long dsDNA (and not a ssDNA oligonucleotide) is used as a donor, transgenic animals provide a second possible source of PCR signals. Among the F1 progeny screened in Figure 4, we observed several transgenic animals, which lost *rde-1(AAA)* sequences within a generation or displayed non-mendelian inheritance patterns. The rate of such events was only slightly decreased when PCR screening primers outside of the donor dsDNA sequence were used.

Mitigating Additional Mutation Events Recovered During Co-Conversion

The observation that only one copy of a target locus is subject to efficient oligonucleotide-templated conversion may prove informative for optimizing conversion by CRISPR/Cas9. Because only a single genomic copy is receptive to templated mutation, increasing Cas9 and/or gRNAs above a certain level may increase the incidence of additional cleavage events, most prominently of deletions that break the non-selected allele, without an increase in conversion frequency. Excessive Cas9 activity may be undesirable for two reasons: (1) more Cas9 activity could be accompanied by an increased propensity for off-target cleavage and (2) untemplated mutations at the unselected copy of marker

locus limit the utility of that marker locus (and strain) for immediate experimentation, including further rounds of CRISPR/Cas9 co-conversion. Under this logic, avoiding excessive Cas9 activity may prove optimal for recovery of desired HR events in the absence of additional mutagenic events.

We carried out a series of experiments at different gRNA plasmid concentrations to assess whether these might affect the balance between wanted and unwanted events. Examining both *rol-6* loci or both *rde-1* loci for several F1 Rol animals from a high gRNA plasmid concentration (50ng/ul of each guide plasmid and 20ng/ul of each template DNA), we found a considerable degree of unwanted deletions and other mutations in the second, unselected allele. Six of 11 co-convertants had additional mutations resembling NHEJ at the non-converted allele at the *rol-6* locus, and 4 of 11 for the rde-1 locus (Figure S2).

A two-fold reduction of the *rde-1* gRNA alone, or both *rol-6* and *rde-1* gRNAs together resulted in a higher number of animals where the templated mutations were recovered over a wild type copy at both *rol-6* and *rde-1* loci. Under all injection conditions the frequency of co-conversion remained high (35-60%), as did the total number of F1 Rol animals. At D718 in *rde-1*, with 25ng/ul of each gRNA plasmid, we observed 16 out of 22 animals were *rol-6(su1006)/+* and 17 out of 22 animals were *rde-1(D718A)/+*. Out of 5 co-convertant animals sequenced for D801A, all were *rol-6(su1006)/+*; *rde-1(D801A)/+*. While further optimization remains to be done (and will possibly be locus and/or gRNA-specific), simply lowering the concentration of gRNA plasmids provides one strategy to reduce the production of additional mutations with no loss in co-conversion frequency or yield of mutants.

Sequence of PCR product used for integration at *rde-1*:

GCCAATGAAAACAGAGGAGCGCAATCTATTATGTACGACGCGACGAAAAATGAATATGCCGTAAGTTTCAGAAAATTGAAAGTTT
TTAAATATCATATTTACAGTTCTACAAAAATTGTACACTAAATACCGGAATCGGTAGATTTGAAATAGCCGCAACAGAAGCGAAGA
ATATGTTTGAACGTCTTCCCGATAAAGAACAAAAAGTCTTAATGTTCATTATCATTTCCAAACGACAACTGAATGCTTACGGTTTTG
TGAAACATTATTGCGATCACACCATCGGTGTAGCTAATCAGCATATTACTTCTGAAACAGTCACAAAAGCTTTGGCATCACTAAGG
CACGAGAAAGGATCAAAACGAATTTTCTATCAAATTGCATTGAAAATCAACGCGAAATTAGGAGGTATCAACCAAGAGCTCGACT
GGTCAGAAATTGCAGAAATATCACCAGAAGAAAAAGAAAGACGGAAAACAATGCCATTAACTATGTATGTTGGAATTGCTGTAAC
TCATCCAACCTCCTACAGTGGAATTGATTATTCTATAGCGGCTGTAGTAGCGAGTATCAATCCAGGTGGAACTATCTATCGAAATA
TGATTGTGACTCAAGAAGAATGTCGTCCCGGTGAGCGTGCAGTGGCTCATGGACGGGAAAGAACAGATATTTTGGAAGCAAAGT
TCGTGAAATTGCTCAGAGAATTCGCAGAAGTGAGTTGTCTTGAGTATTTAAAAGATCTCTGGGATTTTTAATTTTTTTGTAAACTTT
CAGAACAACGACAATCGAGCACCAGCGCATATTGTAGTCTATCGAGCTGGAGTTAGCGATTCGGAGATGCTACGTGTTAGTCATG
ATGAGCTTCGATCTTTAAAAAGCGAAGTAAAACAATTCATGTCGGAACGGGATGGAGAAGATCCAGAGCCGAAGTACACGTTCAT
TGTGATTCAGAAAAGACACAATACACGATTGCTTCGAAGAATGGAAAAAGATAAGCCAGTGGTCAATAAAGATCTTACTCCTGCT
GAAACAGATGTCGCTGTTGCTGCTGTTAAACAATGGGAGGAGGATATGAAAGAAAGCAAAGAAACTGGAATTGTGAACCCATCA
TCCGGAACAACTGTGGATAAACTTATCGTTTCGAAATACAAATTCGATTTTTTCTTGGCATCTCATCATGGTGTCCTTGGTACATCTC
GTCCAGGACATTACACTGTTATGTATGACGATAAAGGAATGAGCCAAGATGAAGTCTATGTAAGCGTTTTGAATAGCAGTTAGCG
ATTTTAGGATTTTGTAATCCGCATATAGTTATTATAAAAAAATGTTTCAGAAAATGACCTACGGACTTGCTTTTCTCTCTGCTAGATG
TCGAAAACCCATCTCGTTGCCTGTTCCGGTTCATTATGCTGCTTTATCATGTGAAAAAGCGAAAGAGCTTTATCGAACTTACAAGGA
ACATTACATCGGTGACTATGCACAGCCACGGACTCGACACGAAATGGAACATTTTCTCCAAACTAACGTGAAGTACCCTGGAATGT
CGTTCGCATAACATTTTGCAAAAGTGTCGCCCGTTTCAATCAAATTTTTCAATTGTAGATATTGTACTTACTTTTTTTTAAAGCCCGG
TTTCAAAAATTCATTCCATGACTAACGTTTTCATAAATTACTTGAAATTTATTCTGTGTTTATTATTTATTACCTCTAAATTTCGTTTTG
AACGTGAGCATCATATCTTAAACTACTTATTGATAACGGTTTCATAAAGATGTTT