

1 **Dissecting quantitative trait nucleotides by saturation genome editing**

2 Kevin R. Roy^{1,2}, Justin D. Smith^{1,2}, Shengdi Li³, Sibylle C. Vonesch^{3,4,5}, Michelle
3 Nguyen^{1,2}, Wallace T. Burnett^{1,2}, Kevin M. Orsley^{1,2}, Cheng-Sheng Lee⁶, James E.
4 Haber⁶, Robert P. St.Onge^{1,7}, and Lars M. Steinmetz^{1,2,3}

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6 1. Stanford Genome Technology Center, Stanford University, Palo Alto,
7 California, USA.

8 2. Department of Genetics, Stanford University School of Medicine, Stanford,
9 California, USA.

10 3. European Molecular Biology Laboratory (EMBL), Genome Biology Unit,
11 Heidelberg, Germany.

12 4. Laboratory for Genome Editing and Systems Genetics, VIB-KU Leuven Center
13 for Microbiology, Leuven, Belgium.

14 5. KU Leuven Center for Microbial and Plant Genetics, Department M2S, Leuven,
15 Belgium.

16 6. Brandeis University, Rosenstiel Basic Medical Sciences Research Center and
17 Department of Biology, Waltham, MA

18 7. Department of Biochemistry, Stanford University School of Medicine,
19 Stanford, California, USA.

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22 Correspondence: Lars M. Steinmetz. Email: larsms@stanford.edu.

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24 **Key words:** CRISPR genome editing, DNA repair, homology-directed repair (HDR),
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26 protein (MCP), plasmid assembly, barcode sequencing (Bar-seq), quantitative
27 genetics, complex traits, quantitative trait loci

28

29 **Abstract**

30 Genome editing technologies have the potential to transform our understanding of
31 how genetic variation gives rise to complex traits through the systematic engineering
32 and phenotypic characterization of genetic variants. However, there has yet to be a
33 system with sufficient efficiency, fidelity, and throughput to comprehensively identify
34 causal variants at the genome scale. Here we explored the ability of templated
35 CRISPR editing systems to install natural variants genome-wide in budding yeast. We
36 optimized several approaches to enhance homology-directed repair (HDR) with donor
37 DNA templates, including donor recruitment to target sites, single-stranded donor
38 production by bacterial retons, and in vivo plasmid assembly. We uncovered unique
39 advantages of each system that we integrated into a single superior system named
40 MAGESTIC 3.0. We used MAGESTIC 3.0 to dissect causal variants residing in 112
41 quantitative trait loci across 32 environmental conditions, revealing an enrichment for
42 missense variants and loci with multiple causal variants. MAGESTIC 3.0 will facilitate
43 the functional analysis of the genome at single-nucleotide resolution and provides a
44 roadmap for improving template-based genome editing systems in other organisms.

45 Introduction

46 Most biological traits are controlled by a complex interplay between an organism's
47 genotype and its environment. A longstanding promise of biology is that with a deep
48 enough understanding of the molecular mechanisms governing quantitative traits, it
49 should be possible to predict phenotypes from genetic and environmental data. To
50 make progress towards this goal, it is necessary to dissect how each locus in the
51 genome contributes to phenotypic diversity across individuals and species. While
52 association and linkage-based studies have identified thousands of loci impacting
53 quantitative traits, they generally lack the resolution to identify the causal variants in
54 each locus as well as the power to detect rare variants. Hence, the causal variants
55 and molecular mechanisms governing most phenotypic variation in natural
56 populations remain obscure.

57 Systematic functional screens of individual genetic variants have the potential
58 to overcome the limitations of traditional mapping approaches¹. Towards this end,
59 we previously developed a high-throughput CRISPR genome editing system based
60 on paired guide RNA/donor DNA templates capable of introducing thousands of
61 genetic variants in parallel in budding yeast termed **Multiplexed Accurate Genome**
62 **Editing with Short, Trackable, Integrated Cellular barcodes** (MAGESTIC)². A key
63 feature of MAGESTIC is a donor recruitment system where a DNA-damage
64 recognizing protein, Fkh1p, fused to the LexA DNA binding domain localizes plasmid
65 donor templates to double-strand breaks to substantially activate homology-directed
66 repair (HDR). Even though donor recruitment substantially increased editing efficiency
67 at individual targets, the overall editing efficiency observed in clones derived from a
68 library of natural variant edits was prohibitively low (~60%) for effective phenotyping².

69 To improve the performance of guide-donor plasmid-based systems for
70 variant screens in the present study, we tested MAGESTIC head-to-head against
71 other library-scale guide-donor systems previously developed in yeast, including
72 genetic inactivation of non-homologous end-joining (NHEJ)³, single-stranded donor
73 DNA synthesis by bacterial retrons (CRISPEY)⁴ and in vivo assembly of linearized
74 donor plasmids⁵. We assessed editing efficiency, fidelity, and survival (i.e. variant
75 representation), as each can have a major impact on the ability to correctly call

76 phenotypes in large complex libraries. We tested a broad panel of target sites
77 consisting of natural variants across the yeast species both as individual edits to
78 measure efficiency and fidelity and in the context of a pooled library to measure
79 editing toxicity and survival. While donor recruitment provided superior editing overall
80 compared to other approaches, each system showed distinct advantages that could
81 be combined into a single, supercharged donor repair system (MAGESTIC 3.0).
82 MAGESTIC 3.0 proved substantially superior to all previous systems and enabled
83 editing all possible single-nucleotide variants across genomic regions (saturation
84 genome editing). As proof of principle, we used this optimized system to map causal
85 variants in 112 quantitative trait loci and found extensive impact of missense variants
86 on phenotypes, as well as an abundance of loci harboring multiple causal variants.

87

88 **Results**

89 **Enhancing homology-directed repair (HDR) for donor-templated CRISPR** 90 **screens**

91 CRISPR guide-donor libraries enable the parallel introduction thousands of
92 programmed edits into a population of cells. Guide-donor DNA pairs (guide-donors)
93 are first synthesized on oligonucleotide arrays, amplified and cloned into plasmid
94 libraries with unique barcode tags, and finally transformed into an isogenic cell
95 population under conditions such that nearly all transformed cells receive a single
96 plasmid (**Fig 1a**)². The designed edit is introduced by CRISPR-mediated cleavage of
97 the target locus followed by HDR with the donor DNA. The barcode tag on the
98 plasmid specifies the edit and allows for reading out variant function in pooled
99 phenotypic screens by sequencing-based barcode counting of strain abundance
100 (e.g. after competitive growth in diverse environmental conditions).

101 A consensus from previous studies developing guide-donor library
102 approaches was that natural HDR efficiencies with plasmid donor DNA are too low
103 for effective screens²⁻⁷. To enhance efficiency, each study employed a different
104 approach, including genetic inactivation of NHEJ (*nej1Δ*)³, in vivo assembly of
105 linearized guide-donor plasmids⁵, recruitment of donor DNA by LexA-Fkh1p
106 (MAGESTIC)², and in vivo production of single-stranded donor DNA with the bacterial

107 Eco1/Ec86 retron system (CRISPEY)⁴ (**Fig 1b**). Each study reported high editing
108 efficiencies (>80-100%) on individual target sites but tested different types of edits at
109 different target sites and measured efficiency via different assays. Therefore, it
110 remains unclear how each of these HDR improvement methods compare with each
111 other on the same set of target sites.

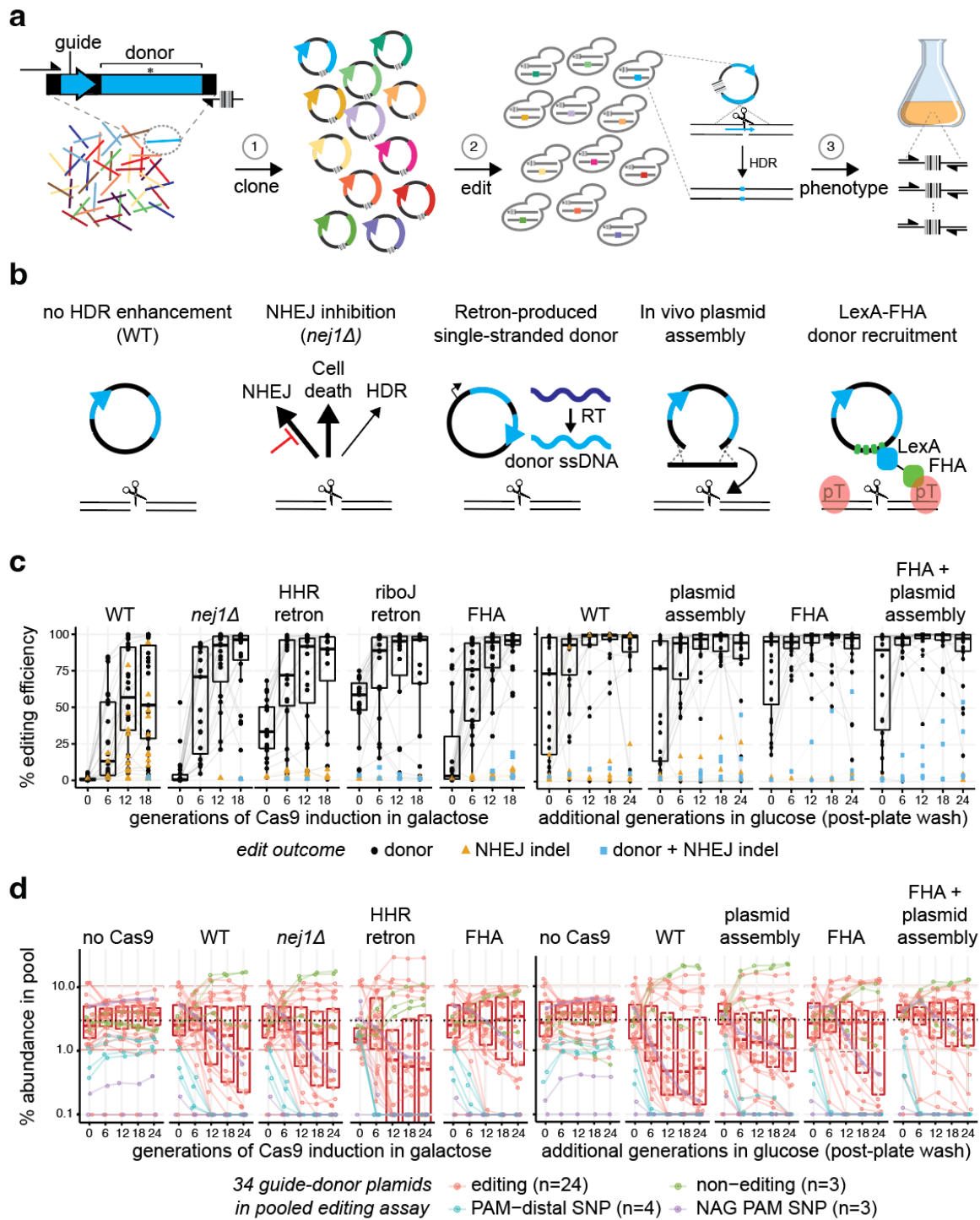
112 First, we explored whether the MAGESTIC donor recruitment system could be
113 improved. The Fkh1p forkhead-associated (FHA) domain binds phosphorylated
114 threonine residues as part of the DNA damage response and is required for
115 localization to DNA breaks⁸. To test whether it is also sufficient, we constructed a
116 minimal fusion protein containing the LexA DNA binding domain and the FHA domain.
117 This minimal fusion localized to the site of HO-induced breaks by microscopy and
118 gave a substantial, consistent boost in HDR editing over the full-length protein in an
119 editing survival assay at two distinct targets in both haploid and diploid yeast cells
120 (**Supp Fig 1**). We also note that the original MAGESTIC system utilized a tRNA-HDV
121 ribozyme promoter for guide expression, and we showed this promoter led to lower
122 editing efficiency for U-rich guide RNA sequences². We hypothesized these were
123 triggering early termination and lower guide levels and lowering efficiency overall, and
124 therefore switched to the *SNR52* (RNA polymerase III) promoter for this study as it
125 has been shown to be less prone to terminate at stretches of T residues⁹.

126 Second, we tested whether the retron editing system could be improved
127 through systematic testing of ribozymes flanking the donor-guide cassette, utilizing
128 the same 18mer *ADE2* guide and donor characterized in the CRISPEY study⁴ for
129 consistency (**Supp Fig 2**). We found that the HDV ribozyme was absolutely required
130 on the 3' end of the guide for detectable editing. Surprisingly, we found that the
131 hammerhead ribozyme (HHR) in the 5' position employed in the CRISPEY retron
132 system⁴ exhibited poor editing efficiency relative to the 5' HDV ribozyme (**Supp Fig**
133 **2c**). As a tandem repeat of HDV in both the 5' and 3' positions would lead to plasmid
134 instability, we explored whether additional ribozymes or RNA processing elements at
135 the 5' position could boost editing similarly to HDV. We found that the riboJ ribozyme
136 improved editing efficiency kinetics substantially over HDV, reaching 90% efficiency
137 after 18 generations, compared to 70% for the 5' HDV and 18% for the 5' HHR (**Supp**
138 **Fig 2c**).

139 Next, we sought to benchmark each system across a broad panel of 24 natural
140 variants, including guide-donors randomly selected from an *RM11* strain variant
141 library as well as guide-donors which gave rise to unedited clones in the previous
142 MAGESTIC system² (**Supplementary table S1**). With galactose induction of Cas9
143 and no HDR enhancement (WT), we found a mean HDR editing efficiency of 62%,
144 with unwanted NHEJ indels on 10 targets ranging from 10 to 95% (**Fig 1c**). Both
145 NHEJ inhibition (*nej1Δ*) and the retron systems prevented indel formation and
146 increased HDR editing efficiency, and the riboJ retron overall showed improved
147 editing kinetics compared to the HHR retron, consistent with the earlier results (**Supp**
148 **Fig 2c**). Overall, LexA-FHA showed superior editing efficiencies with minimal indel
149 formation in galactose. Next, we analyzed editing with constitutive Cas9 expression
150 in glucose media. We found that editing efficiency from colony transformants on agar
151 plates could be substantially improved with additional liquid outgrowth (**Fig 1c**). Even
152 in the absence of an HDR-enhancing system (WT), 19 guide-donors reached ~100%
153 donor editing after 18 generations of additional editing in glucose with only 2 targets
154 showing substantial NHEJ indels, suggesting that constitutive editing in glucose is
155 superior to galactose. The indel formation was mitigated by both the linearized
156 plasmid assembly and LexA-FHA. The LexA-FHA donor recruitment system yielded
157 the highest levels of editing efficiency, especially at the earlier stages of editing (**Fig**
158 **1c**).

159 While editing systems are typically characterized by efficiency and fidelity at
160 individual targets, editing survival is a major factor limiting large-scale CRISPR guide-
161 donor screens^{2,5}. In natural variant libraries, there is further the potential for the guide
162 to re-cleave the edited site and/or the donor plasmid since the typical edit (SNV)
163 results in a single mismatch in the guide region which does not always prevent Cas9
164 cleavage. These issues are important for pooled screens because the ability to
165 measure fitness effects is dependent on the variant starting abundances after editing
166 has gone to completion. To simulate a natural variant library, we pooled together the
167 24 editing guide-donors with three non-functional guide-donors containing truncated
168 guide scaffolds as controls for no editing toxicity and four with SNVs distal to the
169 protospacer adjacent motif (PAM) at positions 15, 16, 17 and 19 not expected to
170 substantially block cleavage² as controls for high editing toxicity. Additionally, we

171 included three guide-donors generating NAG PAMs, which are expected to be
 172 tolerated for Cas9 (re-)cleavage to a variable extent¹⁰. The 34 guide-donor plasmids
 173 were pooled equally and transformed into yeast expressing the different editing
 174 systems.



175

176 **Figure 1.** Enhancing homology-directed repair (HDR) with donor DNA templates for CRISPR screens.

177 **a**, CRISPR screens with guide-donor libraries involve (1) oligonucleotide synthesis and barcoded
178 cloning of paired guide RNA/donor DNA repair templates, (2) cell transformation and CRISPR editing
179 by homology-directed repair (HDR), and (3) characterization of growth phenotypes induced by each
180 variant via barcode sequencing (Bar-seq)-based counting of edited strains.

181 **b**, Previous strategies for enhancing editing efficiency used distinct approaches to improve HDR with
182 donor templates.

183 **c**, The editing efficiency for each system is plotted for a panel of 24 natural variant-targeting guide-
184 donors as a function of the number of generations of editing. The HHR retron corresponds to the
185 published CRISPEY system⁴ and the RiboJ retron is a variant of the CRISPEY system developed in
186 Fig. S2. For systems utilizing induction of Cas9 (left half), cells are transferred from non-inducing
187 (glucose) to inducing (galactose) at 0 generations. For editing systems with constitutively expressed
188 Cas9, the 0-generation time point corresponds to editing observed after colony formation on agar
189 plates. For additional generations of editing outgrowth in glucose media, cells were transferred to
190 liquid media after the transformation (right half). For each of the 24 targets, a ~200 bp region
191 encompassing the edit site was analyzed by on-target NGS. Lines connect the same edit across
192 timepoints. Boxplots show distribution of editing efficiency by donor HDR for all 24 guide-donors (i.e.
193 excluding editing by NHEJ).

194 **d**, Variant abundance during pooled editing. Editing survival was assessed by a competitive growth
195 experiment using a mini-pool consisting of the 24 editing guide-donors (red) assayed in panel c, as
196 well as 3 non-editing cassettes (green), 4 cassettes with PAM-distal SNVs (blue) and 3 cassettes where
197 SNVs result in NAG PAMs (purple). The latter two categories have guides which are expected to cleave
198 the donors at high levels due to tolerance of Cas9 for SNVs at PAM-distal positions and for NAG
199 PAMs. The pool was transformed into (left half) galactose inducible Cas9 systems, induced at time
200 zero by shifting from glucose into galactose, or (right half) constitutive Cas9 systems, where 0g
201 represents a wash of the transformation plate where editing has already begun. The mini pool is
202 constructed initially with all plasmids at near equimolar ratio. Variant ratios were determined at each
203 generation by sequencing of the barcodes in the yeast pools as shown in panel a. Dotted lines indicate
204 3% abundance. Boxplots include only the 24 editing guides.

205 With WT DNA repair, there was a substantial divergence of strain abundance
206 during the editing time course observed with either galactose induction of Cas9 or
207 with constitutively expressed Cas9 in glucose (**Fig 1d**). While NHEJ inhibition and the
208 HHR retron improved editing efficiency, they did not significantly improve variant
209 abundances. Linearized plasmid assembly improved abundances modestly over WT,
210 while LexA-FHA donor recruitment exhibited a strong improvement in overall survival,
211 reducing library skew and enrichment of the non-functional guides considerably
212 compared to the other methods. Strikingly, the combination of donor recruitment and

213 linearized plasmid assembly exhibited an additive effect with improved editing
214 survival compared to either method alone (**Fig 1d**).

215 Inspection of the editing survival curves for the donor recruitment system
216 revealed three distinct classes of abundance trajectories: those with stable
217 abundances, those with moderate dropout rates, and those with high dropout rates
218 matching the profile of the PAM-distal SNVs (**Supp Fig 4a**). The stable class of guide-
219 donors exhibited similar stability in all systems from 6 generations onwards.
220 Interestingly, however, LexA-FHA appeared to have the greatest benefit at the initial
221 stages of editing outgrowth, where significant skew accumulates the WT and plasmid
222 assembly systems (**Supp Fig 4a**, top row). This is consistent with these edits resistant
223 to cleavage by Cas9. By contrast, the moderate dropout class decreased across all
224 systems at each time point (**Supp Fig 4a**, middle row). Intriguingly, the plasmid
225 assembly method appeared to have the greatest benefit for this class of edits,
226 exhibiting a lower rate of dropout than even LexA-FHA. Strikingly, the combination of
227 donor recruitment and plasmid assembly had beneficial effects on both classes,
228 suggesting that these combining orthogonal HDR enhancement strategies is a
229 promising approach for improving library-scale editing (**Supp Fig 4a**).

230 Re-examining editing outcomes stratified by abundance curves revealed a
231 strong relationship between NHEJ indel formation and editing toxicity (**Supp Fig 4b**).
232 This was especially apparent in galactose editing in the absence of HDR
233 enhancement. This is consistent with a model where NHEJ indel formation is a minor
234 outcome relative to perfect DSB repair (either through perfect NHEJ¹¹ or by
235 homologous recombination with sister chromatids) or cell death^{2,12}, but ultimately
236 leads to predominate the editing outcomes of survivors in the absence of HDR
237 enhancement. By contrast, NHEJ indels were only observed to accumulate in 3 and
238 2 cases with plasmid assembly and LexA-FHA, respectively. These occurred at target
239 sites that had initially edited to 100% efficiency with HDR repair (**Supp Fig 4b**).
240 Overall, these results suggest that higher guide efficacy comes at the cost of
241 increased Cas9 tolerance for mismatches and hence lower SNV edit stability.
242 Furthermore, these data underscore the importance of balancing prolonged editing
243 outgrowth with lower efficiency targets while avoiding excessive re-cutting and library

244 dropout of high-efficiency targets, which is achieved with an outgrowth of 6-12
245 generations after colony formation (**Fig 1, Supp Fig 4**).

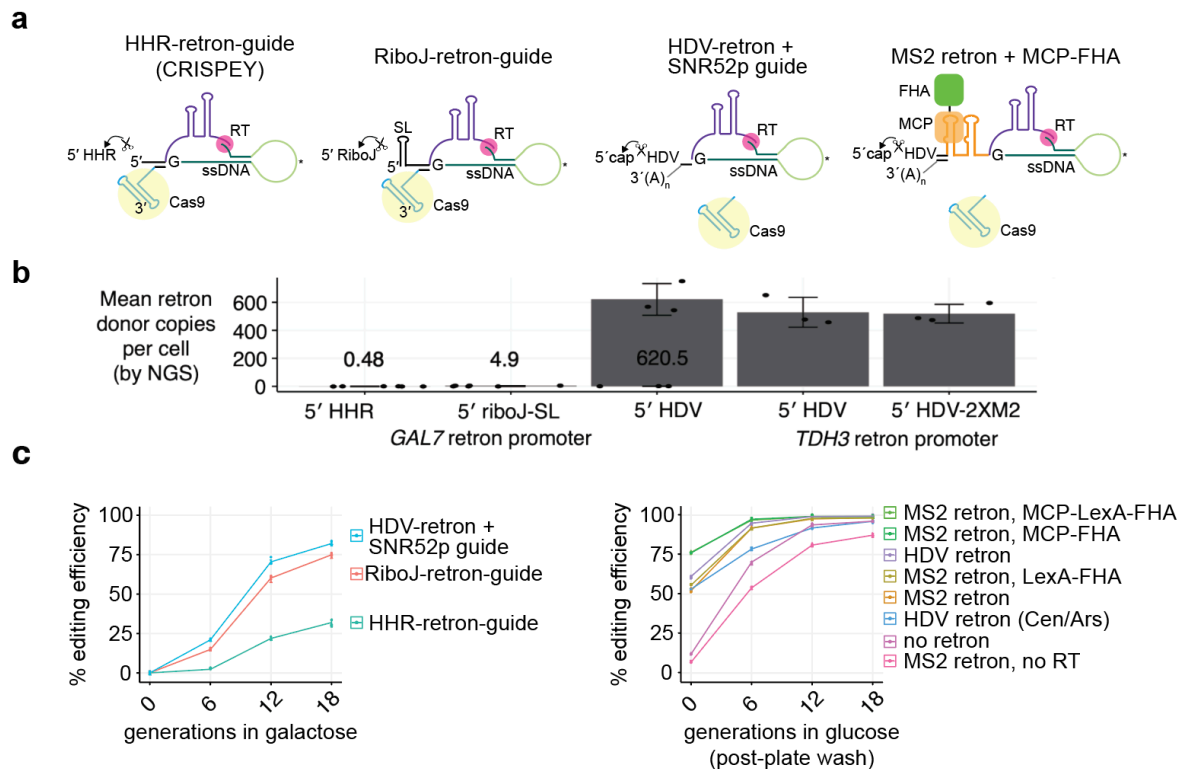
246

247 **Combining an improved retron system with donor recruitment**

248 Inspired by the results obtained from combining donor recruitment and plasmid
249 assembly, we revisited the retron system and looked for ways to further improve and
250 integrate it with the donor recruitment approach. To gain insights into how the
251 different ribozymes impact retron donor DNA output, we used an NGS-based
252 approach to simultaneously amplify donor DNA from the single-stranded retron donor
253 as well as the (unedited) target locus in the genome in the absence of Cas9 (**Supp**
254 **Fig 5**). Surprisingly, the HHR-HDV retron (CRISPEY)⁴ yielded the lowest levels of
255 donor DNA, with less than one donor per genome equivalent (**Supp Fig 5c**). Across
256 all combinations, HHR in the 5' position consistently lowered retron output
257 independent of the 3' ribozyme, and HDV in the 3' position consistently lowered
258 retron output independent of the 5' ribozyme. This contrasts with the editing results,
259 where the HDV in the 3' position was required for detectable editing (**Supp Fig 2**). On
260 the other end of the spectrum, the HDV ribozyme in the 5' position had a dramatic
261 positive effect on retron output, reaching 500-1000 donor ssDNA molecules per cell
262 (**Supp Fig 5**). As the HDV cleaves on its 5' side and the HHR cleaves on its 3' side,
263 the 5' HHR-3' HDV ribozyme arrangement exposes both ends of the retron transcript
264 to cellular exonucleases which would explain the low donor production. This
265 suggests that the retron transcript benefits from extra sequence on the 5' and 3' ends
266 to protect against exonucleases. By contrast, extraneous 5' or 3' sequence inhibits
267 guide activity^{13,14} and is unnecessary for stability due to the protective effect of Cas9
268 binding.

269 Taken together, the results above suggest that separating the guide RNA from
270 the retron donor and expressing each with optimal flanking elements should improve
271 editing. To test this, we expressed the 5' HDV retron donor separately from a guide
272 expressed from the *SNR52* promoter and compared editing efficiency to the HHR
273 and RiboJ retron donor-guides from **Supp Fig 2**. To sensitive the system to detect
274 differences in HDR repair and also to simulate lower efficacy guides observed in

275 libraries, we further truncated the guide RNA from an 18mer to a 17mer. We found
 276 that the 5' HDV retron outperformed both the riboJ and HHR retrons, suggesting that
 277 additional copies of the retron donor are indeed beneficial for HDR and that the
 278 Cas9:guide RNA complex does not need to recruit the retron donor to achieve high
 279 editing efficiency, as has been previously suggested^{4,15,16}.



280

281 **Figure 2.** Recruitment of retron donor DNA using the MS2 system and an MCP-FHA fusion protein.

282 **a**, Different arrangement of retrons tested shown from left to right in order of enhanced retron output
 283 and editing efficiency.

284 **b**, Retron donor cDNA output from each system as measured in **Supp Fig 5**. The levels for HHR and
 285 riboJ retrons are shown above each bar.

286 **c**, Editing efficiency for each retron arrangement shown in **panel a** as a function of generations of
 287 Cas9 and retron induction in galactose (left panel) or generations of liquid growth after colony
 288 formation on agar plates (right panel). The donor and guide are the same characterized in Sharon et
 289 al.⁴ for targeting the yeast *ADE2* gene, where the guide was engineered to have only 18 bp of matching
 290 sequence. To give greater sensitivity towards measuring differences in template HDR rates and to
 291 simulate the weaker guides which would be observed in a genome-wide library, we artificially
 292 weakened this guide with an additional mismatch at position 17. The resulting guide is 5'-
 293 cacTTAACGAAATTGCCCA-3', where lowercase letters denote mismatches to the target site. All
 294 guide-donor plasmids used in the constitutive glucose system are 2 μ (high-copy) plasmids, with the

295 exception of the HDV retron (Cen/Ars) shown blue. All systems in the right panel have the guide RNA
296 expressed under the SNR52 promoter, and all have a *TDH3* promoter-driven retron donor except for
297 the “no retron” system, which consists of a donor without any promoter or flanking retron elements.
298 All constitutive systems express Cas9 from the *TEF1* promoter and the RT from the *ADH1* promoter
299 (except for the no RT control), as well as the FHA fusion protein where indicated. Note that the donor
300 transcribed under the retron promoter but without any retron (no RT control) showed reduced editing,
301 suggesting that transcription through the donor is detrimental for plasmid-based template repair.

302 Despite the ~100-fold improvement in retron cDNA output from the HDV retron
303 over the RiboJ retron, this yielded only modest improvements in editing efficiency
304 (see 6, 12 and 18 generation time points, **Fig 2c**). We reasoned that hundreds or even
305 thousands of donor template might not be enough to saturate the edit locus with
306 template in each cell and that template concentration at the target site is limiting.
307 Therefore, we sought to improve the retron further by recruiting it directly to the site
308 of breaks via the LexA-FHA system. We first explored several methods of introducing
309 LexA repeat structures into a retron construct. Introducing two LexA inverted repeats
310 downstream of the donor DNA increased retron production (**Supp Fig 7**) which
311 manifested in improved editing efficiency and survival (**Supp Fig 8**). However, this
312 effect was independent of the expression of LexA-FHA (**Supp Fig 8**) suggesting that
313 the LexA structure itself was enhancing editing simply through improved retron RNA
314 stability rather than recruitment.

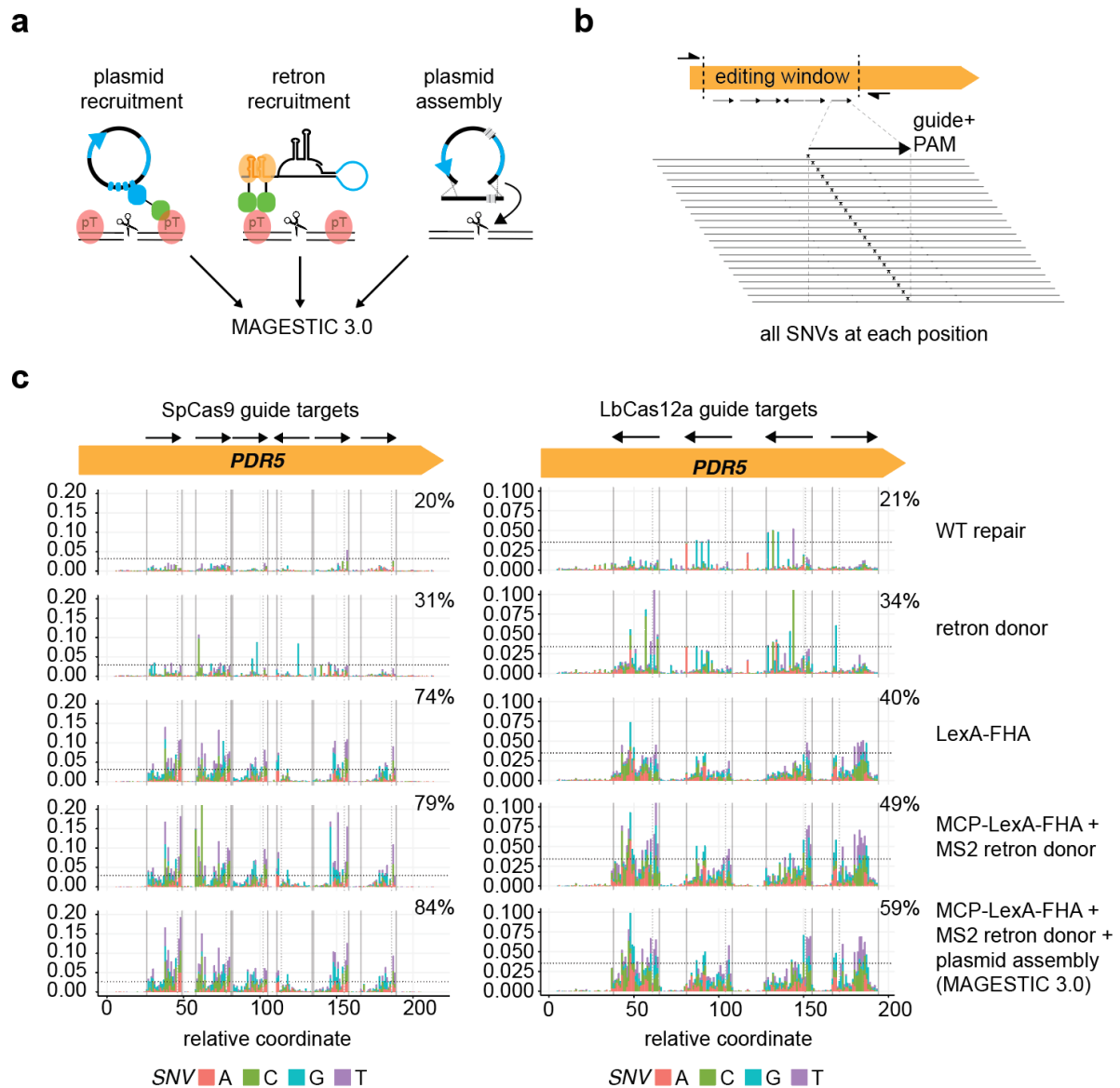
315 To explore another means of retron recruitment, we took advantage of the
316 RNA-DNA hybrid nature of the mature retron product as well as fact the 5' HDV
317 ribozyme remains to the retron transcript after cleavage, protecting the 5' end of the
318 retron RNA through strong secondary structure. We inserted a tandem repeat of MS2
319 stem-loop structures in the retron and constructed an MS2 coat protein (MCP)-FHA
320 fusion (**Supp Fig 9**). In the absence of HDR enhancement (no retron), editing at colony
321 formation was only ~10% (**Fig 2c**). Moving the guide-donor from a single-copy
322 Gen/Ars plasmid to a high-copy 2 μ plasmid alone boosted editing efficiency from 53
323 to 61%. Strikingly, the recruitment of the retron through the MCP-FHA fusion
324 dramatically improved editing efficiency, reaching over 75% at the colony formation
325 stage. Except for the no RT and no retron controls, NHEJ levels in all systems were
326 below 1%, and scaled inversely with improvements in HDR, such that the MCP-FHA
327 fusion showed <0.1% NHEJ indels at the target site (**Supp Fig 8**). While editing with

328 all systems eventually reach close to 100% after 18 generations, the increased donor
329 HDR efficacy results in the need for a shorter editing time course and thus reduced
330 variant skew as shown in **Fig 1** and **Supp Fig 4**. We next tested whether retron
331 recruitment could generate edits by further truncating the *ADE2* guide RNA with
332 mismatches to yield a 16mer, which should lead to little or no cleavage depending on
333 the target site¹⁷. In this condition, the MS2 retrons yielded 3% editing, while a retron
334 construct with the LexA-LexA stem loop with LexA-FHA expression yielded reduced
335 editing compared to a retron-only control (**Supp Fig 9**). We also tested prime editing¹⁸
336 with the same guide lengthened to a full 20mer and the donor edit encoded in the
337 pegRNA tail. This yielded extremely low levels of editing (<1%), suggesting that prime
338 editing is not effective in yeast (**Supp Fig 9**).

339

340 **Saturation genome editing with MAGESTIC 3.0**

341 Next, we explored combining all three systems (plasmid donor recruitment by
342 LexA-FHA, ssDNA retron donor recruitment by MCP-FHA, and plasmid assembly)
343 into a single, supercharged editing system termed MAGESTIC 3.0 (**Fig 3a**). To test
344 the ability of MAGESTIC 3.0 to dissect functional natural variants, we challenged it
345 with an assay designed to edit all potential SNVs across genomic regions. We chose
346 ~200 bp editing windows harboring 6 (SpCas9) or 4 (LbCas12a) non-overlapping
347 guide targets and designed libraries where each guide was paired with a panel of
348 donor DNAs to introduce all 3 SNVs at each position in the 20mer guide sequences
349 and NGG (SpCas9) or TTTV (LbCas12a) PAM sequences (**Fig 3b**). After colony
350 formation, on-target editing outcomes for MAGESTIC 3.0 and each of its sub-
351 systems were assessed by high-throughput sequencing of target region amplicons.
352 In the absence of HDR enhancement, each library exhibited ~20% editing efficiency.
353 This rose dramatically in the MAGESTIC 3.0 system to 84% for the SpCas9 library
354 and 59% for the LbCas12a library (**Fig 3c**). We note that these libraries harbor
355 differing levels of synthesis errors, with the LbCas12a library exhibiting slightly higher
356 levels with a greater fraction of guides with errors, likely explaining the lower editing
357 efficiency.



358

359 **Figure 3.** Saturation genome editing with MAGESTIC 3.0.

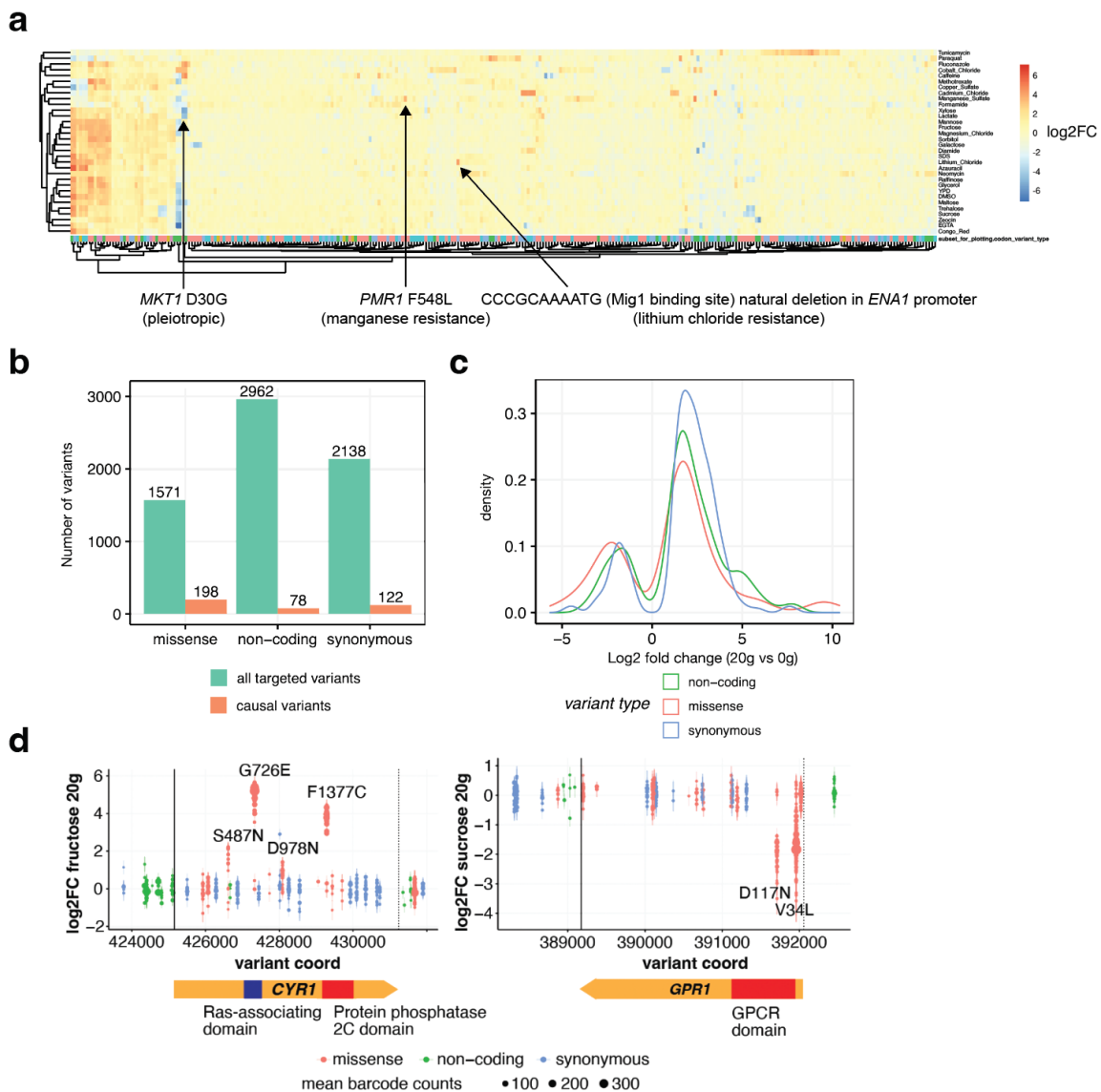
360 **a**, MAGESTIC 3.0 utilizes three major HDR-enhancing technologies, dsDNA plasmid donor recruitment
 361 via LexA sites on the plasmid and the LexA-FHA fusion protein, ssDNA retron donor recruitment via
 362 the MS2 system, and plasmid assembly. The use of an MCP-LexA-FHA protein enables simultaneous
 363 recruitment of plasmids and retron donor to edit sites.

364 **b**, Editing windows with a panel of non-overlapping guides were selected and all possible SNVs across
 365 the guide target region and PAM were engineered into the donor DNAs.

366 **c**, On-target editing rates were quantified by high-throughput sequencing of target region amplicons.
 367 The fraction of reads containing SNVs at each coordinate on the x-axis is plotted on the y-axis with
 368 stacked bars with the colors representing the SNV introduced. The arrows in the editing window signify
 369 the guide and its orientation relative to the target site, with Cas9 guides containing the PAM 3' of the
 370 arrow head (3' end of the guide), and LbCas12a guides containing the PAM 5' of the arrow tail (5' end
 371 of the guide).

372 **Dissecting quantitative trait nucleotides with MAGESTIC 3.0**

373 Finally, we used MAGESTIC 3.0 to edit and phenotype 6,671 variants residing in 112
374 quantitative trait loci (QTL) previously mapped across the genome for 32 conditions¹⁹.
375 As each locus contains one or more causal variants hiding amongst dozens to
376 hundreds of potentially benign variants, we used both SpCas9 and a protospacer-
377 adjacent motif (PAM)-relaxed version of LbCas12a to target the greatest possible
378 fraction of variants in each locus. We screened the variant libraries across the same
379 32 conditions previously tested in the QTL study¹⁹ by using liquid-based pooled
380 growth assays. We sequenced the barcode composition after 20 generations of
381 competitive growth and calculated log₂ fold changes relative to the initial 0-
382 generation time point for each barcode.



383

384 **Figure 4.** Dissecting causal variants residing in 112 QTL across 32 conditions with MAGESTIC 3.0.

385 **a**, A heat map depicting the log₂ fold abundance change for 398 variants which were found to be
386 causal in at least one condition (x-axis), across all 32 conditions (y-axis). Previously reported causal
387 variants in the *MKT1* and *PMR1* genes are highlighted along with a newly uncovered causal variant in
388 the *ENA1* promoter.

389 **b**, Overview of the total number of targeted variants versus the causal variants uncovered in this study
390 stratified by variant type.

391 **c**, The effect sizes for each type of variant are shown as a density plot. Overall missense variants tend
392 to have larger effect sizes than synonymous and non-coding variants.

393 **d**, Log₂ fold changes in barcode abundance after 20 generations of growth in fructose (left panel) and
394 sucrose (right panel) for variants in the *CYR1* and *GPR1* genes, respectively. Each point represents an
395 independently edited and barcoded lineage, and type of variant is color-coded. The size of each point
396 corresponds to mean barcode abundance at both 0- and 20-generation time points.

397 Our results recapitulated previously validated causal variants in these loci (*MKT1*,
398 *PMR1*) and revealed a complex genotype-phenotype map of hundreds of causal
399 natural variants across all loci, with missense variants enriched for effects over non-
400 coding and synonymous variants, as expected (**Fig 4**).

401

402 **Discussion**

403 Association-based studies have been highly successful in quantifying the polygenic
404 nature of most traits²⁰, yet the ability of these approaches to fully dissect the
405 mechanisms driving traits have major limitations²¹. Two prominent limitations involve
406 linkage disequilibrium, which leads to little or no recombination between individual
407 variants in close proximity on the same haplotype, and rare variants, which do not
408 rise to a high enough frequency to give sufficient statistical power for detecting
409 effects. Systematic perturbation approaches such as CRISPR screens have potential
410 to address these limitations and provide a major advance forward in our
411 understanding of complex traits²¹⁻²⁴, by enabling finer-grained dissection of
412 previously mapped QTL and GWAS loci and by discovery of functional variants in
413 previously undetected loci, such as causal variants that are rare or otherwise missed
414 by QTL/GWAS approaches^{1,25,26}.

415 Natural variants pose a significant challenge for CRISPR engineering as the
416 majority are single-nucleotide variants (SNVs). These SNVs must reside within the

417 target sequence of a guide RNA or its protospacer adjacent motif (PAM) to sufficiently
418 block CRISPR cleavage^{2,4}. Therefore, guides typically cannot be preselected (e.g.
419 based on predicted efficacy) due to limited PAM availability. Furthermore, the
420 mismatch tolerance of CRISPR nucleases (e.g. SpCas9) can result in repeated
421 cleavage of the donor template and the target site after editing and lead to significant
422 toxicity (i.e. low editing survival) as we demonstrated in this study. In addition,
423 comprehensive profiling of individual genetic variants at the whole-genome scale
424 requires both high efficiency and fidelity of editing as well as scalable and sensitive
425 approaches to characterize phenotypic effects. While some approaches excel in
426 some areas (e.g. high fidelity of SNV editing with prime editing¹⁸, high efficiency with
427 base editing²⁷, they tend to have drawbacks in other areas (e.g. lower editing
428 efficiency with prime editing, restricted edit types and target range with base editing).
429 In this study, we showed that enhanced HDR-based approaches have the potential
430 to solve these problems by simultaneously achieving higher efficiency, fidelity, and
431 superior variant representation. While budding yeast is well known for its
432 predisposition for higher HDR efficiency than most systems, we show that HDR with
433 the donor DNA template is still a major limiting factor in editing performance in the
434 yeast system. Therefore, the development of the MAGESTIC 3.0 system we outline
435 here provides a roadmap for more efficient harnessing of HDR for variant engineering
436 and functional screens in other systems and suggests that adapting the
437 improvements to retron donor DNA production and recruitment of retron donors to
438 DNA breaks outlined in this study will be fruitful in other species and cell lines.

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447 **Material and methods**

448 **Yeast strains**

449 For the comparison of different editing systems, we used the yKR61 strain, a
450 derivative of the DHY214/ BY-based wild-type strain where several detrimental alleles
451 have been repaired²⁸. For the NHEJ inhibition approach, we knocked out the *NEJ1*
452 gene in yKR61 by utilizing a guide RNA and donor DNA to delete the *NEJ1* open-
453 reading frame to yield yKR139, as previously described². To avoid potential
454 interaction between the *HIS3* marker used in our plasmid assembly approach and the
455 partial HindIII-mediated deletion at the *HIS3* locus present in the BY strain
456 background, we converted the HindIII allele in yKR61 to an entire deletion of the *HIS3*
457 ORF by introduction of a kanMX resistance marker to yield yKR650. For the retron
458 system, we used the ZRS111 strain previously described⁴.

459 **Natural variant guide-donor plasmids**

460 The guide-donor plasmids assayed in Fig 1 are shown in Supplementary Table S1.
461 The guide-donor cassettes were either amplified from previously isolated
462 MAGESTIC-edited clones or from randomly selected from a *RM11* strain natural
463 variant library² and cloned by Gibson assembly into pKR514, a 2 μ (high-copy) plasmid
464 harboring the *SNR52* promoter for guide RNA expression, a 4X tandem array of LexA
465 sites for donor recruitment, and the *FCY1* gene, which is utilized for guide-donor
466 plasmid counterselection after editing. The donors from these plasmids were then
467 used as a template for PCR to generate donor-guide retron constructs for Gibson
468 assembly into either the HHR retron backbone plasmid pKR901, or the RiboJ retron
469 backbone plasmid pKR998. All plasmids were confirmed by Sanger sequencing. The
470 pKR514-based guide donor plasmids were used for all non-retron based editing
471 systems, including the no HDR enhancement control condition. For the plasmid
472 assembly method, the pKR514-based plasmids were cleaved by HindIII, which cuts
473 the *HIS3* ORF in two places. We then amplified a fragment (pF78) spanning these
474 cleavage sites with ~200 bp of overlap on each side to promote in vivo plasmid
475 assembly. For on-target editing efficiency, each guide-donor plasmid was
476 transformed individually in separate transformations. For glucose editing, cells were
477 plated onto CSM-Ura-His agar plates and incubated at 30°C until colony formation.

478 In parallel, transformation aliquots were grown in liquid media to facilitate additional
479 liquid outgrowth passages for all targets and editing systems. For galactose editing,
480 transformation aliquots were first grown in CSM-Ura-His liquid glucose media for two
481 passages to select for transformants and establish time zero samples. For the editing
482 time course, 1.5 uL of culture was transferred to 98.5 uL of fresh media each day to
483 allow for 6 generations of growth in 96-well plates. Genomic DNA was prepared from
484 the cultures and the target sites were amplified for high-throughput Illumina
485 sequencing with 2 x 150 bp reads (Novogene). For the editing survival assays, the
486 plasmids were quantified and pooled together at equimolar ratios prior to
487 transformation. Primers were designed to amplify barcoded donor sequences on the
488 plasmid pools to quantify strain abundance by Illumina sequencing with 2 x 150 bp
489 reads (Novogene).

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491 **Data availability**

492 The raw sequencing data reported in this study are available at the SRA database
493 (<https://www.ncbi.nlm.nih.gov/sra/>) under BioProject accession number
494 PRJNA1067405.

495

496 **Code availability**

497 The scripts and codes used for data analysis in this study are available at GitHub
498 (<https://github.com/k-roy/MAGESTIC>).

499

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507 **Competing Interests**

508 K.R.R., J.D.S., J.E.H., R.P.S, and L.M.S. have filed a patent application based on the
509 MAGESTIC multiplexed editing system and the donor recruitment approach (U.S.
510 provisional application No. 62/559,493, Publication US20200270632A1). K.R.R.,
511 J.D.S., R.P.S, and L.M.S. have filed patent applications on the ribozyme-based
512 methods to enhance retron production (U.S. provisional application No. 63/214,197,
513 WIPO Publication WO2022272293A1) and the retron donor recruitment approach
514 (U.S. provisional application No. 63/214,196, WIPO Publication WO2022272294A1).
515 K.R.R. and L.M.S have filed a patent application based on the MAGESTIC 3.0 system
516 and the integrated plasmid removal system (U.S. provisional application No.
517 63/401,083).

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