[Title]

Impact of Essential Genes on the Success of Genome Editing Experiments Generating 3,313 New Genetically Engineered Mouse Lines

[Authors]

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Abstract

The International Mouse Phenotyping Consortium (IMPC) systematically produces and phenotypes mouse lines with presumptive null mutations to provide insight into gene function. The IMPC now uses the programmable RNA-guided nuclease Cas9 for its increased capacity and flexibility to efficiently generate null alleles in the C57BL/6N strain. In addition to being a valuable novel and accessible research resource, the production of 3,313 knockout mouse lines using comparable protocols provides a rich dataset to analyze experimental and biological variables affecting *in vivo* gene engineering with Cas9. Mouse line production has two critical steps – generation of founders with the desired allele and germline transmission (GLT) of that allele from founders to offspring. A systematic evaluation of the variables impacting success rates identified gene essentiality as the primary factor influencing successful production of null alleles. Collectively, our findings provide best practice recommendations for using Cas9 to generate alleles in mouse essential genes, many of which are orthologs of genes linked to human disease.

Introduction

The International Mouse Phenotyping Consortium (IMPC) systematically generates and phenotypes mouse lines harboring null mutations in protein-coding genes and prioritizes mouse-human orthologs[1,2]. To produce the majority of its null alleles, the IMPC implemented a deletion strategy to remove a critical region (one or more exons shared by all annotated full-length transcripts that when removed will introduce a frameshift and premature termination codon)[3]. Designs were intended to target protein-coding transcripts for nonsense-mediated

77 decay by introducing a premature termination codon >50-55 nt from the final splice acceptor[4].
78 While frameshifts can be achieved using single Cas9-mediated double-strand breaks repaired
79 by non-homologous end joining to introduce small insertions or deletions (indels), exon
80 skipping during splicing can restore the reading frame and partial gene function[5-7]. The deletion
81 approach mitigates the risk of restoring frame, simplifies founder screening, genotyping, and
82 quality control (QC), and resembles the embryonic stem cell-based approaches used for
83 decades to produce null alleles, albeit without selection cassette insertion. To evaluate
84 variables affecting mutant mouse production, we analysed data from 4,874 production
85 attempts on 3,973 unique genes from eight different centres (**Supplementary Table 1**)
86 recorded in the IMPC's production tracking database (GenTaR, formerly iMITS; downloaded
87 2020 Oct 11, www.gentar.org/tracker/#/). Experimental parameters as well as genomic and
88 biological characteristics of targeted genes were evaluated for their effects on mouse line
89 production success.
90
91 **Results**
92 *Experimental parameters affecting mouse line production*
93 Four experimental parameters were assessed for their effects on success: Cas9 delivery
94 method, number of guide RNAs (gRNAs) used, deletion size, and number of founders selected
95 for breeding. We also evaluated the effect of changing parameters in repeated production
96 attempts. Null deletion alleles were generated using Cas9 with guides flanking a critical region
97 containing one or more critical exons[3]. Gene editing reagents were delivered by microinjection
98 (pronuclear or cytoplasmic)[8,9] or electroporation[10-13] to target specific genes in C57BL/6N

zygotes (**Supplementary Fig. 1**). Among unique gene production attempts (*i.e.,* each gene represented only once; **Supplementary Table 2**), the founder rate, measured as the ratio of founders obtained to the number of embryos treated and transferred, was significantly higher using either cytoplasmic injection or electroporation compared to pronuclear injection of zygotes (**Fig. 1a**; $p < 2.22 \times 10^{-16}$, Wilcoxon rank sum test) with no difference between cytoplasmic injection and electroporation (**Fig. 1a**; p=0.26). When we excluded experiments from which no founders were produced, GLT rates by these three delivery methods were all greater than 95% (95.4%, 96.5% and 97.3%, respectively) with no significant difference between them ($p > 0.15$ in pairwise comparisons with Pearson chi-square).

To mitigate the potential risk of low activity or inactive Cas9-guide combinations, several experiments used four guides, two 5' and two 3' flanking the target site. There was no difference in founder rate production (**Fig. 1b**; p=0.82 Wilcoxon rank sum test) or GLT rates of genes edited with two or four guides (GLT 96.8% and 95.7%, respectively, p=0.096 Pearson chi-square). Even with no apparent efficiency gain using additional guides, it is possible that some guides may perform better than others *in vivo* and that inactive or low activity guides may contribute to experimental failure. Using two guides instead of three for four may reduce the small risk of off-target mutagenesis [14-17].

To determine if the size of the deleted region influenced success, we partitioned the expected deletion size defined by the maximum distance between flanking guides into six bins with approximately equal numbers of deletion attempts and observed no significant difference in

121 founder rates (p=0.34, Kruskal-Wallis test for comparing medians of six groups; **Fig. 1c**) or GLT
122 rates (p=0.668 Kruskal-Wallis test; data not shown) for deletion sizes below 1,400 bp. The
123 relatively small number of attempts that attempted to delete segments longer than 1,400 bp
124 precludes conclusive statistical analysis of deletions above that size using this dataset, however
125 decreased efficiency with increased deletion size has been reported[18].
126
127 We next assessed whether the number of founders bred affected GLT rate. GLT breeding
128 founders, regardless of production methods used, resulted in efficient germline editing and
129 breeding a single founder provided a >95% chance of success. The overall high GLT rate was
130 marginally improved by breeding up to three founders, but there was no advantage to breeding
131 more than three founders (**Fig. 1d**).
132
133 While nearly 70% of first Cas9 production attempts were successful, we wanted to understand
134 why 30% of Cas9 production attempts failed to produce GLT of the desired null allele. Many of
135 these experiments were repeated (with or without changing parameters) and only 26.3% of
136 second attempts and 16.5% of third attempts were successful (**Fig. 2a, Supplementary Table 3**).
137 We analysed the set of repeated attempts for 401 genes to determine what, if any, changes to
138 experimental parameters improved success rates. We compared success rates of consecutive
139 attempts for each gene with repeated attempts to test an association between GLT and each of
140 four experimental parameters (delivery method, number of guides, guide sequence, targeted
141 exon(s)). Changing at least one guide sequence, irrespective of whether the critical region
142 targeted changed, showed a statistically significant improvement in GLT rates (54% with no

143 change to guide sequence *cf*. 68.8% with a changed guide sequence, p=0.01 chi-square; **Fig.**
144 **2b**). Changes to other parameters did not significantly affect success rates. A logistic regression
145 model for GLT status conditional on changes in experimental factors also revealed that only
146 changing the guide sequence improved GLT rates (p=0.048, **Table 1**). To evaluate whether these
147 experimental factors interacted to affect GLT rates, we fit an elastic net logistic regression
148 model which performs variable selection and accounts for collinearity through penalization.
149 These regression models provided a 57.58% classification accuracy for GLT status which is only
150 slightly better than a random guess, thus interactions among different parameters could not
151 predict improved experimental success. Therefore, when experiments fail, a significant
152 decrease in success rates after the first attempt should be expected, but changing the guide
153 sequence may improve the likelihood of generating the desired allele compared to making no
154 changes or changing the other parameters examined.
155
156 *Biological parameters affecting successful mouse line production*
157 In considering biological variables that could influence founder and GLT rates, we hypothesized
158 that targeting essential genes (genes whose function are necessary for cell or animal viability[19])
159 could negatively affect founder rates due to the high mutagenic efficiency of Cas9. In support of
160 this, the founder rate obtained for cellular non-essential genes was significantly higher than for
161 cellular essential genes ($p < 2 \times 10^{-16}$ Wilcoxon rank sum test; **Fig. 3a**). Knockout mouse lines
162 (genes for which knockout alleles were successfully produced) were assessed for homozygous
163 viability of the targeted allele by the IMPC[20]. Alleles identified as homozygous lethal after
164 production, so called lethal genes which include both cellular and developmental essential

165 genes[19], had significantly lower founder rates than alleles of non-lethal genes ($p=2.2 \times 10^{-11}$
166 Wilcoxon rank sum test; **Fig. 3b**). Similarly, the birth rates for essential and lethal genes during
167 founder production attempts were lower than that for non-essential and non-lethal genes
168 (**Supplementary Fig. 2**). Lower birth rates may reflect a loss of mutant embryos during
169 gestation or shortly after birth due to effects of the introduced mutation. When repeated
170 attempts were classified by gene essentiality[19] 72.2% of first attempts were successful for
171 cellular non-essential genes compared to 52.6% for cellular essential genes (**Fig. 3c**). In addition,
172 a larger percentage of cellular non-essential genes than essential genes were successful for
173 each subsequent attempt, albeit with decreased success rates for each subsequent attempt for
174 the same gene.
175
176 There was a small, but not statistically significant, difference in GLT rates between cellular non-
177 essential and cellular essential genes (96.8% and 95.2%, respectively; p=0.15 Pearson Chi-
178 square) among experiments that generated founders. This may be a result of fewer founders
179 obtained and therefore fewer bred for cellular essential genes compared to non-essential
180 genes. Alternatively, this may have occurred if haplo-insufficient genes were targeted and
181 produced mosaic founders that could not propagate mutant progeny that reached genotyping
182 age.
183
184 To assess the influence of additional biological variables on founder and GLT rates, we applied a
185 general linear model (GLM) to test the association of several factors including embryonic
186 expression (GEO GSE11224)[21], observed/expected loss-of-function (o/e) score, probability of

187 loss-of-function (pLI) score[22], chromosome position, histone methylation and acetylation (as a
188 proxy for chromatin accessibility), cytogenic banding (a second proxy for chromatin
189 accessibility), and human ortholog disease annotation in the Online Mendelian Inheritance in
190 Man (OMIM) database [23] (**Supplementary Table 4**). When gene essentiality was excluded from
191 analysis, only embryonic expression was significantly associated with low founder rates (odds
192 ratio (OR)=0.95, p=0.011). However, when essentiality was included, it was the only predictor of
193 experimental failure (OR=0.89, p=2.06 x 10^{-11}; **Fig. 3d**, **Table 2, Supplementary Table 5)**. The
194 proportion of essential genes in each experimental parameter grouping did not vary in a way
195 that may have confounded the results of those analyses (**Supplementary Tables 6** and **7**).
196
197 After successful production of a founder, subsequent GLT rates were very high (**Fig. 1d**). Among
198 74 genes for which the reason for GLT failure was readily determined the majority failed GLT
199 because founders died before breeding, founders failed to produce progeny, or founders
200 propagated only wild-type progeny that reached genotyping age (**Fig. 4**). The remaining genes
201 failed primarily because the desired null allele could not be validated in N1 progeny (*e.g.,*
202 partial deletion or no frameshift recovered). Infertile founders and transmission of only wild-
203 type progeny might be due to founder mosaicism with only wild-type cells contributing to the
204 germline or mutation effects on gametogenesis with only wild-type cells able to produce
205 functional germ cells. Moreover, transmission of only wild-type alleles could indicate the
206 presence of haplo-insufficient alleles for some genes causing mutant progeny to die before
207 genotyping. These data support the hypothesis that a substantial subset of mutations may fail

208 GLT due to negative effects on viability or fertility and are consistent with the significant effect
209 of gene essentiality on founder production (**Table 2**).
210
211 **Discussion**
212 The analysis of our large, multi-centre dataset identified several variables that affect Cas9
213 mutagenesis success and provides the basis for recommendations for genome editing in mouse
214 zygotes. For deletion alleles, Cas9 ribonucleoprotein (RNP) electroporation is the most
215 accessible and scalable method, providing equivalent performance to cytoplasmic
216 microinjection of Cas9 mRNA and gRNAs. GLT rates are high for Cas9 generated founders and
217 there is no apparent advantage to breeding more than three founders. Most attempts are
218 successful the first time, but for attempts that fail to produce founders, changing guide
219 sequences may be beneficial when repeating production for a given gene. The activity of Cas9-
220 guide RNA combinations can be assessed *in vivo* (*e.g.*, by zygote treatment, *in vitro* culture, and
221 blastocyst genotyping[24]). Exploring the known biology of the gene, such as predicted effects on
222 phenotype (*e.g.*, disease association of human ortholog), viability, and/or fertility as well as
223 essentiality scores from publicly available sources (as in [19]), can assist with experimental design.
224 Successful targeting of essential genes may require methods that promote founder
225 heterozygosity (*e.g.,* lower Cas9 concentrations) or mosaicism (*e.g.,* delivering reagents to one
226 blastomere of a 2-cell embryo by microinjection). In some cases, conditional alleles[25,26] may be
227 required instead of constitutive knockout alleles to generate a mouse line in which homozygous
228 loss of function may be examined in specific tissues or developmental stages.

229 The effect of cell essentiality or lethality can apply to generating lines with disease-associated
230 variants such as single nucleotide variants. Given the high proportion of essential genes among
231 human disease genes[19,27], generating mouse models of human disease may require preserving
232 one copy of the wild-type allele, that is producing heterozygous founders or conditional variant
233 alleles, to establish a new mouse line. Overall, the use of Cas9 is a robust and flexible method to
234 generate gene-edited mouse lines. Improving model production success rates by applying our
235 recommendations can reduce the number of animals used to generate new models, consistent
236 with the ethical principles of the 3R's[28].

[Materials & Methods]

Mouse strains: All null allele mouse lines were produced in the C57BL/6N strain background available from Charles River or the Jackson Laboratory (**Supplementary Methods Table 1**). All live animal protocols conformed to the applicable standards for the ethical use of animals in research at the respective facilities with detailed ethics statements found in **Supplementary Methods Table 1** for each production centre. Animal welfare was regularly monitored.

Allele design: Null alleles were designed such that the mutations resulting from Cas9 endonuclease activity and double-strand break (DSB) repair caused a reading frameshift in protein-coding transcripts and introduced a premature stop codon. This required the identification of a critical region for each targeted gene. A critical region was defined as one or more exons that when frameshifted or deleted resulted in a frameshift in the open reading frames of all known full-length protein-coding transcripts (per Ensembl build 38)[3]. For the majority of designs, the premature stop codon was predicted to be in the first half of the protein-coding open reading frame and to target transcripts for nonsense-mediated decay[4,29]. Such alleles are considered presumptive nulls. Three major categories of alleles were evaluated in this study. Exon deletion (exdel) alleles resulting from NHEJ-mediated repair of Cas9-induced DSBs flanking the exon(s) within a gene's critical region. Intra-exon deletion (intra-exdel) alleles resulting from repair of Cas9-induced paired DSBs within a single exon in the target gene's critical region, used for example, when all exons of a gene are in the same frame precluding an exon deletion strategy or for single exon genes. Inter-exon deletion (inter-exdel) alleles resulting from repair of Cas9-induced paired DSBs in two or more different (often sequential) exons in a gene's critical region, used for example, to delete a functional domain that spans

multiple exons or when specific gRNAs in appropriate intron locations could not be identified. Alleles that did not result in a frameshift failed quality control (QC) metrics and were not maintained. Exceptions were made when the deletion was still in frame, but either removed critical protein domains (*e.g.*, zinc fingers from a zinc-finger protein) or a substantial fraction of the protein-coding sequence; these alleles were also deemed to be presumptive null allele.

sgRNA selection: Guide RNA (gRNA) spacer sequences were selected using either the CRISPR design tool[30], the Wellcome Sanger Genome Editing (WGE) Tool[31], CRISPOR[32], CRISPRTools[33], CHOPCHOP[34], or FORCAST[35]. Suitable gRNA spacer sequences were selected to minimize predicted off-target mutagenesis using specificity scores >65 when available and/or sequences with at least 3 mismatches for all predicted off-target sites. Multiple guides were used to generate deletion alleles, with either two, three, or four guides (2G, 3G, or 4G, respectively) with two or more guides flanking the target critical region. In the 3G approach, the middle guide could be within an exon and result in the deletion in conjunction with either the upstream or downstream guide, removing either the splice acceptor or donor from the critical region, resulting in mis-splicing and the introduction of a frameshift.

sgRNA synthesis: sgRNAs were synthesized by either subcloning sgRNA spacer sequences and *in vitro* transcription (plasmid-IVT), PCR and *in vitro* transcription (PCR-IVT)[36], gBlock synthesis and *in vitro* transcription (gBlock-IVT), or by primer extension and *in vitro* transcription (PE-IVT)[36]. Alternatively, sgRNAs were purchased from commercial suppliers. See **Supplementary Methods Table 1** for centre-specific reagent details.

PCR-IVT: DNA templates for PCR-IVT were produced using overlapping oligonucleotides in a high-fidelity PCR reaction[37] or using a plasmid template (Addgene #42230[38]) and appropriate

primers[36]. PCR amplicons were purified using the Monarch PCR & DNA cleanup kit (New England BioLabs T1030) or the QIAQuick PCR purification kit (Qiagen 28104) and used as a template for *in vitro* transcription of the sgRNA with the T7 MEGAshortscript™ Kit (ThermoFisher AM1354).

Plasmid-IVT: Overlapping oligonucleotides with *Bsa*I appendages to facilitate standard sticky ended cloning into a T7 expression plasmid (a kind gift from Sebastian Gerety, based upon[39]) were purchased annealed. Alternatively, annealed oligonucleotides were cloned into plasmid DR274 (Addgene #42250[40]). Plasmid DNA was extracted using the QIAGEN Plasmid Plus 96 Kit (Qiagen 16181) and guide cloning confirmed by Sanger sequencing. The DNA was then linearized and used as a template for T7 RNA *in vitro* transcription using the T7 MEGAshortscript™ Kit (ThermoFisher AM1354) or Thermo T7 RNA polymerase (TOYOBO, TRL-201).

gBlock-IVT: sgRNAs were synthesized directly from gBlock® DNA (Integrated DNA Technologies) templates containing the T7 promoter using the HiScribe™ T7 High Yield RNA Synthesis Kit (New England BioLabs E2050) following manufacturer's instructions for sgRNA synthesis.

PE-IVT: The EnGen sgRNA Synthesis Kit (New England BioLabs E3322) was used for PE-IVT per the kit protocol, but with incubation at 37ºC for 60-90 minutes prior to DNAse treatment.

For some 3G and 4G designs, up to two primers (*e.g.* both upstream gRNAs or both downstream gRNAs) were pooled at appropriate final concentrations before PCR or PE-IVT.

After *in vitro* transcription, sgRNA was purified using the RNA Clean & Concentrator-25 (Zymo Research R1017) or the MEGAclear Transcription Clean-Up Kit (ThermoFisher AM1908). All samples were analyzed by Nanodrop to determine A260/280 and A260/230 ratios (≥1.9 to pass

quality control). The integrity and size of sgRNA was assessed by agarose gel electrophoresis, Agilent Bioanalyzer, Agilent RNA Tape or the Qiaxcel Advanced System (RNA QC V2.0). Synthesized sgRNAs were stored at -80ºC in elution buffer or stored as ammonium acetate precipitates in ethanol at -20ºC. Before use, sgRNAs were either thawed on ice or pelleted, air dried, and resuspended in RNAse-free MI buffer.

Cas9: Cas9 mRNA was purchased (**Supplementary Methods Table 1**) or transcribed in-house[41]. Cas9 protein was purchased from commercial suppliers. See **Supplementary Methods Table 1** for centre-specific reagent details.

Injection mix preparation: Injection mixes were prepared essentially as previously reported[36] with or without filtration prior to injection. For mRNA microinjection, injection mixes consisted of Cas9 mRNA and sgRNA in microinjection buffer (**Supplementary Methods Table 1)**. Concentrations for each production attempt are shown in **Supplementary Table S8**. For Cas9 protein microinjection, Cas9 ribnucleoprotein (RNP) complexes were produced by mixing the Cas9 protein with sgRNA at 5X the concentration shown in **Supplementary Methods Table S1** in RNP injection buffer and incubating at 37ºC or room temperature for 10 minutes. The RNP mix was then diluted with 4 volumes of RNP injection buffer prior to injection. See **Supplementary Methods Table 1** for centre-specific reagent details.

Electroporation mix preparation: Electroporation mixes were prepared essentially as previously reported[11-13,36]. Electroporation mixes consisted of Cas9 protein and sgRNA in RNP electroporation buffer (**Supplementary Methods Table 1**) at 2X the concentrations shown in **Supplementary Table S8**, incubated at 37ºC or room temperature for 5-15 minutes, and placed on ice until electroporation. Immediately before electroporation, RNP was diluted with an equal

volume of Opti-MEM (ThermoFisher 31985062). See **Supplementary Methods Table 1** for centre-specific reagent details.

Generation of embryos by mating: C57BL/6N female mice, 3-6 weeks old, were injected with 5 IU/mouse of pregnant mare serum, followed 46-48 hr later with 5 IU/mouse of human chorionic gonadotropin. The females were then mated overnight with C57BL/6N males. Fertilized oocytes were collected from females with copulatory plugs the following morning at 0.5 days post-coitum (dpc). Oviducts were dissected and cumulus masses from these were released and treated with hyaluronidase. Fertilized 1-cell embryos were selected and maintained at 37ºC in media prior to microinjection or electroporation.

Microinjection of Cas9 reagents: The number of embryos injected and the injection route (pronuclear or cytoplasmic) for each experiment is in **Supplementary Table S8**. Pronuclear microinjections were performed following standard protocols[8,42]. Cytoplasmic injections were performed essentially as in [9]. Visible movement of the cytoplasm indicated successful injection. Injected zygotes were transferred into pseudopregnant females (see **Supplementary Methods Table 1**) on the afternoon of the injection or after overnight culture (recorded for each production attempt in **Supplementary Table S8**), with 12-15 or 20-28 zygotes per unilateral or bilateral transfer into pseudopregnant females, respectively.

Electroporation of Cas9 reagents: Electroporation was performed essentially as described[11-13,36]. At some centres, zygotes were briefly treated with Acid Tyrode's solution (Sigma-Aldrich T1788). After acid treatment, embryos were rinsed at least 3 times with the final rinse in Opti-MEM. For electroporation, embryos were transferred into a 1:1 mixture of Cas9 RNP and Opti-MEM or Opti-MEM when RNP were formed in Opti-MEM. For each production attempt,

electroporation pulses are in **Supplementary Table S8**. After electroporation the embryos were rinsed and transferred into pseudopregnant recipients the same day or after overnight culture (as recorded for each production attempt in **Supplementary Table S8**). Centre-specific details for buffers used are in **Supplementary Methods Table 1**.

Genotyping: Genomic DNA was prepared from ear punch or tail biopsies of two- to three-week-old pups (see **Supplementary Methods Table 1** for reagents) using commercial kits or previously described protocols[43,44]. DNA was amplified by standard end-point PCR or quantitative PCR (qPCR). End-point PCR assays were designed to produce differently sized amplicons. To detect wild-type alleles, one primer was designed outside of the deletion target sequence and the second primer designed within the deletion target sequence such that amplicons are only produced from wild-type alleles. To detect deletion alleles, primers were designed to flank the predicted deletion junction. Amplification can result in two amplicons – a shorter amplicon representing the deletion allele and a larger amplicon representing the wild-type allele, if PCR conditions allow the amplification of the larger amplicon. Three-primer designs use a common primer outside of the deletion for both amplicons. PCR products were visualized using the Caliper LabChip GX system, QIAxcel Advanced, or agarose gel electrophoresis. Sequences are available upon request from the relevant production centre.

In some cases, gene-specific 'loss of WT allele' (LoA) qPCR assays were designed to the region of the genome predicted to be deleted[45,46]. Deletion alleles will not amplify a product at the target site such that homozygous or hemizygous X-linked male deletions would have a copy number of 0, heterozygous a copy number of 1 and mosaic animals a copy number between 1 and 2 for autosomal alleles or between 0 and 1 in hemizygous X-linked alleles in males. These assays

allowed estimation of the level of mosacism in founder animals. Mice showing the greatest loss of allele were selected for breeding to confirm germline transmission. Sequences for loss-of-allele assays are available upon request from the relevant production centres.

Once germline transmission was confirmed, mice were genotyped with either end-point PCR or probe-based LoA assays. Seep **Supplementary Methods Table 1** for centre-specific genotyping methods.

Germline Transmission Test Breeding: Founders born from microinjection or electroporation experiments that carried the desired allele based on genotyping results were pair-mated to C57BL/6N mice. N1 pups were screened with the same genotyping assay used to identify founders. Deletion amplicons from deletion-positive N1 mice were subjected to Sanger sequencing (with or without subcloning) and other occasionally other quality control measures.

Copy number assessment: When warranted, to assess whether the excised genomic fragment from deletion alleles re-inserted into the genome, DNA from N1 mice was purified using the NucleoSpin Tissue Kit (Machery-Nagel 740453) and subjected to digital droplet PCR (ddPCR) at The Centre for Applied Genomics (Toronto, Canada), the Mary Lyon Centre (Didcot, UK), or the Mouse Biology Program (University of California, Davis). The ddPCR assays were designed such that the amplification primers and probes were entirely contained within the target deletion fragment. For heterozygous N1 mice, a copy number equal to 1 (+/-0.2) was considered a pass; for hemizygous X-linked male mice, a copy number of 0 to 0.2 was considered a pass.

Data download and filtering: A complete data set of Cas9-mediated mouse production attempts was downloaded on October 11, 2020 from the International Mouse Phenotyping Consortium production tracking database (formerly iMITS and now GenTaR; 'Cas9 Micro-

Injection Excel download'). This data included all Cas9-based production attempts as of that date. A production attempt was defined as the treatment of embryos to introduce Cas9 and guide RNAs to direct genome editing, subsequent embryo transfer, birth and screening of pups born from the embryo transfer, and subsequent breeding of mutant founders to obtain germline transmission of the desired edited allele. The data was filtered to remove attempts labeled as "private", as "experimental", or producing an allele other than a null allele, those with a status "Microinjection in Progress", embryo transfer day of "Next Day", none or >1000 embryos injected, incomplete information (e.g. number of founders not set, incomplete quality control information), and/or attempts that targeted non-protein coding genes. These data were further limited to attempts from production centres that had reported germline transmission for at least 50 unique genes for each of one or more of the analyzed methods (Cas9 mRNA pronuclear microinjection, Cas9 mRNA cytoplasmic injection, Cas9 RNP electroporation). This comprised the complete data set for analysis (**Supplementary Table 8**).

To define the set of unique gene experiments (i.e., each gene represented only once in the data set), the earliest attempt with germline transmission of the desired allele (Status = Genotype confirmed) for successful genes or the latest unsuccessful attempt (Status = Micro-injection aborted) was kept so that each gene was represented by a single attempt. If all attempts had a status of "Founder obtained", the most recent was kept. However, if no attempts for a given gene were successful in this filtered dataset, the larger IMPC dataset was queried to see if a successful attempt existed in the pre-filtered dataset (e.g., at another IMPC production centre or as the result of technology development activities at a given centre). Successful production at another centre or through technology development activities could have resulted in aborting

the production attempt in our filtered dataset, rather than failure of a complete experiment, or that technical issues rather than the parameters studied here resulted in failure, so these attempts were excluded from analysis.

For repeat attempt analysis, all attempts at the same production centre for genes that had more than one attempt were identified. This data set was then filtered to remove attempts in progress (Status = “Microinjection in progress” or “Founders obtained”). The remaining attempts were sorted in chronological order by microinjection [electroporation] date. If the first attempt for a given gene was successful, the set of attempts for that gene was removed from the repeat attempt analysis. Similarly, if an attempt was aborted within 6 weeks of a successful germline transmission attempt, it was removed since it may have been aborted because germline transmission had already been obtained, rather than having “failed”. Finally, if there was no GLT in any attempt at one centre, but successful GLT at another centre, the set of failed attempts was removed from the repeat dataset. The resulting data set comprised the repeat dataset (**Supplementary Table 3**).

Data Annotation: Genes targeted for mouse line production attempts in **Supplementary Table 2** were annotated with derived parameters including bins for Cas9 mRNA and protein concentration, gRNA cut sites and predicted deletion sizes, percentage of embryos that survived to transfer of those treated (injected or electroporated), birth rate (number of pups born divided by embryos transferred), founder rate (number of founders born divided by embryos transferred), number of founders selected for breeding. Repeat attempts (**Supplementary Table 3**) were annotated with whether the Cas9 type (mRNA vs. protein), amount of Cas9, delivery of reagents (injection versus electroporation), or gRNA locations

435 (sequences) changed between sequential attempts. All filtering and annotation of the data was
436 performed in Python3.8.5 using packages numPy1.2.1[47] and pandas1.2.2[48].
437 Genes for each attempt were annotated (**Supplementary Table 4**) with their viability (as
438 annotated at the IMPC – viable or homozygous lethal), human orthologs and cell essentiality of
439 human orthologous genes[19], embryonic expression (GEO GSE11224)[21], length, GC content,
440 number of CpG sites, and CpG percentage (**Supplementary Table 2**). The human orthologs'
441 probability of being loss-of-function intolerant (pLI) and observed / expected (oe) mutation rate
442 was retrieved from gnomAD[22]. Additional annotations were added for analysis of biological
443 variables affecting success. Annotation details are in **Supplementary Table 2**.
444 **Statistical Analysis:** The primary outcomes were the founder rate and the germline
445 transmission status. The founder rate had a right-skewed distribution with a range [0,0.5].
446 Hence, comparisons of the founder rate across different categories of biological or
447 experimental factors were conducted using nonparametric tests. For pairwise comparisons, the
448 Wilcoxon rank sum test[49] was used and when there were more than two categories the Kruskal-
449 Wallis test[50] was employed. The biological factors considered in the comparisons were the gene
450 essentiality (essential versus non-essential) and gene lethality (lethal versus non-lethal). The
451 experimental factors were the delivery method (three categories), number of gRNAs used (2
452 versus 4), deletion size (six categories), and number of founders selected for breeding (four
453 categories). Since the GLT status is binary (yes versus no), comparisons of the GLT rate
454 (proportion of genes with GLT) across different categories of biological or experimental factors
455 were performed using the Pearson chi-square test[51]. In the case of multiple pairwise
456 comparisons, correction for multiple testing was done using Holm's method[52]. Evaluation of

success of repeated attempts was based on descriptive summaries, mainly calculation of relevant proportions. The assessment of the impact of changing experimental factors to the success of gene editing in repeated attempts was conducted using logistic regression with the GLT status as the binary response and changes in the delivery method (change versus no change), number of gRNAs used (decrease, no change, increase), deletion size (change versus no change) and number of founders selected for breeding (change versus no change) as categorical covariates. All statistical analyses were performed using the R statistical programing software[53], along with the packages `ggplot2`[54] for figures, `tidyverse`[55] for data manipulations and `effects`[56,57] for effect plots.

The general linear models of biological variables were fit using the glm function in the R 3.6.2 native stats package (https://rdocumentation.org/packages/stats/versions/3.6.2) using the factors in **Supplementary Table 4** and with founder rate as the dependent variable.

All code can be found at https://github.com/The-Centre-for-Phenogenomics/IMPC-Cas9-Production.

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664

665 **Table 1.** Logistical regression model for GLT status between production attempts conditional on
666 experimental parameters

Predictors	Odds Ratio	CI	p-value
(Intercept)	1.18	0.96-1.47	0.136
ΔDelivery method	0.74	0.45-1.21	0.222
ΔDecrease no. guides	1.88	0.92-4.07	0.092
ΔIncrease no. guides	0.86	0.37-2.03	0.731
ΔGuide sequence	1.88	1.02-3.58	0.048
ΔTarget exon	1.22	0.44-3.58	0.709

Observations: 498

667

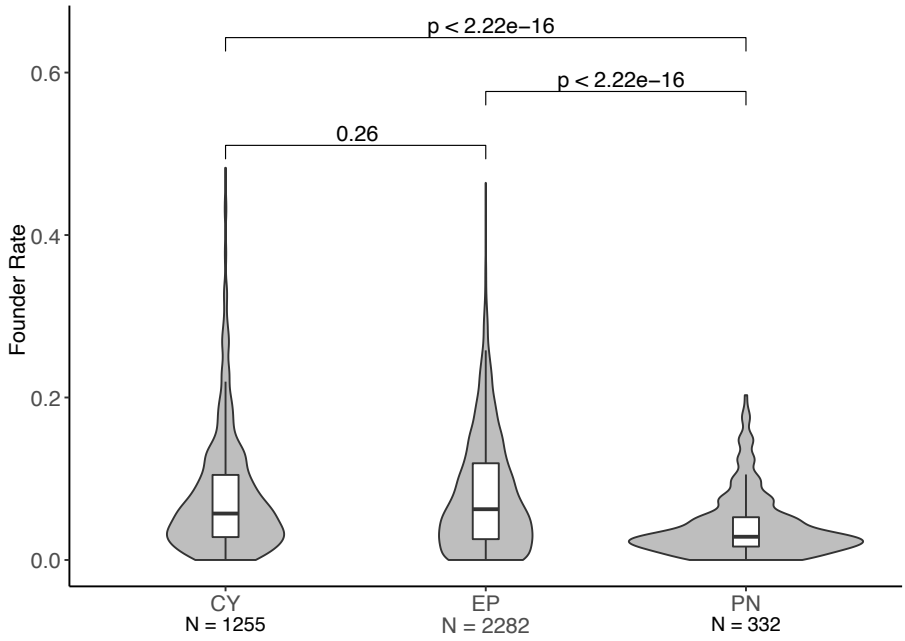
668 **Table 2.** General linear model for successful founder production conditional on biological
669 factors annotated for a gene

Variable	Without Essentiality		With Essentiality	
	Odds Ratio	p-value	Odds Ratio	p-value
(Intercept)	2.50	2.0×10^{-16}	2.52	2.22×10^{-16}
Essential	NA	NA	0.89	2.06×10^{-11}
Embryonic expression	0.95	0.011	0.97	0.219
pLI score	0.97	0.101	0.97	0.183
o/e score	1.00	0.987	1.00	0.887
Chromosome position	1.01	0.647	1.01	0.653
Acetylated gene	0.98	0.193	0.99	0.450
Methylated gene	1.03	0.137	1.01	0.383
Gram positive stain	1.00	0.938	1.00	0.891
OMIM annotation	0.99	0.412	0.98	0.220

Observations: 3209

670

671 a



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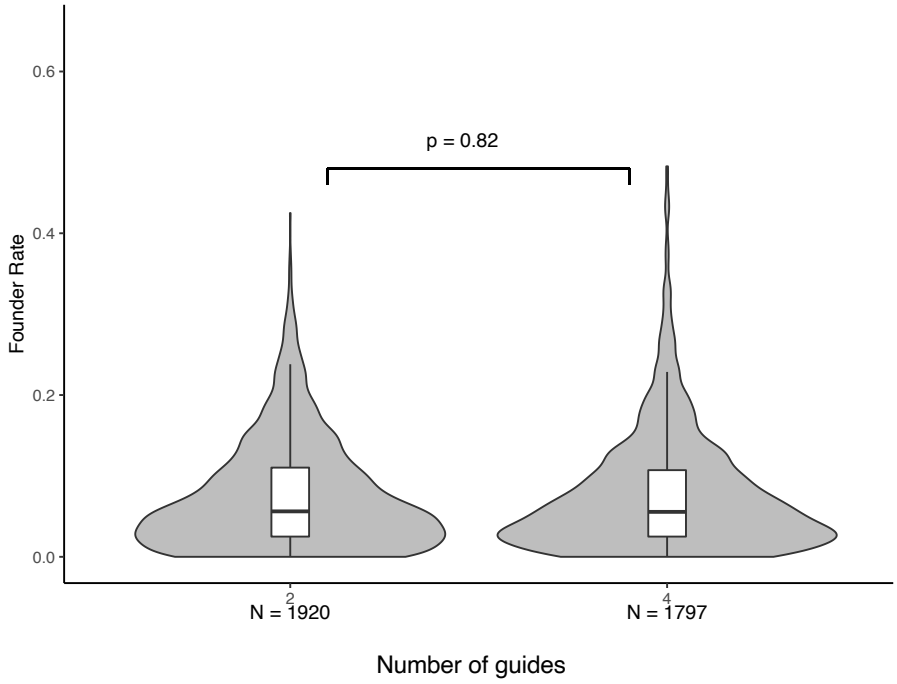
673 b



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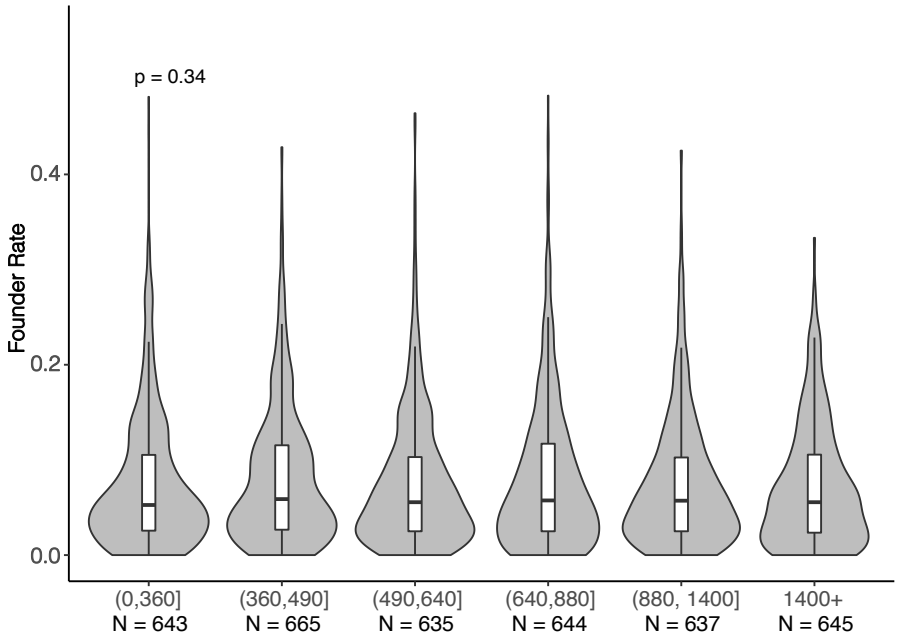

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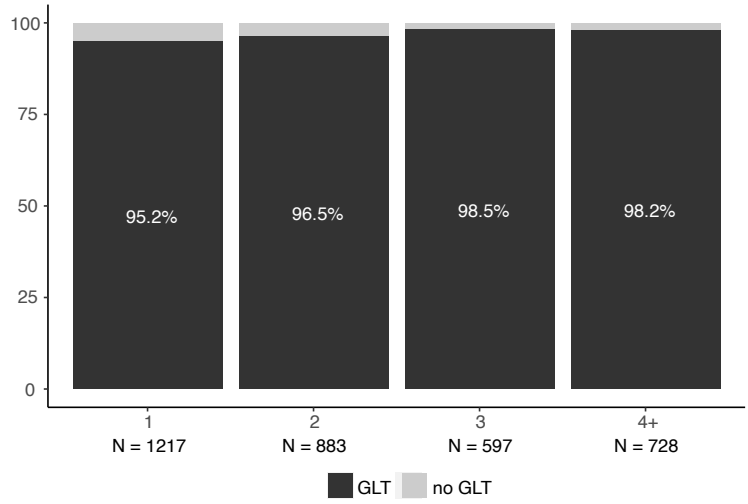


679
680 **Figure 1.** Experimental parameters affecting Cas9-mediated mutant mouse line production. **a**.

681 Founder rates from experiments with different methods of reagent delivery. CY, cytoplasmic

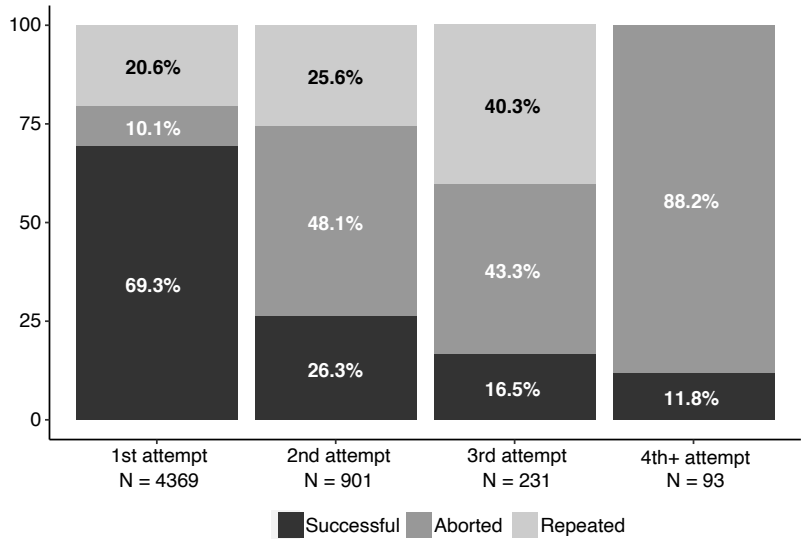
682 injection; EP, electroporation; PN, pronuclear injection. **b**. Founder rates from experiments

683 using two (2) or four (4) guide RNAs designed to produce deletion alleles. **c.** Founder rates from

684 experiments with Cas9 guide RNAs designed to delete different sizes of critical regions

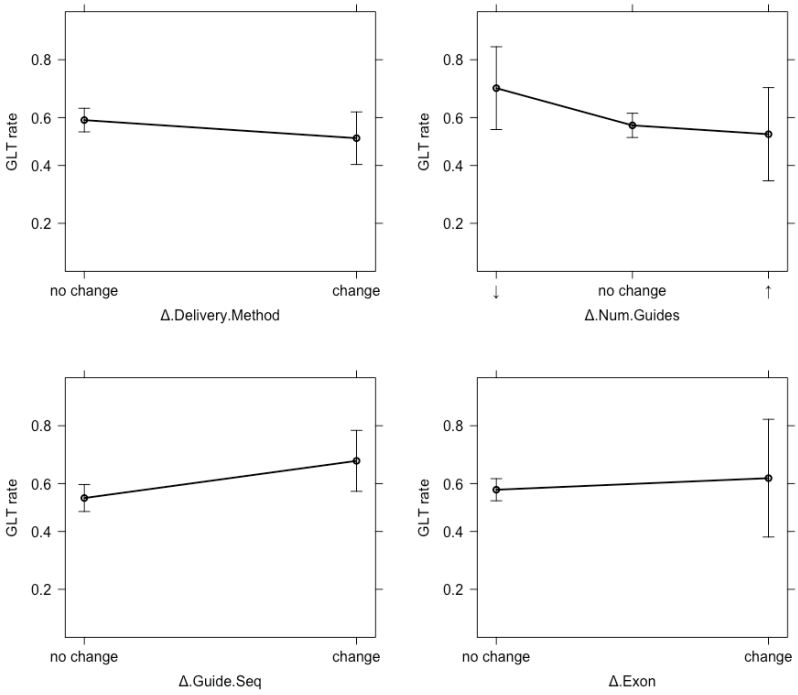
685 (genomic DNA). Each bin has ~640 unique gene deletion attempts. **d**. Percentage of genes with
686 GLT of the desired deletion allele after breeding one (1), two (2), three (3) or four or more (4+)
687 founders. Pairwise comparison of GLT rates using the Holm method showed a significant
688 difference only when breeding one founder was compared to breeding three or four founders
689 ($p = 0.004$ for both comparisons). Unique gene attempts are the first attempt with GLT of the
690 desired allele or the last of a set of unsuccessful attempts for each gene. See materials and
691 methods for a complete description of data filtering. GLT, germline transmission.
692

693 a



694

695 b



696

697 **Figure 2.** Percentage of genes with GLT of the desired allele. **a**. Percentage of genes with GLT of

698 the desired deletion allele (successful), were abandoned with no additional attempts (aborted),

699 or were repeated in subsequent experiments (repeated). **b**. Effect plots from the linear

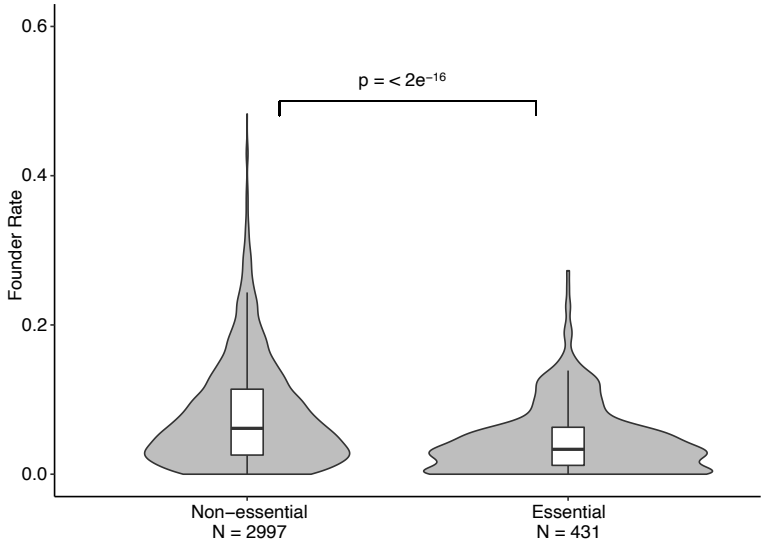
700 regression model. A negative slope indicates decreased GLT rates and a positive slope indicates

701 increased GLT rates in subsequent attempts after the indicated parameter changed. See

702 materials and methods for a complete description of data filtering.

703

704 a



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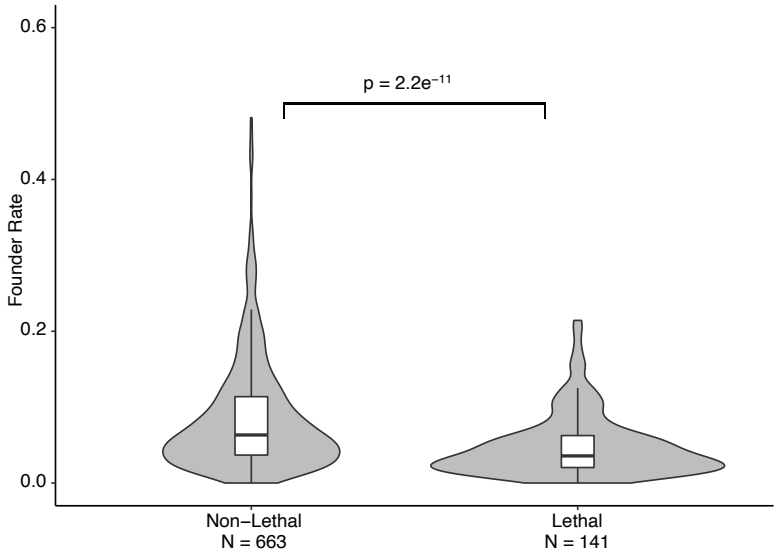

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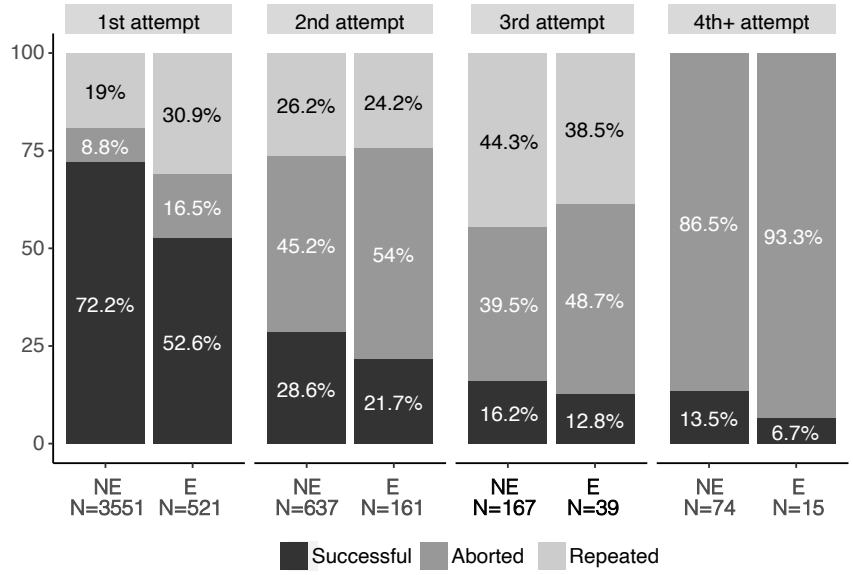

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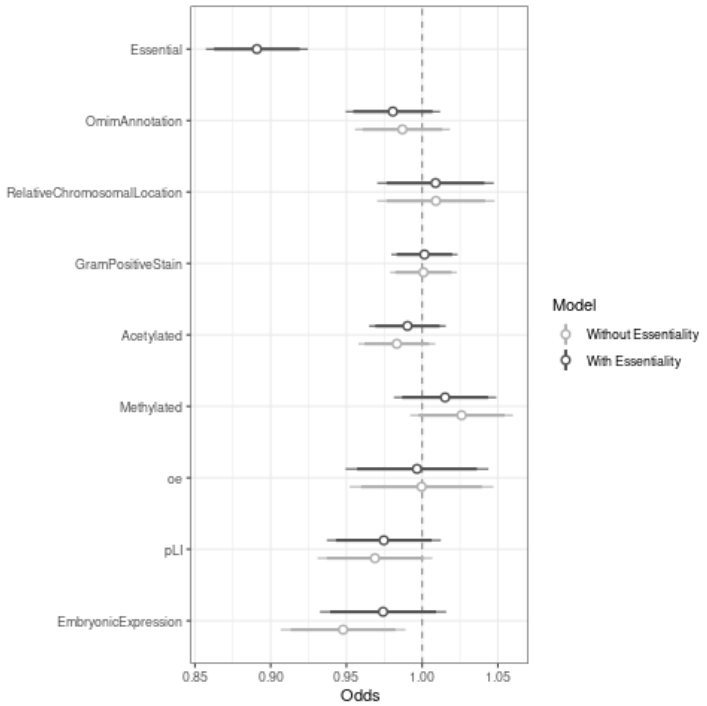

Figure 3. Effects of biological variables on founder and GLT rates for null allele production. **a**. Founder rates of cellular non-essential and essential genes. **b**. Founder rates of homozygous lethal and non-lethal genes. **c**. GLT of null alleles for essential (E) and non-essential (NE) genes with multiple attempts to produce a null allele. **d**. General linear model (GLM) showing the

718 association of each variable with the success of the attempt to generate founders. An odds
719 ratio below 1 is associated with a reduced probability of success, an odds ratio above 1 is
720 associated with an improved probability of success, and an odds ratio of 1 is associated with no
721 effect on success. **Table 2** has the odds ratios and p-values for each variable, with and without
722 essentiality in the model, that assess the significance of the difference of the estimate from
723 zero. **Supplementary Table 5** has the full model output. Each attempt represents a unique gene
724 with the first attempt that successfully generated the desired allele or the last unsuccessful
725 attempt for each gene used for analysis. See materials and methods for a complete description
726 of data filtering.
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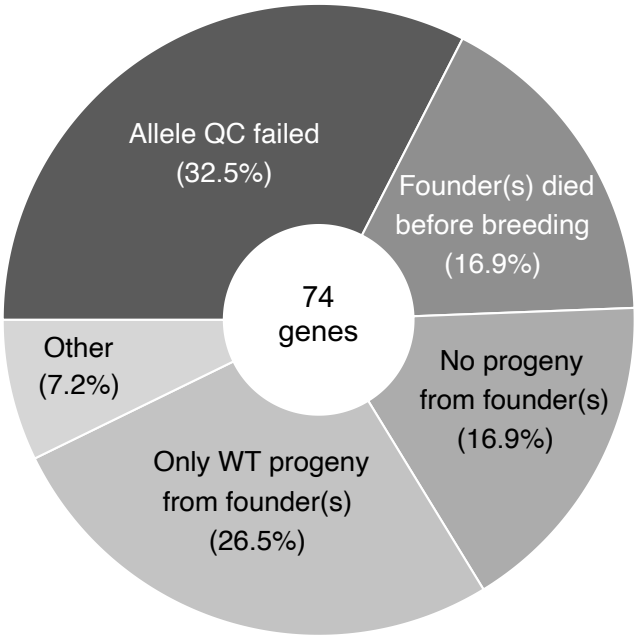

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730 **Figure 4.** Summary of reasons founders failed to transmit a quality-controlled deletion allele to

731 subsequent generations to establish a knock-out mouse line.