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Impact of essential genes on the success of genome editing experiments generating 3313 new genetically engineered mouse lines

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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 3313 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

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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.

Keywords Cas9, Genome editing, Mouse, Knockout

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 decay by introducing a premature termination codon > 50–55 nt from the final splice acceptor[4]. While frameshifts can be achieved using single Cas9-mediated double-strand breaks repaired by non-homologous end joining to introduce small insertions or deletions (indels), exon skipping during splicing can restore the reading frame and partial gene function[5–7]. The deletion approach mitigates the risk of restoring frame, simplifies founder screening, genotyping, and quality control (QC), and resembles the embryonic stem cell-based approaches used for decades to produce null alleles, albeit without selection cassette insertion. To evaluate variables affecting mutant mouse production, we analysed data from 4874 production attempts on 4186 unique genes from eight different centres (Supplementary Table 1) recorded in the IMPC's production tracking database (GenTaR, formerly iMITS; downloaded 2020 Oct 11, www.gentar.org/tracker/#/). Experimental parameters as well as genomic and biological characteristics of targeted genes were evaluated for their effects on mouse line production success.

Results

Experimental parameters affecting mouse line production

Four experimental parameters were assessed for their effects on success: Cas9 delivery method, number of guide RNAs (gRNAs) used, intended deletion size, and number of founders selected for breeding. We also evaluated the effect of changing parameters in repeated production attempts. Null deletion alleles were generated using Cas9 with guides flanking a critical region containing one or more critical exons[3]. Gene editing reagents were delivered by microinjection (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; $p = 0.26$, Wilcoxon rank sum test). 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 test).

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 targeted critical region. 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 test). 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 or four may reduce the small risk of off-target mutagenesis[14–17].

To determine if the intended 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 founder rates ($p = 0.34$, Kruskal–Wallis test for comparing medians of six groups; Fig. 1c) or GLT rates ($p = 0.668$ Kruskal–Wallis test; data not shown) for deletion sizes below 1400 bp. The relatively small number of attempts that tried to delete segments longer than 1400 bp precludes conclusive statistical analysis of deletions above that size using this dataset, however decreased efficiency with increased deletion size has been reported[18].

We next assessed whether the number of founders bred affected GLT rates. GLT test breeding founders, regardless of production methods used, resulted in efficient germline transmission of the edited allele and breeding a single founder provided a > 95% chance of success. The overall high GLT rate was marginally improved by breeding up to three founders (95.2% *cf.* > 98%, $p = 0.0004$ using Pearson's chi-square test with Holm's correction), but there was no advantage to breeding more than three founders (Fig. 1d).

While nearly 80% of first Cas9 production attempts were successful, we wanted to understand why 20% of Cas9 production attempts failed to produce GLT of the desired null allele. Approximately half of these experiments were repeated (with or without changing parameters) with a success rate of 58.8% for second attempts and 51.5% for third attempts (Fig. 2, Supplementary Table 3). The cumulative success rate increased from 78.2 to 84.3% after the second attempts for failed genes, but only marginally thereafter (Supplementary Fig. 3). Correlated with this marginal increase is a reduced number of genes that underwent third and fourth or more attempts. Next, we analysed the set of repeated attempts corresponding to 403 genes to determine what, if any, changes to experimental parameters improved success rates. We compared success rates of consecutive attempts for each gene with repeated attempts to test an association between GLT and each of four experimental parameters

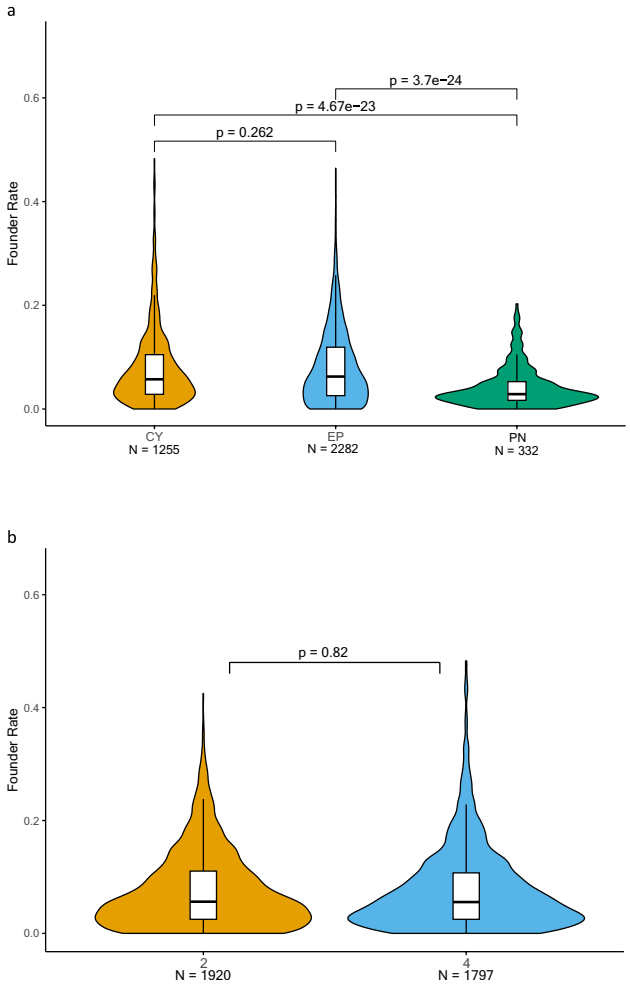

Fig. 1. Experimental parameters affecting Cas9-mediated mutant mouse line production. (**a**) Violin plot for founder rates from experiments with different methods of reagent delivery (CY, cytoplasmic injection; EP, electroporation; PN, pronuclear injection). Reported are the *p*-values (*p*) of pairwise comparisons using the Wilcoxon rank sum test, the number of genes (N), and corresponding medians in boxplots. (**b**) Violin plot for founder rates from experiments using two (2) or four (4) guide RNAs designed to produce deletion alleles. Reported is the *p*-value of pairwise comparison using the Wilcoxon rank sum test, the number of genes (N) and corresponding medians in boxplots. (**c**) Violin plot for founder rates from experiments with Cas9 guide RNAs designed to delete different sizes (in bp) of critical regions (genomic DNA). Each bin has ~ 640 unique gene deletion attempts. Reported is the *p*-value (*p*) of the overall comparison using the Kruskal–Wallis test. (**d**) Barplot showing the percentage of genes with GLT of the desired deletion allele after breeding one (1), two (2), three (3) or four or more (4 +) founders. Pairwise comparison of GLT rates using the Pearson chi-square test with Holm's correction showed a significant difference only when breeding one founder was compared to breeding three or four founders (** $p = 0.004$ for each comparison, 1 vs. 3 and 1 vs. 4 +). No difference between 1 vs.2 or 3 vs. 4 + was observed. Unique gene attempts are the first attempt with GLT of the desired allele or the last of a set of unsuccessful attempts for each gene. See materials and methods for a complete description of data filtering. GLT, germline transmission.

(delivery method, number of guides, guide sequence, targeted exon(s)). Changes to any of these parameters did not significantly affect success rates of repeated attempts (Table 1). To evaluate whether these experimental factors interacted to affect GLT rates, we fit an elastic net logistic regression model which performs variable selection and accounts for collinearity through penalization. These regression models provided a 57.58% classification accuracy for GLT status which is only slightly better than a random guess, thus interactions among different parameters could not predict improved experimental success. Therefore, when experiments fail, a significant decrease in success rates after the first attempt should be expected. One possible explanation to account for no editing is reduced or absent guide activity. Thus, evaluation of guide activity (*e.g.*, by PCR sequence across the target cut sites in presumed wild-type pups or embryo treatment, in vitro culture, and blastocyst genotyping) for failed attempts prior to repeating the attempt can provide assurance that failure was not due to inactive guides.

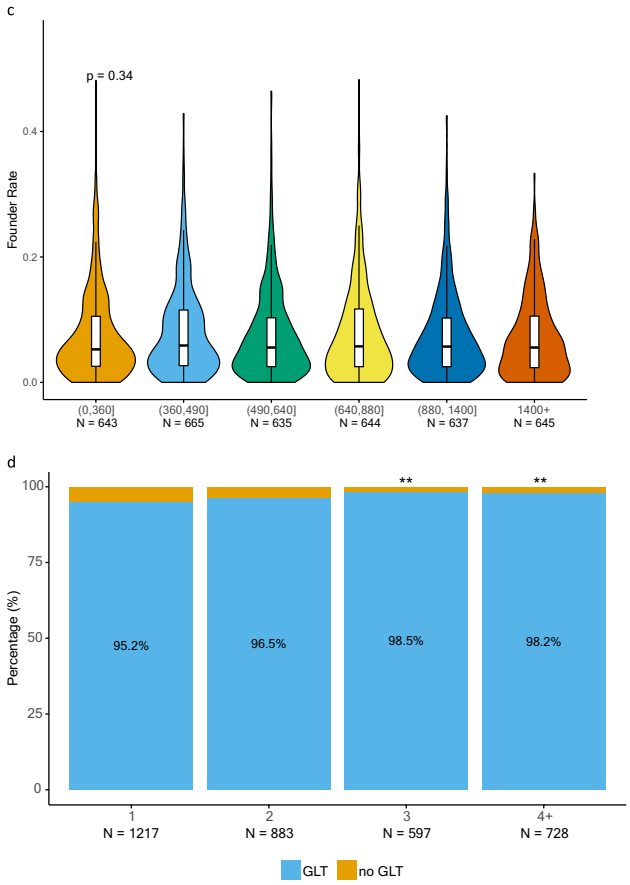

Fig. 1. (continued)

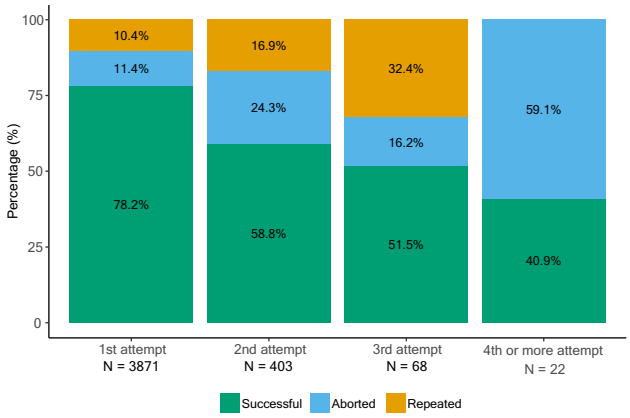

Fig. 2. Percentage of genes with GLT of the desired allele. Proportions represent genes with GLT of the desired deletion allele (successful), were abandoned with no additional attempts (aborted), or were repeated in subsequent experiments (repeated).

Biological parameters affecting successful mouse line production

In considering biological variables that could influence founder and GLT rates, we hypothesized that targeting essential genes (genes whose functions are necessary for cell or animal viability[19]) could negatively affect founder rates due to the high editing efficiency of Cas9. In support of this, the founder rate obtained for cellular non-essential genes was significantly higher than for cellular essential genes ($p < 2 \times 10^{-16}$ Wilcoxon rank sum test; Fig. 3a). Knockout mouse lines (genes for which knockout alleles were successfully produced) were assessed for homozygous viability of the targeted allele by the IMPC[20]. Alleles identified as homozygous lethal after production, so called lethal genes which include both cellular and developmental essential genes[19], had significantly lower founder rates than alleles of non-lethal genes ($p = 2.2 \times 10^{-11}$ Wilcoxon rank sum test; Fig. 3b). Similarly, the birth rates for essential and lethal genes during founder production attempts were lower than that

Predictors	Odds Ratio	CI	*p*-value	adjusted *p*-value
(Intercept)	1.18	0.96–1.47	0.136	0.545
ΔDelivery method	0.74	0.45–1.21	0.222	0.666
ΔDecrease no. guides	1.88	0.92–4.07	0.092	0.462
ΔIncrease no. guides	0.86	0.37–2.03	0.731	1.000
ΔGuide sequence	1.88	1.02–3.58	0.048	0.287
ΔTarget exon	1.22	0.44–3.58	0.709	1.000
Observations: 498				

Table 1. Logistic regression model for GLT status between production attempts conditional on experimental parameters.

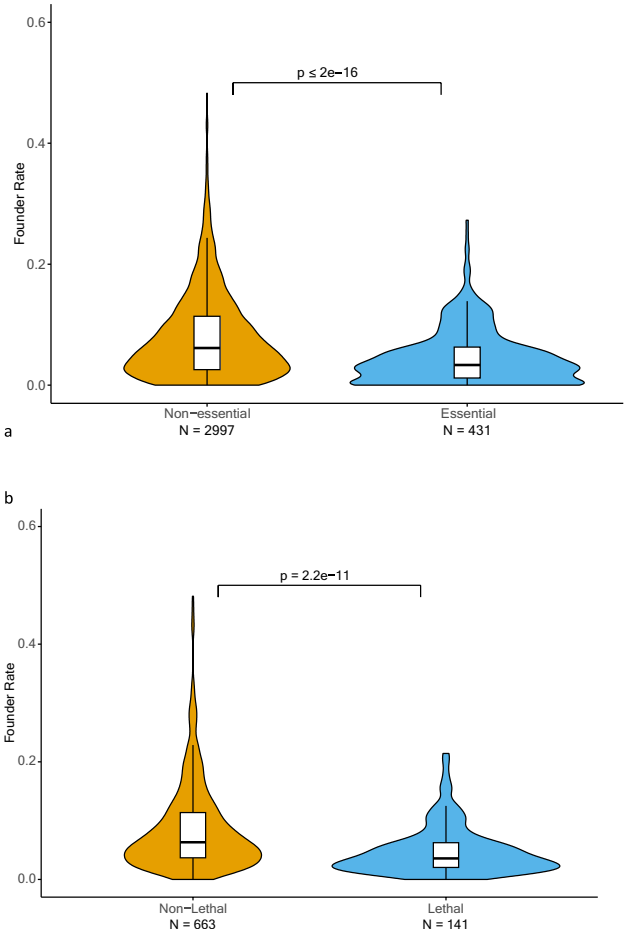

Fig. 3. Effects of biological variables on founder and GLT rates for null allele production. (**a**) Violin plot of founder rates of cellular non-essential and essential genes. Reported are the *p*-value (*p*) of the pairwise comparison using the Wilcoxon rank sum test, the number of genes (N), and corresponding medians in boxplots. (**b**) Violin plot for founder rates of homozygous lethal and non-lethal genes. Reported are the *p*-value (*p*) of the pairwise comparison using the Wilcoxon rank sum test, the number of genes (N), and corresponding medians in boxplots. (**c**) Stacked bar chart showing GLT of null alleles for essential and non-essential genes with multiple attempts to produce a null allele. (**d**) Logistic regression model showing the association of each variable with the success of the attempt to generate founders. An odds ratio below 1 is associated with a reduced probability of success, an odds ratio above 1 is associated with an improved probability of success, and an odds ratio of 1 is associated with no effect on success. Table 2 has the odds ratios and *p*-values for each variable, with and without essentiality in the model, that assess the significance of the difference of the estimate from zero. Supplementary Table 5 has the full model output. Each attempt represents a unique gene with the first attempt that successfully generated the desired allele or the last unsuccessful attempt for each gene used for analysis. See materials and methods for a complete description of data filtering.

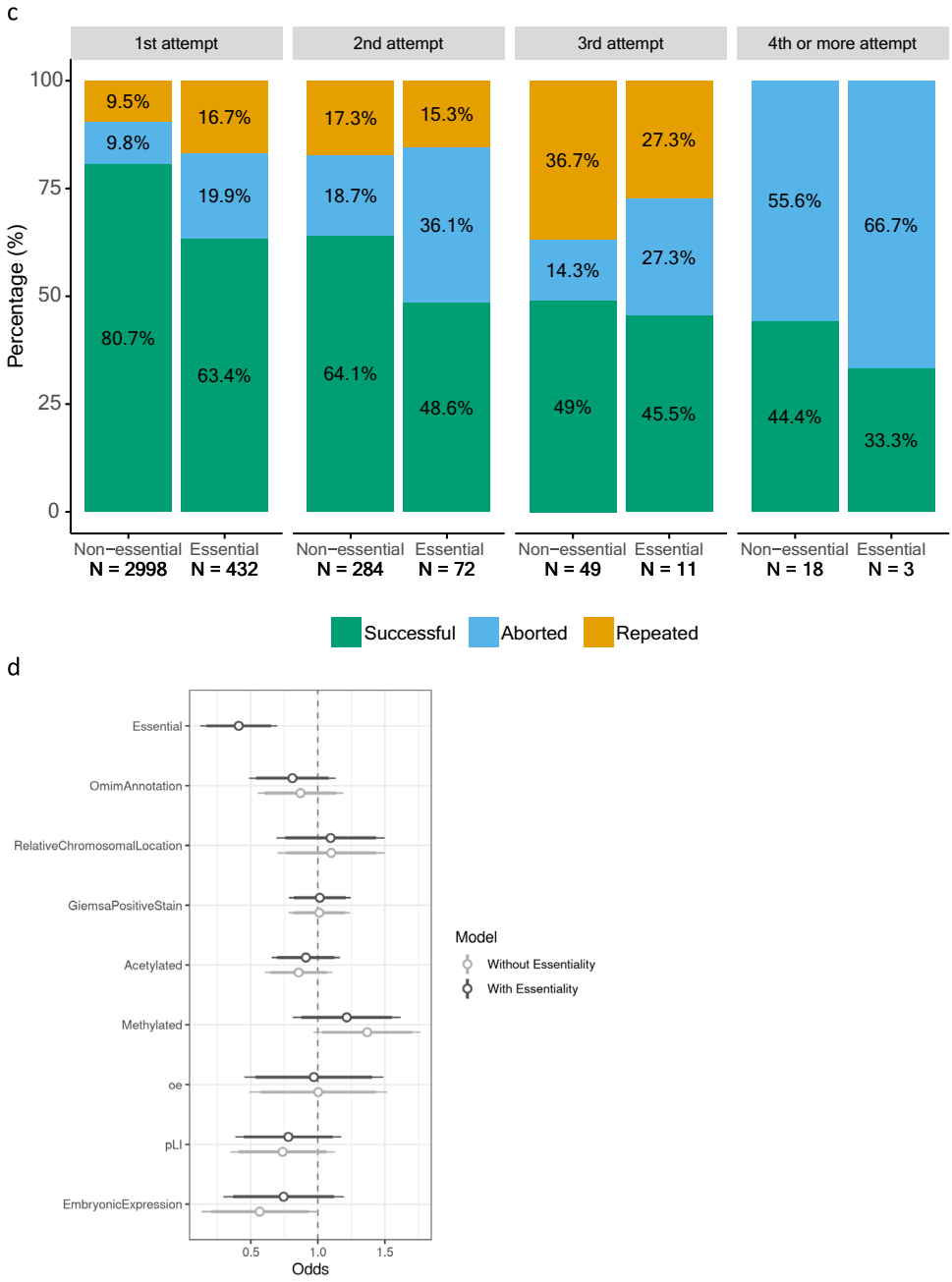

Fig. 3. (continued)

for non-essential and non-lethal genes (Supplementary Fig. 2). A plausible explanation to account for the lower birth rates observed for essential and lethal genes is a loss of embryos with biallelic editing during gestation or shortly after birth due to loss of gene function. When repeated attempts were classified by gene essentiality[19], 80.7% of first attempts were successful for cellular non-essential genes compared to 63.4% for cellular essential genes (Fig. 3c). In addition, a larger percentage of cellular non-essential genes than essential genes were successful for each subsequent attempt, albeit with decreased success rates for each subsequent attempt for the same set of genes. No difference in GLT rates between cellular non-essential and cellular essential genes (96.8% and 95.2%, respectively; $p = 0.15$ Pearson Chi-square) among experiments that generated founders was observed.

To assess the influence of additional biological variables on founder and GLT rates, we applied a logistic regression model to test the association of several factors including embryonic expression (GEO GSE11224)[21], observed/expected loss-of-function (o/e) score and probability of loss-of-function (pLI) score[22] of human orthologs, as well as chromosome position and histones marked by methylation and acetylation (as a proxy for chromatin state regarding transcriptional repression or activation, respectively). We also included Giemsa stain cytogenic banding (a proxy for chromatin structure, euchromatin *vs*. heterochromatin) and human ortholog disease annotation in the Online Mendelian Inheritance in Man (OMIM) database[23] (Supplementary Table 4). Cellular essentiality was the only predictor of experimental failure (OR = 0.41, $p = 1.23 \times 10^{-9}$, p-adj $= 1.11 \times 10^{-8}$)

Variable	Without essentiality			With essentiality		
	Odds Ratio	*p*-value	adjusted *p*-value	Odds Ratio	*p*-value	adjusted *p*-value
(Intercept)	11.97	2.0×10^{-16}	2.7×10^{-19}	12.09	2.22×10^{-16}	5.3×10^{-19}
Essential	NA	NA	NA	0.41	1.23×10^{-9}	1.11×10^{-8}
Embryonic expression	0.57	0.010	0.081	0.74	0.199	1.00
pLI score	0.74	0.126	0.867	0.78	0.218	1.00
o/e score	1.00	0.992	1.00	0.97	0.910	1.00
Chromosome position	1.10	0.640	1.00	1.09	0.658	1.00
Acetylated gene	0.86	0.229	1.00	0.91	0.472	1.00
Methylated gene	1.37	0.124	0.866	1.21	0.341	1.00
Giemsa positive stain	1.01	0.926	1.00	1.01	0.901	1.00
OMIM annotation	0.87	0.397	1.00	0.81	0.203	1.00
Observations: 3209						

Table 2. Logistic regression model for successful founder production conditional on biological factors annotated for a gene.

with no other annotated biological property being significantly associated with success, whether or not essentiality was included in the model (Fig. 3d, Table 2, Supplementary Table 5). The proportion of essential genes in each experimental parameter grouping did not vary in a way that may have confounded the results of these analyses (Supplementary Tables 6 and 7).

After successful production of a founder, subsequent GLT rates were very high (Fig. 1d). Among 74 genes for which the reason for GLT failure was readily determined, the majority of lines failed GLT because all founders died before breeding, all founders failed to produce progeny, or all founders for a given gene deletion produced only wild-type progeny when genotyped at post-natal day (P) 13–17 (Fig. 4). The remaining genes failed primarily because the desired null allele could not be validated in N1 progeny (*e.g.,* partial deletion or no frameshift recovered). Infertile founders and transmission of only wild-type progeny might be due to founder mosaicism with only wild-type cells contributing to the germline or mutation effects on gametogenesis with only wild-type cells able to produce functional germ cells. Moreover, transmission of only wild-type alleles could indicate the presence of haplo-insufficient alleles for some genes causing progeny with loss-of-function alleles to die before genotyping. These data support the hypothesis that a substantial subset of loss-of-function edits may fail GLT due to negative effects on viability or fertility and are consistent with the significant effect of gene essentiality on founder production (Table 2).

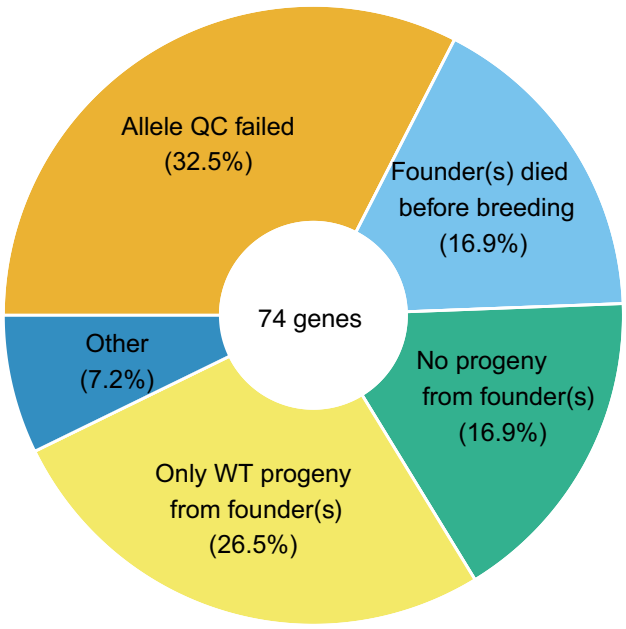

Fig. 4. Summary of reasons accounting for germline transmission failures with founders that failed to transmit a quality-controlled deletion allele to the N1 generation and thus failed to establish a knockout mouse line.

Discussion

The analysis of our large, multi-centre dataset identified several variables that affect Cas9 editing success and provides the basis for recommendations for genome editing in mouse zygotes. For deletion alleles, Cas9 ribonucleoprotein (RNP) electroporation is the most accessible and scalable method, providing equivalent performance to cytoplasmic microinjection of Cas9 mRNA and gRNAs. Cas9 concentration is correlated with delivery method, with higher concentrations used for electroporation than for microinjection. Thus, there are several covariates associated with Cas9 concentration, and due to practical constraints associated with large-scale production pipelines we were unable to systematically evaluate these variables. Currently, it is not possible to analyze each variable independently from our data. However, previously published work demonstrated that no statistically significant difference in founder rates was observed with up to twofold changes in Cas9 concentrations[10]. Our data support that the methodologies reported here are robust and work well across a range of concentrations. At this time, we recommend that concentrations should be evaluated at individual centres to establish the optimal concentration(s) relative to delivery methods used.

GLT rates are high for Cas9 generated founders and there is no apparent advantage to breeding more than three founders. Most attempts are successful the first time, but for attempts that fail to produce founders, evaluating guide activity prior to repeating production for a given gene may be beneficial. The activity of Cas9-guide RNA combinations can be assessed in vivo (*e.g.*, by sequencing across target cut sites in presumed wild-type pups, or by zygote treatment, in vitro culture, and blastocyst genotyping[24]). Exploring the known biology of the gene, in particular its essentiality score from publicly available sources (as in[19]), can assist with experimental design. Other factors affecting GLT rates include founder mosaicism which may influence germline transmission rates. Even when treating zygotes, Cas9 activity can result in multiple alleles being transmitted and tissue biopsies used for genotyping may not completely represent the allele content of the germline. In cases with suspected mosaicism, testing germline allele content is possible with male founders by cryopreserving and genotyping sperm[10], enabling one to differentiate the effects of lethality, haploinsufficiency, and mosaicism on germline transmission when it fails.

We did not observe significant effects of chromosomal position, histone modification status (as indicated by histone acetylation or methylation marks), euchromatin or heterochromatin (as indicated by Giesma staining), intolerance for loss-of-function in human orthologs or disease association on experimental success. Founder rates being independent of chromatin state and structure is consistent with deep learning approaches to predict Cas9 activity in cell lines[25]. Given the interdependence of two guides to generate a deletion allele, it is not possible to use our deletion efficiency data to test correlations between predicted activity based on cell line data and our in vivo editing efficiencies. The lack of correlation between pLI and o/e scores as well as OMIM disease associations on experimental success was unexpected since cellular essential genes are usually intolerant to loss-of-function variation and represent a high proportion of disease genes[19,26]. This emphasizes the importance of the data from large cellular essentiality screens in predicting success.

The reduced founder rate observed for essential and lethal genes likely reflects a loss of founders with biallelic editing. Nearly all cellular essential genes result in embryonic lethality as homozygotes, but some lethal genes, the so-called developmental essential genes, are not essential for cellular function but rather essential for the development of tissues and/or organs necessary for embryonic development and survival[19]. Successful targeting of essential or lethal genes may require methods that promote founder heterozygosity (*e.g.,* lower Cas9 concentrations) or mosaicism (*e.g.,* delivering reagents to one blastomere of a 2-cell embryo by microinjection to promote mosaicism and enable embryo development). In some cases, conditional alleles[27,28] may be required instead of constitutive knockout alleles to generate a mouse line in which heterozygous and/or homozygous loss of function may be examined in specific tissues or developmental stages.

The effects of cell essentiality or lethality may also be observed when generating disease-associated variants, such as single nucleotide variants. Efficient Cas9-mediated editing can result in a biallelic disease variants or biallelic editing where one allele contains the disease-associated variant and the other allele is a loss-of-function indel effectively generating a biallelic loss-of-function founder. Given the high proportion of essential genes among human disease genes[19,26], generating mouse models of human disease may also require preserving one copy of the wild-type allele to establish a new mouse line. Overall, the use of Cas9 is a robust and flexible method to generate gene-edited mouse lines. Improving model production success rates with rational experimental design and consideration of gene essentiality can reduce the number of animals used to generate new models, consistent with the principles of the 3R's[29].

Materials and methods

Mouse strains

All null allele mouse lines were produced in the C57BL/6N strain background available from Charles River, Taconic, the Jackson Laboratory, or bred in house (Supplementary Information 3). All live animal protocols conformed to the applicable standards for the ethical use of animals in research at the respective facilities. At the Baylor College of Medicine, all mice were maintained in an AAALAC-accredited animal facility. All studies were reviewed and approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine and followed the National Institutes of Health guidelines for the Care and Use of Laboratory Animals. At the Czech Centre for Phenogenomics, animal protocols were approved by the Institutional Animal Care and Use Committee and The Czech Academy of Sciences ethics committee. All procedures were conducted on animals at Czech Centre for Phenogenomics under project licenses 115/2016 and 93/2020 in accord with national guidelines and regulations. At the Institut Clinique de la Souris, mice were generated, bred and maintained in the animal facility of PHENOMIN-ICS which is accredited by the French Ministry for Superior Education and Research and the French Ministry of Agriculture (agreement #A67-218-37) and in accordance with the Directive of the

European Parliament: 2010/63/EU, revising/replacing Directive 86/609/EEC and with French Law (Decree n° 2013-118 01 and its supporting annexes entered into legislation 01 February 2013) relative with the protection of animals used in scientific experimentation. All animal experiments were approved by local ethical committees (Approval Committee: Com'Eth N°17 and French Ministry for Superior Education and Research). Approval licenses: internal numbers 2012-009 & 2014-024. Approval licenses: MESR: APAFIS#4789–2016040511578546 and supervised in compliance with the European Community guidelines for laboratory animal care and use. At the Jackson Laboratory, all animals were maintained in an AAALAC-accredited animal facility at The Jackson Laboratory and all procedures were performed in accordance with the Animal Welfare Act and the AVMA Guidelines on Euthanasia, in compliance with the ILAR Guide for Care and Use of Laboratory Animals, and with prior approval from The Jackson Laboratory Animal Care and Use Committee. At MRC Harwell, animals were maintained at the Mary Lyon Centre and all procedures on animals were performed following approval by the MRC-Harwell's Animal Welfare and Ethical Review Body (AWERB) under Home Office licensed authority. Establishment licence number X9BFFDAE2 and project licence numbers PP9563804 (rederivation), P05C608B0 (microinjection) & PE40B1D0F (breeding). The use of animals in this study was in accordance with the UK Home Office regulations under the Animals (Scientific Procedures) Act 1986. At The Centre for Phenogenomics, all procedures involving animals were performed in compliance with the Animals for Research Act of Ontario and the Guidelines of the Canadian Council on Animal Care (CCAC). All live animal protocols were reviewed and approved by The Centre for Phenogenomics Animal Care Committee and followed CCAC guidelines contained in the Guide to Care and Use of Experimental Animals, vol 1, 2nd edition and CCAC guidelines on Transgenic Animals (1997) under protocols 0008, 0084, 0153, and 0275. The Centre for Phenogenomics maintains a CCAC Certificate of GAP – Good Animal Practice® and is accredited by the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA). At the Wellcome Trust Sanger Institute, all procedures on animals were performed following approval by the Sanger Institute Animal Welfare and Ethical Review Body (AWERB) under Home Office licensed authority, Establishment licence number X3A0ED725, project licence number P96810DE8. The use of animals in this study was carried out in accordance with the UK Home Office regulations under the Animals (Scientific Procedures) Act 1986. At the University of California, Davis (UC Davis), all animal use was conducted in accordance with the 1966 Animal Welfare Act and the 2013 AVMA Guidelines for the Euthanasia of Animals. All studies were done consistent with the 8th Revision to the ILAR Guide for the Care and Use of Laboratory Animals and in compliance with and with prior approval from the UC Davis institutional animal care and use committee (IACUC). The UC Davis has been accredited by AAALAC continuously since 1966. Animal welfare was regularly monitored with every effort made to minimize the number of animals used and to mitigate adverse welfare effects. All methods are reported in accordance with the ARRIVE guidelines for producing genetically engineered animals within the context of the International Mouse Phenotyping Consortium (IMPC). See the Supplementary Information 3 for additional information.

Mouse strain availability

All strains in this study are available through public repositories. Repository ordering information for the strains, along with the phenotyping data collected by the IMPC, can be accessed at www.mousephenotype.org using the relevant gene symbol or MGI identifier as a search term on the "Genes" tab. Bulk data download is also possible using links found at this URL. We have included individual URLs for each gene project in Supplementary Information 2.

Allele design

Null alleles were designed such that the mutations resulting from Cas9 endonuclease activity and double-strand break (DSB) repair were predicted to cause a frameshift in protein-coding transcripts resulting in a premature stop codon. This design 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 most 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[4,30]. 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[31], the Wellcome Sanger Genome Editing (WGE) Tool[32], CRISPOR[33], CRISPRTools[34], CHOPCHOP[35], or FORCAST[36]. Suitable gRNA spacer sequences were selected to minimize predicted Cas9-induced off-target variation using specificity scores > 65 when available and/or sequences with at least 3 mismatches for all predicted off-target sites. These parameters have been validated to generate minimal Cas9-mediated off-target sequence variation[16]. 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)[37], gBlock synthesis and in vitro transcription (gBlock-IVT), or by primer extension and in vitro transcription (PE-IVT)[37]. Alternatively, sgRNAs were purchased from commercial suppliers. See Supplementary Information 3 for centre-specific reagent details.

PCR-IVT

DNA templates for PCR-IVT were produced using overlapping oligonucleotides in a high-fidelity PCR reaction[38] or using a plasmid template (Addgene #42230[39]) and appropriate primers[37]. PCR amplicons were purified using the Monarch PCR & DNA cleanup kit (New England BioLabs T1030) or the QIAQuick PCR purification kit (Qiagen 28,104) 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[40]) were purchased annealed. Alternatively, annealed oligonucleotides were cloned into plasmid DR274 (Addgene #42250[41]). Plasmid DNA was extracted using the QIAGEN Plasmid Plus 96 Kit (Qiagen 16,181) 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 37 °C for 60–90 min 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 − 80 °C in elution buffer or stored as ammonium acetate precipitates in ethanol at − 20 °C. 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 Information 3) or transcribed in-house[42]. Cas9 protein was purchased from commercial suppliers. See Supplementary Information 3 for centre-specific reagent details.

Injection mix preparation

Injection mixes were prepared essentially as previously reported[37] with or without filtration prior to injection. For mRNA microinjection, injection mixes consisted of Cas9 mRNA and sgRNA in microinjection buffer (Supplementary Information 3). Concentrations for each production attempt are shown in Supplementary Information 2. For Cas9 protein microinjection, Cas9 ribnucleoprotein (RNP) complexes were produced by mixing the Cas9 protein with sgRNA at 5X the concentration shown in Supplementary Information 3 in RNP injection buffer and incubating at 37 °C or room temperature for 10 min. The RNP mix was then diluted with 4 volumes of RNP injection buffer prior to injection. See Supplementary Information 3 for centre-specific reagent details.

Electroporation mix preparation

Electroporation mixes were prepared essentially as previously reported[11–13,37]. Electroporation mixes consisted of Cas9 protein and sgRNA in RNP electroporation buffer (Supplementary Information 3) at 2X the concentrations shown in Supplementary Information 2, incubated at 37 °C or room temperature for 5–15 min, and placed on ice until electroporation. Immediately before electroporation, RNP was diluted with an equal volume of Opti-MEM (ThermoFisher 31985062). See Supplementary Information 3 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 h 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 37 °C 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 Information 2. Pronuclear microinjections were performed following standard protocols[8,43]. Cytoplasmic injections were performed essentially as in[9]. Visible movement of the cytoplasm indicated successful injection. Injected zygotes were transferred into pseudopregnant females (Supplementary Information 3) on the afternoon of the injection or after overnight culture (recorded for each production attempt in Supplementary Information 2), 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[11–13,37]. 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 Information 2. 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 Information 2). Centre-specific details for buffers used are in Supplementary Information 3.

Genotyping

Genomic DNA was prepared from ear punch or tail biopsies of 2- to 3-week-old pups (see Supplementary Information 3 for reagents) using commercial kits or previously described protocols[44,45]. 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[46,47]. 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 for 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. See Supplementary Information 3 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 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 0–0.2 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 Information 2).

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 vs. electroporation), or gRNA locations (sequences) changed between sequential attempts. All filtering and annotation of the data was performed in Python3.8.5 using packages numPy1.2.1[48] and pandas1.2.2[49].

Genes for each attempt were annotated (Supplementary Table 4) with their viability (as annotated at the IMPC – viable or homozygous lethal), human orthologs and cell essentiality of human orthologous genes[19], embryonic expression (GEO GSE11224)[21], length, GC content, number of CpG sites, and CpG percentage (Supplementary Table 2). The human orthologs' probability of being loss-of-function intolerant (pLI) and observed / expected (oe) mutation rate was retrieved from gnomAD[22]. Additional annotations were added for analysis of biological variables affecting success. Annotation details are in Supplementary Table 2.

Statistical analysis

The primary outcomes were the founder rate and the germline transmission status. The founder rate had a right-skewed distribution with a range [0,0.5]. Hence, comparisons of the founder rate across different categories of biological or experimental factors were conducted using nonparametric tests. For pairwise comparisons, the Wilcoxon rank sum test[50] was used and when there were more than two categories the Kruskal–Wallis test[51] was employed. The biological factors considered in the comparisons were the gene essentiality (essential vs. non-essential) and gene lethality (lethal vs. non-lethal). The experimental factors were the delivery method (three categories), number of gRNAs used (2 vs. 4), deletion size (six categories), and number of founders selected for breeding (four categories). Since the GLT status is binary (yes vs. no), comparisons of the GLT rate (proportion of genes with GLT) across different categories of biological or experimental factors were performed using the Pearson chi-square test[52]. In the case of multiple pairwise comparisons, correction for multiple testing was done using Holm's method[53]. 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 a logistic regression model with the GLT status as the binary response and changes in the delivery method (change vs. no change), number of gRNAs used (decrease, no change, increase), deletion size (change vs. no change) and number of founders selected for breeding (change vs. no change) as categorical covariates. All statistical analyses were performed using the R statistical programing software[54], along with the packages `ggplot2`[55] for figures, `tidyverse`[56] for data manipulations.

The logistic regression models of biological variables were fit using logistic regression from 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 the success or failure in founder production as the dependent variable.

Data availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

Code availability

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

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Author contributions

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Competing interests

The authors declare no competing interests.

Additional information

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