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Response to "Unexpected mutations after CRISPR-Cas9 editing *in vivo*"

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To the editor: Schaefer *et al.* recently stated that CRISPR-Cas9 nuclease can induce off-target alterations at genomic loci that do not resemble the intended on-target site.[1] This new proposed CRISPR-Cas9 off-target activity runs contrary to previously published work (performed mostly in cells but also in mice)[2–6] and, if the authors are correct, could have profound implications for research and therapeutic applications. However, here we demonstrate that the simplest interpretation of Schaefer *et al.*'s data is that the two CRISPR-Cas9-treated mice are more closely related genetically to each other than to the control mouse. This strongly suggests that the so-called "unexpected mutations" simply represent shared SNPs and indels that existed prior to nuclease treatment.

Schaefer *et al.*'s conclusion that the sequence variants shared by the genome-edited F03 and F05 mice (and not found in the control untreated FVB mouse) are caused by CRISPR-Cas9 critically depends upon the assumption that all of these mice were initially genetically

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Conflict of interest statement
J.K.J. has financial interests in Beam Therapeutics, Editas Medicine, Monitor Biotechnologies, Pairwise Plants, Poseida Therapeutics, and Transposagen Biopharmaceuticals. M.J.A. has financial interests in Monitor Biotechnologies. J.K.J.'s and M.J.A.'s interests were reviewed and are managed by Massachusetts General Hospital and Partners HealthCare in accordance with their conflict of interest policies.

Data availability statement. Sequencing data as part of the original study was accessed from available at SRA accessions SRR5450996-SRR5450998. Source data for Figure 1 is available as part of our online resource: http://aryee.mgh.harvard.edu/crispr_mutation_reanalysis

Code availability statement. Code and data to reproduce our analysis can be visualized and downloaded here: http://aryee.mgh.harvard.edu/crispr_mutation_reanalysis

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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identical. If this clonality assumption were true, one would expect that all three mice should be nearly identical for common variants found in dbSNP (a hypothetical result represented in Fig. 1a). However, after genotyping these mice with GATK best practices, we identified a total of 31,079 high-quality variants at dbSNP loci that were concordant in two mice but distinct from the third when examining all possible pairwise combinations (Fig. 1b; Supplementary Note 1). Furthermore, 33–46% of these high-confidence genotyped variants in each mouse are heterozygous (Supplementary Table 1), which the authors have argued should not exist in highly inbred mice[7]. Thus, the three mice are neither clonal nor completely isogenic. Even under a more realistic and relaxed equal distance model that allows rare and private mutations (Fig. 1c), our re-analysis still reveals that the F03 and F05 mice are genetically more closely related to each other than to the control FVB mouse (Fig. 1b,d; Supplementary Note 2).

Even if one were to assume that the variants in question were induced by CRISPR-Cas9, it is difficult to reconcile the off-target activity proposed by Schaefer *et al.* with our current understanding of how this nuclease functions. We confirmed the authors' claim that no DNA sequences resembling the on-target site can be found near the sequence variants that they attribute to CRISPR-Cas9 (Supplementary Fig. 2; Supplementary Note 3). Additionally, we could not find an alternative consensus DNA motif at or near the locations of these variants that might be recognized by the CRISPR-Cas9 nuclease (Supplementary Fig. 3; Supplementary Note 4). This makes it hard to envision any reasonable mechanism for how CRISPR-Cas9 could direct alterations to the same genomic loci in the two mice. Furthermore, given the well-established variability of indel mutations induced by CRISPR-Cas9 at any given cleavage site,[8] we calculate that the probability that these proposed Cas9-induced changes would be exactly the same at a large number of loci (as observed in Schaefer *et al.*'s data; Supplementary Fig. 4) is less than 1 in 10^{12} under even the most generous assumptions (Binomial Test; Supplementary Fig. 5; Supplementary Note 5).

Based on the analyses described above and further common variant analyses (Supplementary Fig. 6, 7; Supplementary Note 6), the simplest explanation of Schaefer *et al.*'s results is that the CRISPR-treated F03 and F05 embryos already harbored these shared private SNPs and indels prior to nuclease treatment whereas the control mouse did not. This alternative explanation avoids the need to postulate a new CRISPR-Cas9 activity that has not been previously observed and that is inconsistent with previously reported observations about how it functions. Schaefer et al. mistakenly assumed that association meant causality, but this can lead to erroneous conclusions. For example, our analysis shows an equally high percentage of heterozygous variants in the control mouse that are not present in the two nuclease-treated mice, but we would certainly not attribute these to mutations induced by the lack of CRISPR-Cas9 treatment in the control mouse.

In summary, our analyses of the primary data demonstrate that the original conclusions by Schaefer et al. are not supported by their existing data. In addition, given our current understanding of CRISPR-Cas9 function based on the published literature, it seems exceedingly unlikely that the new activities proposed by Schaefer et al. would be proven true even if one were to perform additional WGS experiments with appropriate and important controls missing from their original study.

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Supplementary Material

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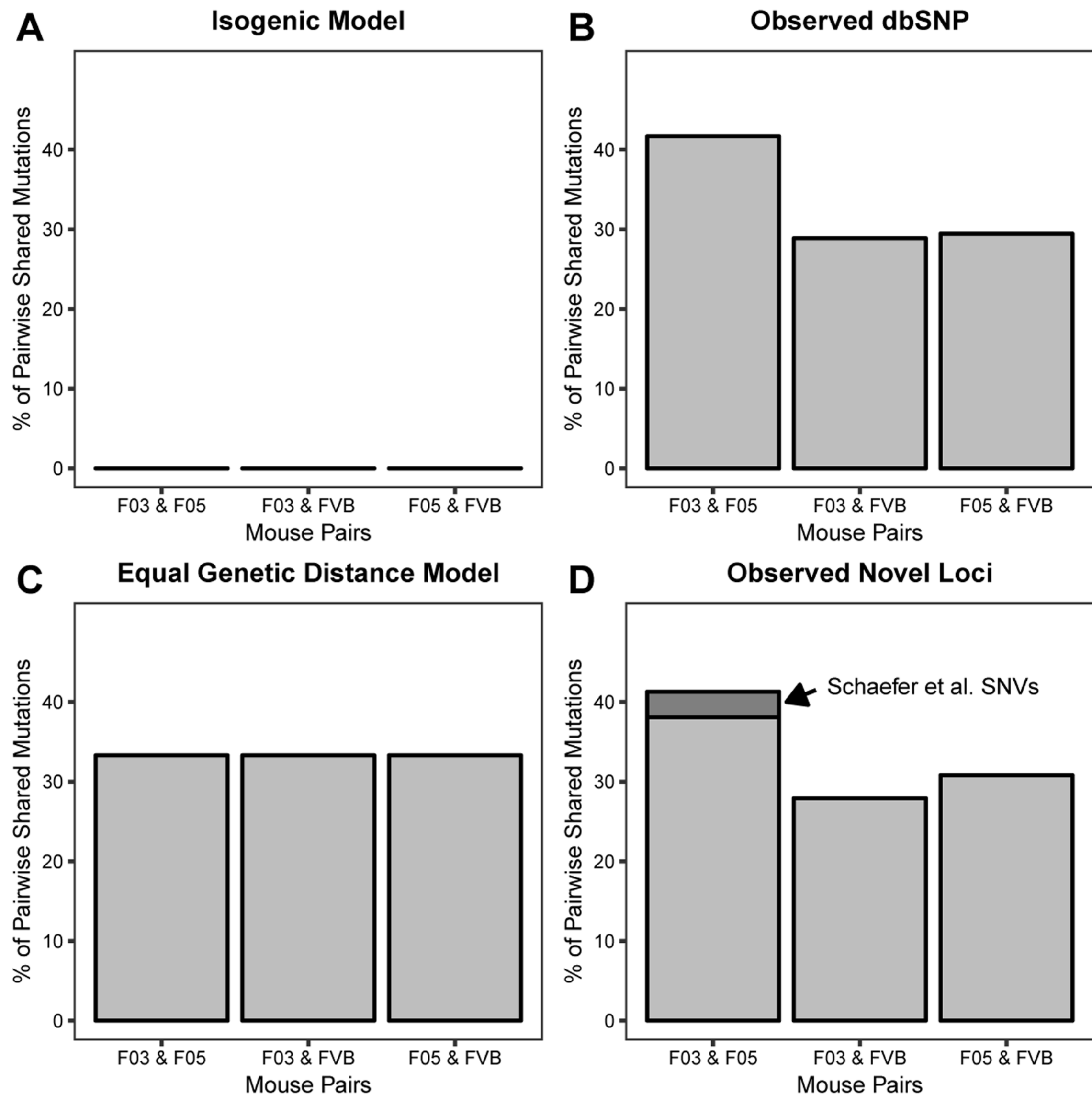

Figure 1. Measures of genetic relatedness in the F03, F05 and FVB mice.

(a) Isogenic model of 3 mice with no private mutations within or shared between mice. (b) observed dbSNP in F03 and F05 mice (n = 31,079). (c) an isogenic system assumes the number of loci with shared genotypes is nearly identical for all mice (d) the observed data demonstrates a clear departure from this equal genetic model at common variants and other non-dbSNP loci (n = 38,981). The variants previously reported by Schaefer *et al.* (dark gray) represent only a small subset of the genotypes common to F03 and F05 but distinct from FVB at non-dbSNP sites. The observed ratios in B and D cannot be distinguished from each other (p = 0.304; two-sided Fisher's Exact Test), but each represent a significant departure ($p < 2.2 \times 10^{-16}$; Chi-Squared Test) from the equal genetic distance model (C) required to attribute differential SNVs to Cas9 activity.