The experimental design and data interpretation in "Unexpected mutations after CRISPR–Cas9 editing *in vivo*" by Schaefer et al. are insufficient to support the conclusions drawn by the authors

To the Editor: The recent correspondence to the Editor of Nature Methods by Schaefer et. al.[1] has garnered significant attention since its publication as a result of its strong conclusions that contradict numerous publications in the field using similar analytical approaches and methods[2-4]. The authors suggest that the CRISPR-Cas9 system is highly mutagenic in genomic regions not expected to be targeted by the gRNA. We believe that the conclusions drawn from this study are unsubstantiated by the disclosed experiments as they were designed and carried out. Further, it is impossible to ascribe the observed differences in the subject mice to the effects of CRISPR *per se*. The genetic differences seen in this comparative analysis were likely present prior to editing with CRISPR.

In our view, the experiments, observations, and subsequent assertions in Schaefer et al.[1] can be summarized as follows. Two mice created using CRISPR-based genome editing in the zygote stage, when compared to a single "co-housed FVB/NJ mice without CRISPR-mediated correction", showed a significant number of single nucleotide variants (SNVs) and insertions and deletions (indels) across the genome. The number of mutations common to the two independently generated CRISPR edited mice was 1,397 SNVs and 117 indels. Surprisingly, these apparent mutations all arose from sequences in the genome that contain poor homology to the gRNA (between 5% – 65%). Furthermore, none of the 50 closest, predicted off-target sites (based on gRNA sequence homology) had any observed activity (SNVs or indels). The authors speculate that there is an unreported activity where "certain sgRNAs may target loci independently of their target *in vivo*."

Our opinion that the conclusions drawn from this study are unsubstantiated by the disclosed experiments and that it is impossible to ascribe the observed differences in the subject mice to the effects of CRISPR *per se* are based upon the following observations:

Firstly, the overall number of the study subjects is low (n = 2 treated mice and n = 1 untreated mouse) and the depth of sequencing applied to the treated and untreated mice is not equivalent. An underpowered study may prove limiting when attempting to understand statistical reproducibility and reliability of scientific observations.

Secondly, the selection of a co-housed mouse (as opposed to the parents or *bona fide* littermates) as the control is insufficient to attribute the observed differences between the treated mice and control mouse to CRISPR. The design of the experiment makes it impossible for the authors to rule out the possibility that the reported genomic differences between the experimental animals and the single control existed prior to experimental manipulation with CRISPR. In fact, published literature has shown that differences in the genomes of *littermates*

analyzed by whole genome sequencing (WGS) can be significant (985 SNVs were identified)[5]. These differences are attributed to private mutations propagated by normal Mendelian inheritance within a breeding colony. In Oey et al., further analysis of the parents by sequencing methods confirm the vast majority of these SNVs were present in the parents and a small minority arose as private variations in the progeny[5].

In order to control for the reality that inbred mice are not perfectly identical at the nucleotide level, an appropriately controlled experiment would include essential components such as 1) sequencing of the parent animals to ascertain the input genome sequences going into the experiment, 2) breeding out the CRISPR edited mice to remove chimerism, and 3) generating and characterizing mice using identical methodology derived from the same experimental protocol, but lacking key individual components, to rule out the possibility that the method itself was mutagenic. More specifically, mice generated with plasmid (encoding the sgRNA) + single stranded DNA oligonucleotide (ssODN) donor DNA + Cas9 protein should be compared to mice generated with plasmid + ssODN donor, plasmid + Cas9 protein, and ssODN donor + Cas9 protein. This would control for the possibility that either of these components individually, or the process of generating the mice, was inherently mutagenic. A similar study[2] has been published in the same journal using appropriate controls and finding significantly lower SNVs and indels suggesting experimental differences, and not CRISPR, are likely causes of the recent observations of Schaefer et al. [1]

Furthermore, we would highlight the following observations reported in the Schaefer et al. [1] communication:

The specific gRNA used in the disclosed experiments, when run through gRNA specificity prediction algorithms, shows a high propensity for off targets, identifying 1 off-target site that differs from the mouse genome by 1 nucleotide match, 1 off-target site that differs from the mouse genome by 2 nucleotide matches, and 24 off-target sites that differ from the mouse genome by 3 nucleotide matches. While perhaps acceptable for research purposes, a gRNA with a predicted high off-target profile would be immediately excluded as a therapeutic candidate. Despite the high propensity for off target activity we found it surprising that this gRNA showed none of the predicted off-targets using the methods employed in this study underscoring the importance of both predicting and testing empirically for off-target activity.

Most exonic SNVs found in the two CRISPR edited mice (Supplemental tables 1 and 2) were not only shared between these mice, despite the assertion that the SNVs were created in separate zygotes, but also exhibited identical nucleotide changes in both position and nucleotide composition. Furthermore, the 'normal' to 'mutant' allele count ratio was almost identical despite these being mosaic animals. Both animals were either homozygous or heterozygous for the same nucleotide change at the same genomic position. *This strongly suggests the vast majority of these mutations were present in the animals of origin. The odds of the exact nucleotide changes occurring in the exact same position of the exact same gene at the exact same ratios in almost every case are effectively zero.*

To summarize, our opinion is that the authors failed to sufficiently control the reported study in such a way that one could conclude that CRISPR induces the observed mutations. In our view, the genetic differences seen in this comparative analysis were likely present prior to editing with CRISPR. We encourage the authors to follow up with an appropriately controlled experiment as understanding and controlling the specificity of CRISPR technology is essential for research and critical for therapeutic development. We are firmly committed to a rigorous, objective, and comprehensive assessment of specificity in our own work and seek to advance a shared understanding in the field of how to best assess this critical parameter for bringing CRISPR-based medicines to patients with genetically-defined or genetically-treatable diseases.

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