Supplementary Discussion

***Overview of previous studies examining off-target mutations* in vivo**

As noted in the main text, previously published studies that have reported no or very few off-target mutations *in vivo* used approaches that were not validated to effectively identify these mutations[11-14, 22-34]. Most of these reports used *in silico* approaches that are known to miss *bona fide* off-target sites in cells, making their efficacy for identifying these sites *in vivo* uncertain. Three of these studies[12-14] used the cell-based GUIDE-seq method[10] to determine what sites to examine *in vivo* and only one found a single low-level off-target (frequency ~1%); however, GUIDE-seq was performed on surrogate cells in culture in these experiments, a strategy that may miss off-target sites that occur in the actual target tissue *in vivo*. Given the capability of VIVO to identify off-target sites *in vivo*, it may be of interest to use the method to re-examine previously published *in vivo* off-target profiles of nucleases assessed by other methods to see if additional unwanted alterations are or are not elucidated.

Potential explanations for relatively greater reductions in plasma Pcsk9 protein levels compared to efficiencies of* Pcsk9 *genetic mutation

Previous studies have also shown anenhanced reduction of plasma Pcsk9 relative to the frequency of *Pcsk9* genetic alteration[11, 33]. This phenomenon may be due to decreased PCSK9 level in plasma following genome editing leads to an effective increase in the concentration of available LDL receptor (**LDLR**) at the cell surface (Pcsk9 binds to LDLR and they are internalized together). Thus, as a result of increased LDLR availability, there might then be an additional reduction in PCSK9 plasma levels. Alternatively, the actual frequency of *Pcsk9* genetic alteration

might be higher than what is observed because adenoviral vectors infect only hepatocytes but indels were measured on genomic DNA isolated from all cell types in the liver.

***CIRCLE-seq identifies a super-set of the off-target sites mutagenized* in vivo**

In our VIVO experiments, we believe that CIRCLE-seq identified a superset of all potential off-target cleavage sites that includes a subset of *bona fide* off-target sites actually mutagenized *in vivo*. This observation is consistent with previously published work in which CIRCLE-seq also identified supersets of off-target sites that included those actually mutagenized in cells expressing different CRISPR-Cas9 nucleases[8]. This high sensitivity for identifying *bona fide* off-target sites is most likely due to the very high protein concentrations used in the CIRCLE-seq assay. Specifically, the nuclease is present at a ten-fold molar excess over the total concentration of circularized genomic DNA (**Methods**), a condition that is not achievable in cells. As such, we envision that at least some (or, in cases like gM or gMH where very few closely related sites are present, perhaps all) of the sites cleaved *in vitro* in the CIRCLE-seq assay might not show significant evidence of indels in living cells. Consistent with this, in our previous work testing sites identified from CIRCLE-seq for off-target cleavage in human cells in culture, we found that as lower concentrations of nuclease were used, bona fide off-target sites (that had been verified in human cells) were still effectively identified whereas fewer of the sites that were not verified in human cells were observed (see Supplementary Fig. 12b in our earlier CIRCLE-seq report[8]). Taken together, these data support the idea that high nuclease concentration *in vitro* accounts for the higher sensitivity of CIRCLE-seq for identifying off-target

sites, although the presence of chromatin might also explain why some sites are not mutated in cells *in vivo* or in culture.

Reduction of* in vivo *off-target effects using previously published strategies

Although this current study used wild-type SpCas9, it will be of interest in future studies to assess whether other gRNAs that exhibit off-target mutations *in vivo* with SpCas9 can have these off-targets reduced to undetectable levels by using high-fidelity CRISPR-Cas nuclease variants[35-37] and/or by delivery of Cas9 mRNA/gRNA or ribonucleoprotein complexes rather than DNA[38].

Extension of VIVO to CRISPR-Cas variants and orthologues, other gene-editing nuclease platforms, various delivery methods, and non-mammalian organisms

VIVO should be useful for evaluating the *in vivo* specificities of a wide variety of nucleases in various organisms. Beyond evaluating wild-type SpCas9, the method could also be used to assess the *in vivo* specificities of engineered SpCas9 high-fidelity[35-37] or PAM recognition[39, 40] variants and of other naturally occurring Cas9 orthologues (e.g., Cas9 from *Staphylococcus aureus*[33]). With some minor modification of the CIRCLE-seq protocol, we envision that VIVO could be used with other types of gene-editing nucleases such as zinc finger nucleases, meganucleases, transcription activator-like effector nucleases, and CRISPR-Cpf1/Cas12a nucleases. Although we used adenovirus in this proof-of-concept study to achieve efficient liver delivery because it provided an effective means of transient transgene expression *in vivo* that peaks about 1 week and is limited in duration to about 2-3 weeks[41], VIVO could also be used with other more clinically relevant viral or non-viral delivery strategies (e.g., retroviral and

lentiviral vectors, adeno-associated virus, lipid nanoparticles). The method could also be used to compare and assess the specificities observed with these different nuclease and delivery platforms[42]. Finally, we envision that the VIVO approach should also be generalizable to other non-mammalian organisms (e.g., insects and plants).

References for Supplementary Discussion

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