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BRIEF REPORT

Sequencing data from Massachusetts General Hospital shows Cas9 integration into the genome, highlighting a serious hazard in gene-editing therapeutics [version 1; peer review: 2 approved with reservations]

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Abstract

The ability to edit a specific gene within our genomes using guided-nucleases (Cas9/ZFN/TALEN - CaZiTa) presents huge opportunities for curing many genetic disorders. Delivery of this 'drug' within cells is a critical step for such therapies. The ability of recombinant adeno-associated virus (rAAV) to enter cells makes it a perfect choice as a vector for gene therapy. A plasmid comprising the rAAV, the CaZiTa, guide RNAs (for CRISPR) is expected to enter the cell, edit the target gene(s), remain episomal, and thus fade away with time. However, the rather obvious danger of integration of the plasmid into the genome, if the episomal hypothesis is incorrect, is under-reported. A recent report has highlighted that bacterial genes from a plasmid were integrated into bovine genomes. Massachusetts General Hospital has recently published data on CRISPR edits (Accid:PRJNA563918), noting 'high levels of AAV integration (up to 47%) into Cas9-induced double-strand breaks'. However, there is no mention of Cas9 integration. Here, the same data from Massachusetts General Hospital shows Cas9 integration in the exact edit sites provided for two genes - TMC1 and DMD. Also, there is a mis-annotation of one sample as 'no gRNA', since Cas9 integrations have been detected in that sample. This is an important distinction between AAV and CaZiTa integration: while AAV integration can be tolerated, Cas9 integration is a huge, and unacceptable, danger.

Keywords

Gene-editing, CRISPR-Cas, AAV, plasmid integration

Open Peer Review

Approval Status

	1	2
version 1		
04 Nov 2019	view	view
<ol style="list-style-type: none"> 1. Rakesh Chatrikhi , University of Pennsylvania, Philadelphia, USA 2. Saravanabhavan Thangavel , Christian Medical College Vellore Association, Vellore, India 		
Any reports and responses or comments on the article can be found at the end of the article.		

Corresponding author: Sandeep Chakraborty (sanchak@gmail.com)

Author roles: Chakraborty S: Conceptualization, Methodology, Software, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

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Introduction

Nuclease based gene-editing techniques (Cas9/ZFN/TALEN - CaZiTa) introduce a double stranded break at a specified location with the guide of DNA-binding proteins (ZFN/TALEN) or RNA (CRISPR)¹. Delivery within cells is a critical step for such gene-therapies². The ability of recombinant adeno-associated virus (rAAV) to enter cells makes it a perfect choice as a vector for gene therapy³. A plasmid comprising the rAAV, CaZiTa, guide RNAs (for CRISPR), etc. is expected to enter the cell, edit, and remain episomal⁴. However, the rather obvious danger of integration of the plasmid into the genome is ignored. A recent pre-print⁵ has highlighted that bacterial genes from the template plasmid (pCR2.1-TOPO) has integrated into bovine genomes⁶.

Recently, Massachusetts General Hospital published data on CRISPR edits (Accid:PRJNA563918), and concluded that 'AAV integration should be recognised as a common outcome for applications that utilize AAV for genome editing'⁷. However, there was no report of Cas9 integration. Here, data showing Cas9 integrating in the exact edit sites is shown. The same caveat applies to all three CaZiTa gene-therapies¹.

Methods

Sequencing data was obtained from BioProject accession number, PRJNA563918, using SRA download tools (<https://github.com/ncbi/sra-tools/wiki>). This project aims at finding "the genomic consequences of transduction with AAV vectors encoding CRISPR-Cas nucleases"⁷.

The reads were unquified, split into open-reading frames using the getorf (version:6.6.0.0) program from the EMBOSS suite (<http://emboss.sourceforge.net/download/>)⁸.

Command: "getorf -find 1 -sequence <infile> -outseq <output> -minsize 10".

Exact 10-kmer were aligned to the Cas9 protein (Accid: AGZ01981.1, 1417aa). Subsequently, these reads were then aligned to the gene of interest - TMC1 (Accid:NG_008213.1) or DMD (Accid:NM_001314034.1). The online BLAST interface using the default settings (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to generate images of integration with the genes of interest⁹. The number of reads with integration is conservative, since reads with less than a 10-kmer match were ignored. The AAV genome used was Accid:MK163936.1. Sequences that integrate Cas9/AAV with TMC1 and DMD are provided in *Extended data*: TMC1.cas9.fa/TMC1.aav.fa and DMD.cas9.fa/DMD.aav.fa, respectively.

Results and discussion

Cas9 integration in transmembrane channel like 1 (TMC1)

TMC1, a transmembrane protein, is required for proper functioning of cochlear hair cells, and has been implicated in hearing loss and prelingual deafness¹⁰. In mice, this gene is located in chr19 (Accid:NG_008213.1)). **Table 1** lists the samples sequenced for targeting this gene. As expected, the non-injected controls have no Cas9 reads. The sample SRR10068671

(marked with triple asterisks) has probably been mis-annotated as one with "no gRNA", since Cas9 integrations are detected in that sample. From the Cas9 integration site, the guide RNA (gRNA) for the TMC1 gene can be deduced to be "CATGGTAATGTCCCTCCTGGGGA", although this information is not yet available. The sequences for these reads are provided as extended data. As a specific example, the sequence (SRR10068639.63180.1) encodes the 26aa peptide= SPEKLLMYHHPQTYQKLKLIMEQYG from Cas9 and is merged with the TMC1 gene (**Figure 1**).

Cas9 integration in dystrophin

Duchenne muscular dystrophy (DMD), a genetic disorder associated with progressive muscle degeneration (dystrophy), is caused by aberrations in the dystrophin protein, located on chromosome X in humans and mice¹¹. According to the data (Accid: PRJNA563918), different exons and introns have been targeted using two different variations of the Cas9 - SpCas9 and SaCas9 - which differ in certain characteristics¹². SaCas9 does seem empirically to have more samples without any integration, but that might just be random. The sequences for these reads are provided as extended data. As a specific example, the sequence (SRR10068622.33932.1) encodes the 12aa peptide=LDATLIHQSIITG from Cas9 and is merged with the DMD gene (**Figure 2**).

AAV integration in TMC1 and DMD

The integration of AAV into the genome has already been noted⁷. This has been replicated in this study as well. The AAV genome used was Accid:MK163936.1. The reads can be found in *Extended data*: TMC1.aav.fa (N=5000) and DMD.aav.fa (N=14000).

Conclusions

Gene-editing based therapies provide revolutionary hope for curing many diseases. However, ensuring safety of any such endeavours must be paramount to avoid doing more harm^{13,14}. Plasmid integration is one such potential hazard⁵. Recently, Massachusetts General Hospital reported high-levels of *in vivo* AAV into the genome while providing sequencing data (Accid: PRJNA563918), and concluded that 'AAV integration should be recognized as a common outcome for applications that utilize AAV for genome editing'⁷. In this study, in addition to AAV, Cas9 integration is shown in the same samples. The same caveat applies to gene-therapies using CaZiTa¹. Off-target edits (OTE) are an important aspect of CRISPR-cas gene-editing¹⁵⁻¹⁹. Such integrations, found by targeting amplicon sequencing, will only get worse due to OTEs, which are hard to detect²⁰⁻²². Another problem is pre-existing immunity to Cas9 proteins^{23,24}. This problem can be mitigated by sending the CaZiTa as protein²⁵, but that would seriously restrict the use-cases, and also suffer from OTEs, large deletions, complex rearrangements and translocations²⁶, or even including fragments from exosomes²⁷. This is an important distinction between AAV and CaZiTa integration, since AAV integration can be tolerated (integration at chromosome breakage points²⁸, though there is debate on its role in hepatocellular carcinoma^{29,30}), Cas9 integration is a huge, and unacceptable

Table 1. Integration of Cas9 into the edited gene. The paired reads are clubbed into a single file, and then unclubbed. Open reading frames from these are then matched to Cas9 proteins for 10 aa, and subsequently these reads are matched to the gene of interest (TMC1 or DMD), helping identify the exact edit site. The sample SRR10068671 (marked with triple asterisks) has probably been mis-annotated as one with "no gRNA", since Cas9 integrations are detected in that sample. These integrations happen both for SpCas9 and SaCas9. From the Cas9 integration site, the gRNA for the TMC1 gene can be deduced to be "CATGGTAATGTCCCTCCTGGGGA", although this information is not yet available. CN, cortical neuron; CA: cochlea, adult.

Gene	Accid	Description	NREADS	NUNIQ	NCas9	NTarget
	SRR10068641	<i>In vivo</i> CA non injected control	140156	12184	0	0
	SRR10068642	<i>In vivo</i> CA non injected control	160880	9511	0	0
	SRR10068670	CN neonatal no gRNA	164390	7079	0	0
***	SRR10068671	CN neonatal no gRNA	259600	10794	10	10
	SRR10068648	CN neonatal 2	243658	16919	90	77
TMC1	SRR10068659	CN neonatal 1	220840	14033	108	98
	SRR10068666	CN neonatal 2	230024	20975	772	682
	SRR10068667	CN neonatal 1	208190	17984	598	546
	SRR10068636	<i>In vivo</i> CA 4	187904	16498	0	0
	SRR10068638	<i>In vivo</i> CA 3	117948	7866	2	1
	SRR10068639	<i>In vivo</i> CA 2	222014	15268	10	8
	SRR10068640	<i>In vivo</i> CA 1	136336	9321	4	2
	SRR10068621	<i>In vivo</i> exon 53	324432	13477	2	2
	SRR10068622	<i>In vivo</i> exon 53	285824	12335	7	5
	SRR10068623	<i>In vivo</i> intron51-53	382044	18977	312	238
	SRR10068624	<i>In vivo</i> intron51-53	370728	19900	286	238
	SRR10068625	<i>In vivo</i> intron 53	289852	10673	6	6
DMD	SRR10068627	<i>In vivo</i> intron 53	253460	9596	14	12
SpCas9	SRR10068628	<i>In vivo</i> intron 51	388128	16116	4	3
	SRR10068629	<i>In vivo</i> intron 51	267736	10710	7	6
	SRR10068630	<i>In vivo</i> intron51-53	336368	12750	48	5
	SRR10068631	<i>In vivo</i> intron51-53	472724	20169	48	4
DMD	SRR10068632	<i>In vivo</i> intron 53	221456	7786	0	0
SaCas9	SRR10068633	<i>In vivo</i> intron 53	237768	8136	0	0
	SRR10068634	<i>In vivo</i> intron 51	428600	17698	0	0

Figure 1. Specific example of a sequence in the TMC1 gene which has Cas9 integrated. The sequence (SRR10068639.63180.1) encodes the 26aa peptide=SPEKLLMYHDPQTYQKLKLIMEQYG from Cas9 and is merged with the TMC1 gene.

Figure 2. Specific example of a sequence in the DMD gene which has Cas9 integrated. The sequence (SRR10068622.33932.1) encodes the 12aa peptide=LDATLIHQSTIG from Cas9 and is merged with the DMD gene.

danger. After integration, individuals may be expressing Cas9, and that is fraught with genotoxic danger.

Data availability

Underlying data

Raw sequence reads from Massachusetts General Hospital, Accession number PRJNA563918, <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA563918/>

Extended data

Zenodo: Sequencing data from Massachusetts General Hospital shows Cas9 integration into the genome, highlighting a serious

hazard in gene-editing therapeutics, <https://doi.org/10.5281/zenodo.3460305>³¹

This project contains the following extended data:

- DMD.aav.fa
- DMD.cas9.fa
- TMC1.aav.fa
- TMC1.cas9.fa

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CC-BY 4.0).

References

- Gaj T, Gersbach CA, Barbas CF 3rd: **ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering.** *Trends Biotechnol.* 2013; **31**(7): 397–405. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- LaFontaine JS, Fathe K, Smyth HD: **Delivery and therapeutic applications of gene editing technologies ZFNs, TALENs, and CRISPR/Cas9.** *Int J Pharm.* 2015; **494**(1): 180–194. [PubMed Abstract](#) | [Publisher Full Text](#)
- Pillay S, Zou W, Cheng F, et al.: **Adeno-associated Virus (AAV) Serotypes Have Distinctive Interactions with Domains of the Cellular AAV Receptor.** *J Virol.* 2017; **91**(18): pii: e00391-17. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Naso MF, Tomkowicz B, Perry WL 3rd, et al.: **Adeno-Associated Virus (AAV) as a Vector for Gene Therapy.** *BioDrugs.* 2017; **31**(4): 317–334. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Norris AL, Lee SS, Greenlees KJ, et al.: **Template plasmid integration in germline genome-edited cattle.** *bioRxiv.* 2019; 715482. [Publisher Full Text](#)
- Carlson DF, Lancto CA, Zang B, et al.: **Production of hornless dairy cattle from genome-edited cell lines.** *Nat Biotechnol.* 2016; **34**(5): 479–81. [PubMed Abstract](#) | [Publisher Full Text](#)
- Hanlon KS, Kleinstiver BP, Garcia SP, et al.: **High levels of AAV vector integration into CRISPR-induced DNA breaks.** *Nat Commun.* 2019; **10**: 4439. [Publisher Full Text](#)
- Rice P, Longden I, Bleasby A: **EMBOSS: the European Molecular Biology Open Software Suite.** *Trends Genet.* 2000; **16**(6): 276–277. [PubMed Abstract](#) | [Publisher Full Text](#)
- Altschul SF, Madden TL, Schaffer AA, et al.: **Gapped BLAST and PSI-BLAST: a new generation of protein database search programs.** *Nucleic Acids Res.* 1997; **25**(17): 3389–3402. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)

10. Nakanishi H, Kurima K, Kawashima Y, *et al.*: **Mutations of TMC1 cause deafness by disrupting mechanoelectrical transduction.** *Auris Nasus Larynx*. 2014; **41**(5): 399–408.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
11. Hoffman EP, Brown RH Jr, Kunkel LM: **Dystrophin: the protein product of the Duchenne muscular dystrophy locus.** *Cell*. 1987; **51**(6): 919–928.
[PubMed Abstract](#) | [Publisher Full Text](#)
12. Xie H, Tang L, He X, *et al.*: **SaCas9 Requires 5'-NNGRRT-3' PAM for Sufficient Cleavage and Possesses Higher Cleavage Activity than SpCas9 or FnCpf1 in Human Cells.** *Biotechnol J*. 2018; **13**(4): e1700561.
[PubMed Abstract](#) | [Publisher Full Text](#)
13. Couzin J, Kaiser J: **Gene therapy. As Gelsinger case ends, gene therapy suffers another blow.** 2005; **307**(5712): 1028.
[PubMed Abstract](#) | [Publisher Full Text](#)
14. Saâda-Bouid E, Defauchaux C, Karabadjian A, *et al.*: **Hyperprogression during anti-PD-1/PD-L1 therapy in patients with recurrent and/or metastatic head and neck squamous cell carcinoma.** *Ann Oncol*. 2017; **28**(7): 1605–1611.
[PubMed Abstract](#) | [Publisher Full Text](#)
15. Fu Y, Foden JA, Khayter C, *et al.*: **High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells.** *Nat Biotechnol*. 2013; **31**(9): 822–826.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
16. Pattanayak V, Lin S, Guiling JP, *et al.*: **High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity.** *Nat Biotechnol*. 2013; **31**(9): 839–43.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
17. Kescu C, Arslan S, Singh R, *et al.*: **Genome-wide analysis reveals characteristics of off-target sites bound by the Cas9 endonuclease.** *Nat Biotechnol*. 2014; **32**(7): 677–83.
[PubMed Abstract](#) | [Publisher Full Text](#)
18. Chakraborty S: **Inconclusive studies on possible CRISPR-Cas off-targets should moderate expectations about enzymes that have evolved to be non-specific.** *J Biosci*. 2018; **43**(2): 225–228.
[PubMed Abstract](#) | [Publisher Full Text](#)
19. Chakraborty S: **CRISPR-Cas off-target detection using Oxford Nanopore sequencing - is the mitochondrial genome more vulnerable to off-targets?** *bioRxiv*. 2019; 741322.
[Publisher Full Text](#)
20. Wienert B, Wyman SK, Richardson CD, *et al.*: **Unbiased detection of CRISPR off-targets *in vivo* using DISCOVER-Seq.** *Science*. 2019; **364**(6437): 286–289.
[PubMed Abstract](#) | [Free Full Text](#)
21. Tsai SQ, Nguyen NT, Malagon-Lopez J, *et al.*: **CIRCLE-seq: a highly sensitive *in vitro* screen for genome-wide CRISPR-Cas9 nuclease off-targets.** *Nat Methods*. 2017; **14**(6): 607–614.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
22. Tsai SQ, Zheng Z, Nguyen NT, *et al.*: **GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases.** *Nat Biotechnol*. 2015; **33**(2): 187–197.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
23. Charlesworth CT, Deshpande PS, Dever DP, *et al.*: **Identification of pre-existing adaptive immunity to cas9 proteins in humans.** *bioRxiv*. 2018; 243345.
[Publisher Full Text](#)
24. Ferdosi SR, Ewaisha R, Moghadam F, *et al.*: **Multifunctional CRISPR-Cas9 with engineered immunosilenced human T cell epitopes.** *Nat Commun*. 2019; **10**(1): 1842.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
25. Kim S, Kim D, Cho SW, *et al.*: **Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins.** *Genome Res*. 2014; **24**(6): 1012–1019.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
26. Kosicki M, Tomberg K, Bradley A: **Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements.** *Nat Biotechnol*. 2018; **36**(8): 765–771.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
27. Ono R, Yasuhiko Y, Aisaki Ki, *et al.*: **Exosome-mediated horizontal gene transfer occurs in double-strand break repair during genome editing.** *Commun Biol*. 2019; **2**: 57.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
28. Miller DG, Petek LM, Russell DW: **Adeno-associated virus vectors integrate at chromosome breakage sites.** *Nat Genet*. 2004; **36**(7): 767–73.
[PubMed Abstract](#) | [Publisher Full Text](#)
29. Nault JC, Datta S, Imbeaud S, *et al.*: **AAV2 and Hepatocellular Carcinoma.** *Hum Gene Ther*. 2016; **27**(3): 211–213.
[PubMed Abstract](#) | [Publisher Full Text](#)
30. Berns KI, Byrne BJ, Flotte TR, *et al.*: **Adeno-Associated Virus Type 2 and Hepatocellular Carcinoma?** *Hum Gene Ther*. 2015; **26**(12): 779–781.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
31. Chakraborty S: **Sequencing data from Massachusetts General Hospital shows Cas9 integration into the genome, highlighting a serious hazard in gene-editing therapeutics.** 2019.
<http://www.doi.org/10.5281/zenodo.3460305>

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Version 1

Reviewer Report 14 April 2023

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Saravanabhavan Thangavel

Centre for stem cell research (CSCR), a unit of inStem Bengaluru, Christian Medical College Vellore Association, Vellore, Tamil Nadu, India

Recombinant adeno-associated virus (rAAV) is one of the tools to deliver Cas9 into the target cells. The author uses the publicly available dataset containing rAAV delivered Cas9 edited cells to analyse the genomic regions around the cutsite. While it has previously been demonstrated that the AAV genome can integrate into the cutsite, this study detects a 12 to 26 a.a peptide from cas9 integrating into the cutsite.

This discovery is significant because integration of the Cas9 sequence has never been demonstrated before, and the consequences of integration and long-term expression of Cas9 are unknown. This emphasizes the importance of carefully selecting a delivery vehicle for Cas9.

- It's not clear from the writing that the Cas9 is targeted towards TMC1, DMD.
- It's not clear that the sequence of saCas9 used for probing
- The authors also miss to cite an important work from Naldini's lab showing sequestering of AAV genome into the cutsite when AAV6 was used as a HDR donor.
- The possibility of sgRNA or its scaffold integrating into the cutsite should be discussed as well.
- The methodology used for detecting the Cas9 sequence can be more elaborate

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Genome editing, Hematopoietic stem cell gene therapy

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 06 February 2020

<https://doi.org/10.5256/f1000research.22812.r59005>

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Rakesh Chatrikhi

Department of Biochemistry and Biophysics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Chakraborty studied sequencing data on CRISPR-induced DNA edits from Massachusetts General Hospital to show Cas9 integration into the genome. The author analyzed previously published data on CRISPR-induced DNA edits to highlight integration of Cas9 into the genome, which was not reported in the previously published study that generated the sequencing data. This study highlights risks involved in using gene-editing techniques such as CRISPR-Cas9 based system and raises important caveats that need to be considered when using the technology for therapeutics. However, the author needs to address the following points to strengthen the manuscript:

1. The author needs to quantitatively compare the levels of AAV and Cas9 integration and whether it is statistically significant. This is important because the author is using the same samples and sequencing data to discuss Cas9 integration into the genome, which was not reported earlier and thus required to effectively highlight the results in this study. The author needs to make a clear comparison of AAV and Cas9 integration from the data in the Results and Discussion section.

2. The author needs to support the argument that Cas9 integration is a serious hazard and is a huge unacceptable danger. This is important because the author argues that Cas9 integration is hazardous throughout the manuscript (title, abstract and conclusion). The author needs to include multiple references to previous work in the literature that have shown or highlighted the toxicity of Cas9 integration into the genome and that it is hazardous.
3. In the Conclusions section, the author needs to briefly discuss the alternative approaches that could be used to avoid Cas9 integration. For example, the author needs to elaborate on the use of purified Cas9 ribonucleoproteins as a substitute to plasmids. The author included a sentence on this topic in the Conclusions section, but need to elaborate on the advantages of using Cas9 ribonucleoproteins. For example, in addition to avoiding Cas9 integration, this method has higher efficiency (Farboud B et al., J Vis Exp, 2018), fewer off-targets (Liang X et al., Journal of Biotech, 2015; Kim S et al., Genome Res, 2014 - Ref 25 in this study)^{1,2}.
4. The author needs to include the rationale for focusing on the genes TMC1 and DMD. Were those genes studied in the previous study that generated the data (Ref 7)? This needs to be clearly stated. If there are more genes studied in Ref 7, then the author needs to include the rationale of choosing these two genes over other genes in the study.
5. The Introduction section could also be strengthened by briefly discussing if there are any similar studies carried out previously in the literature studying the level of Cas9 integration into the genome and their limitations.

References

1. Liang X, Potter J, Kumar S, Zou Y, et al.: Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection. *J Biotechnol.* 2015; **208**: 44-53 [PubMed Abstract](#) | [Publisher Full Text](#)
2. Kim S, Kim D, Cho SW, Kim J, et al.: Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. *Genome Res.* 2014; **24** (6): 1012-9 [PubMed Abstract](#) | [Publisher Full Text](#)

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: T cell Biology; RNA Biology; Cancer Biology; Molecular Biology; Regulation of gene expression; Protein-RNA interactions; Biochemistry and Molecular Biophysics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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