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Engineering an RNA-based tissue-specific platform for genetic editing through use of a miRNA-enabled Cas12a

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ABSTRACT

The capacity to edit genomes using CRISPR-Cas systems holds immense potential for countless genetic-based diseases. However, one significant impediment preventing broad therapeutic utilization is *in vivo* delivery. While genetic editing at a single cell level *in vitro* can be achieved with high efficiency, the capacity to utilize these same biologic tools in a desired tissue *in vivo* remains challenging. Non-integrating RNA virus-based vectors constitute efficient platforms for transgene expression and surpass several barriers to *in vivo* delivery. However, the broad tissue tropism of viral vectors raises the concern for off-target effects. Moreover, prolonged expression of the Cas proteins, regardless of delivery method, can accumulate aberrant RNAs leading to unwanted immunological responses. In an effort to circumvent these shortcomings, here we describe a versatile RNA virus-based technology that can achieve cell-specific activity and self-inactivation by combining host microRNA (miRNA) biology with the CRISPR-Cas12a RNA-guided nuclease. Exploiting the RNase activity of Cas12a, we generated a vector that self-inactivates upon delivery of Cas12a and an accompanying CRISPR RNA (crRNA). Furthermore, we show that maturation of the crRNA can be made dependent on cell-specific miRNAs, which confers cell-specificity. We demonstrate that this genetic editing circuit delivers diminished yet sufficient levels of Cas12a to achieve effective genome editing whilst inducing a minimal immunological response. It can also function in a cell-specific manner thereby facilitating *in vivo* editing and mitigating the risk of unwanted, off-target effects.

INTRODUCTION

The CRISPR-Cas systems of archaea and many bacteria are sequence-specific adaptive defense systems that have evolved to cleave foreign nucleic acid[1]. This defense system is dependent on acquisition and integration of foreign DNA protospacers in a process generally referred to as adaptation[2]. Once integrated, expression of the so-called protospacers generate a precursor CRISPR RNA (pre-crRNA) which is further processed and matured to produce crRNA – comprised of a 5' direct repeat and the spacer. Finally, crRNA is bound by a Cas nuclease to elicit interference on incoming DNA as defined by complementarity of its guide RNA. Moreover, as the protospacer DNA is inherited, adaptation of a single prokaryotic cell can result in Lamarckian evolution for its offspring[3].

While most of the Cas-nucleases only possess RNA-guided DNase activity, Cas12a also has RNase function[4]. The RNase function is responsible for processing the pre-crRNA by cleaving direct repeat sequences that flank the protospacer[4]. The crRNA that is generated as a result of these processing events is sufficient for instilling specificity onto the DNase activity of Cas12a. Similar to Cas9, Cas12a has also been repurposed as a eukaryotic gene editor[5-8]. However, as Cas12a biology is still in its infancy, its optimization lags behind that of Cas9. Despite this, the ability of Cas12a to process its own crRNA enables one to use it to generate the crRNA from diverse types of RNA so long as it is flanked by direct repeats[9]. This activity not only allows one to generate multiple crRNAs for any number of targets, but it has also enabled the generation of mRNAs that both code for Cas12a and the desired guides on a single transcript[10]. This

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51   is in contrast to the most commonly applied Cas9 editing tool which demands a

52   separate DNA dependent RNA polymerase for production of Cas9 and the single guide

53   RNA[6,11].

54

55   Despite the immense potential of both the Cas9 and Cas12a systems, one significant

56   impediment remains delivery of these large proteins alongside the desired crRNA(s).

57   This challenge is formidable, especially when one wishes to efficiently edit a large

58   number of cells to repair a genetic defect *in vivo*. This problem is further confounded by

59   the fact that maintaining Cas expression for longer periods of time can result in the

60   generation of off-target effects, aberrant RNAs, chromosomal translocations, and/or

61   removal of the Cas-expressing cells[12,13]. Given these challenges, the most optimal

62   genetic editors would be delivered with the efficiency of a virus in a manner that was

63   free of any possibility of genomic integration and would function only in a desired cell

64   type for the time required to achieve editing. To this end, we designed a self-

65   inactivating RNA that expresses Cas12a and processes its own crRNA in a cell-specific

66   manner that is amenable to RNA virus-based delivery.

67

68   Having some parallels with the CRISPR-Cas platform, host miRNAs are rooted in

69   defense systems albeit optimized for the targeting of RNA, rather than DNA[14].

70   Generally described as RNA interference (RNAi), this defense system acquires double

71   stranded RNA (dsRNA) fragments from incoming virus and processes them into small

72   interfering RNAs (siRNAs) similar in length than that of crRNAs. Furthermore, like

73   crRNAs, siRNAs provide specificity to an otherwise non-specific nuclease. In the case

74   of RNAi, siRNAs are bound by a family of Argonaute (AGO) proteins with the capacity to

75   cleave target mRNA in a sequence-specific manner[15]. The emergence of this system

76   was so evolutionarily successful, that the genes involved in this biology duplicated and

77   generated a similar small RNA based system commonly referred to as microRNAs

78   (miRNAs)[16]. Unlike the small RNA-based antiviral systems, miRNAs derive from the

79   host genome but are ultimately processed and utilized in a similar function, even

80   sharing some overlap with the processing machinery of the Type II CRISPR-Cas

81   system[17]. The general repurposing of this biology is believed to have happened

82   independently in plants and animals but, in both examples, the biogenesis of miRNA-

83   mediated regulation enabled another level of transcriptional control which coincided with

84   multicellularity[16].

85

86   One significant difference between antiviral RNAi and host miRNA biology is the

87   complementarity between the small RNA and its target(s). In contrast to antiviral RNAi,

88   miRNAs do not generally bind with perfect complementarity to their targets and

89   therefore do not induce cleavage, owing to the fact that they are derived from host

90   genes[16]. As a result, miRNAs are often considered 'fine-tuners' of host biology and are

91   thought to act over the course of days or weeks[18]. In contrast, virus-derived siRNAs

92   can engage their target with complete complementarity, as they derive from the

93   pathogen itself, and thus enable both cleavage and the recycling of the small RNA –

94   achieving silencing within hours[16]. However, as this difference in biology is almost

95   completely defined by complementarity, if one introduces a perfect binding site for a

96   given miRNA into a transcript, it will undergo potent silencing more akin to the normal

97   activity of antiviral RNAi[19]. This exploitation of miRNA biology therefore enables one to

98   generate viruses or virus-based vectors which can be ubiquitously targeted or function

99   in a species-, tissue-, cell-, or even signaling-specifc manner based on endogenous

100   miRNA expression[20-26].

101

102   In an effort to generate an RNA-based DNA editor that functions in a cell-specific

103   manner that would be amenable for *in vivo* use, we combined CRISPR-Cas and miRNA

104   biology. In brief, we utilize the fact that Cas12a processes its own pre-crRNA to make a

105   vector that delivers both Cas12a and crRNA and in doing so, inactivates itself. To this

106   end, we encode crRNAs in the 3'-UTR of Cas12a and show that it leads to self-

107   cleavage of its own transcript. Moreover, we demonstrate that delivery of this self-

108   inactivating construct is sufficient to achieve efficient gene editing. And lastly, we show

109   that processing of the pre-crRNA can be made to be dependent on miRNA-expression

110   thereby conferring cell-type specificity on our editing platform.

111

RESULTS

113   In an effort to build a single mRNA transcript that could both self-inactivate and function

114   in a cell-specific manner, we designed a construct encoding an enhanced green

115   fluorescent protein (GFP) and an HA epitope-tagged Cas12a separated by a P2A

116   peptide site[27] with its targeting instructions in the 3' UTR (Fig. 1a). To achieve self-

117   inactivation, we incorporated a crRNA in the 3' untranslated region (UTR) of the GFP-

118   Cas12a mRNA that consisted of a direct repeat and a spacer (DR). Accurate

119   processing of this crRNA relies on a second motif found 3' of the spacer comprising a

120   perfect miRNA target (miR-T) site. In the presence of the cognate miRNA, Ago2 as part

121   of the RNA induced silencing complex (RISC), will be recruited and result in 3' cleavage

122   of the crRNA[19].   As miRNAs can be cell-specific, this synthetic construct would

123   inactivate itself ubiquitously while only generating functional crRNA in a desired cell type

124   where the cognate miRNA is present (Fig. 1a).

125

126   To initially characterize self-inactivation, we experimented with variations on the DR

127   design to ascertain the relationship between Cas12a engagement and loss of GFP

128   expression (Fig. 1b). To this end, we utilized either canonical direct repeats, direct

129   repeats that would be poorly or unable to be cleaved by Cas12a (A18G and UUAA,

130   respectively), or one in which the direct repeats were disrupted altogether (scrambled;

131   scrbl)[9,28]. To determine how these constructs would function, they were introduced into

132   fibroblasts and monitored for GFP expression by both fluorescence microscopy and

133   western blot (Fig. 1b-c). These data demonstrated that the GFP expression from the

134   construct containing canonical direct repeats showed only low levels of fluorescence or

135   expression by western blot which could also be correlated with HA-Cas12a expression.

136   When the direct repeats were comprised of the A18G sites, fluorescence increased as

137   compared to canonical sites. This enhanced expression could also be further

138   corroborated by western blot analysis of both GFP and HA-Cas12a suggesting self-

139   inactivation was diminished with the A18G sites. When the direct repeats were made to

140   be uncleavable by Cas12a (UUAA), GFP expression was comparable to a construct

141   lacking any direct repeats (scrbl). These data could be further validated by small RNA

142   northern blot which indicated that wild type direct repeats, and to a lesser extent A18G,

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143   was capable of generating a visible crRNA, inversely correlating to the GFP and HA-

144   Cas12a protein levels (Fig. 1c-d).

145

146   We next sought to make correct processing of the crRNA dependent on miRNA-

147   mediated cleavage to enable cell-specificity. To this end, we replaced the 3' direct

148   repeat with two target sites for an endogenously expressed miRNA, miR-106a or an

149   irrelevant control target sequence (ctrl-T) while keeping a wild-type direct repeat on the

150   5' end to mediate self-inactivation (Fig. 1b). GFP and Cas12a protein expression from

151   these two constructs were comparable to that containing two canonical direct repeats

152   indicating that a single direct repeat is sufficient for self-inactivation (Fig. 1b-c). In

153   contrast, the crRNA is no longer processed when the 3' direct repeat is replaced with

154   the control miRNA target sequence (ctrl-T) indicating a lack of cleavage (Fig. 1d).

155   However, when ctrl-T is replaced with target sites corresponding to miR-106a, which is

156   abundantly expressed in fibroblasts, we observe a distinct product corresponding to

157   Ago2-based cleavage (Fig. 1d). As Ago2 cleaves its target site between bases 10-11[16],

158   the resulting crRNA contains an extended 3' terminus which should not impede Cas12a

159   targeting or specificity[29].

160

161   To ascertain whether the product of 5' direct repeat and a 3' miRNA cleavage site

162   remains functional, we next expressed variants of our RNA construct that encoded a

163   crRNA targeting beta 2 microglobulin (B2M).   In comparing transcripts lacking direct

164   repeats (scrbl), having both direct repeats, or containing a 5' direct repeat with either a

165   control 3' target sequence (ctrl) or miR-106a 3' sites we observe loss of MHC Class I, a

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166   proxy for B2M targeting, only in conditions in which the 3' end of the spacer contained a

167   wild type direct repeat or the miR-106a target sites (Fig. 1d). These data demonstrate a

168   ~14% reduction of MHC1 with the canonical Cas12a targeting system which increases

169   to greater than 30% targeting in the presence of miR-106a despite the extended crRNA

170   (Fig. 1d-e). Together, these data suggest that miRNA biology can be exploited in

171   conjunction with Cas12a-based processing to generate a single RNA capable of both

172   self-inactivation and cell-specific targeting.

173

174   Given the capacity of a single transcript to both yield a functioning Cas12a editing

175   platform and undergo self-inactivation, we next assessed whether this biologic circuit

176   could be applied to a viral modality that would be most amenable to *in vivo* delivery. For

177   safety reasons[30], this work was done with an RNA virus incapable of spread and

178   containing a scrambled crRNA with no complementarity to a genomic sequence.

179   Utilizing only the RNA dependent RNA polymerase (RdRp) of Nodamura virus and the

180   5' and 3' uncoding material required for RdRp recognition as described elsewhere[31], we

181   generated a self-replicating RNA (herein referred to as a replicon) to express GFP,

182   Cas12a, and a 3' crRNA-containing UTR (Fig. 2a). As observed from our original

183   plasmid design, launching of this replicon showed self-inactivation was also achieved

184   using a viral-based delivery system (Fig. 2b). Comparable to what was observed using

185   DNA, the RNA-based circuit equivalent showed self-targeting with canonical direct

186   repeats (either one or two) with an intermediate phenotype observed for the A18G

187   variant.

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189   To determine whether the transcriptional response to our self-inactivating circuit would

190   be amenable to *in vivo* use, we next performed bulk RNA sequencing to ascertain the

191   transcriptional response to Cas12a expression and/or crRNA processing. To this end,

192   we first compared the expression of Cas12a that was capable of self-inactivation and

193   compared it to one incapable thereof. Surprisingly, this sequencing data set revealed

194   that in contrast to sustained expression of Cas12a alone, the self-inactivating plasmid

195   resulted in a significant number of differentially expressed genes (DEGs) (Fig. 3a and

196   Supplementary Table 1). All upregulated genes with a log2fold change greater than 1

197   and an adjusted p-value less than 0.01 were annotated as belonging to the interferon

198   response (Supplementary Table 1). These data would indicated that Cas12a

199   processing of its own RNA results in a significant accumulation of aberrant RNA

200   capable of inducing the host antiviral defenses. In contrast, this same comparison using

201   the replicon-based platform yielded no DEGs (Fig. 3b). To determine if the lack of an

202   interferon signature in response to the replicon-based platform was simply the result of

203   having it generated in both conditions as a result of RdRp activity, we next compared

204   the plasmid-based Cas12a system with processable crRNA to the equivalent replicon

205   platform (Fig. 3c). This comparison yielded a larger number of DEGs but the interferon

206   signature remained limited to plasmid-based delivery of Cas12a and crRNA

207   demonstrating that the replicon self-inactivation is potent enough to prevent a cellular

208   antiviral response (Supplementary Table 2). This was further corroborated by replicon

209   read numbers which show that self-inactivation prevents any accumulation of either

210   positive or negative sense transcripts that might otherwise serve as pathogen

211   associated molecular patterns (Fig. 3d).

212

213 **DISCUSSION**

214 Here we present data demonstrating that RNA-based platforms can be designed to

215 support safe and effective genetic editing. Based on the dual RNase and DNase

216 properties of Cas12a, we show that RNA constructs can be engineered to be self-

217 targeting. This attribute ensures that Cas12a and crRNA expression is temporal,

218 thereby minimizing off-target editing and the accumulation of aberrant and potentially

219 inflammatory RNA. In addition, self-inactivation also allows one to adopt a RNA virus-

220 based delivery system, as self-cleavage minimizes cytotoxicity and ensures no

221 persistence or genomic integration.

222

223 Having shown that an RNA-based replicon can deliver a self-inactivated Cas12a and

224 crRNA in the absence of a transcriptional response, we next sought to engineer this

225 construct so that it would only function in a desired cell type. In general, nucleic acid-

226 based therapeutics and gene therapy vectors rely on promoter elements that are

227 uniquely specific to a desired cell type. While this strategy has achieved some

228 noteworthy successes, use of DNA as a vector introduces other unwanted issues

229 including the need for entry into the nucleus and the possibility of genomic integration.

230 RNA-based vectors mitigate this risk by having no DNA phase and performing all of

231 their function in the cytoplasm[32]. Given these attributes, we chose to adapt miRNA-

232 based targeting as a means of instilling cell-specific activity. Here we show that the

233 addition of a perfect complementary miRNA target site can replace the 3' direct repeat

234 needed to liberate a desired crRNA and thus render its biology cell specific. Together

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235   with the knowledge that every tissue or cell-type has a unique miRNA profile, these data

236   suggest that one can engineer an RNA-based vector to efficiently enter the cytoplasm

237   and then function only in those cells where editing is desired.

238

239   The completion of the human genome project ushered in hope that treating genetic

240   diseases would soon become possible[33]. This dream was further fueled by the

241   discovery and repurposing of the bacterial and archaeal CRISPR-Cas immune systems

242   which provided unprecedented genome editing capabilities[1]. Indeed, recent efforts

243   have suggested that this promise is closer to reality than ever before as demonstrated

244   through the *ex vivo* editing of human T cells for therapeutic purposes[13].   While this

245   latter accomplishment may provide countless genetic treatments, the full potential of

246   CRISPR-based therapeutic still will require a vehicle for *in vivo* editing. Here we provide

247   a platform to enable this next advancement. By exploiting the biology of RNA viruses,

248   Cas12a, and miRNAs, here we demonstrate that one can design a single RNA that

249   could be systemically delivered with high efficiency but only function for a set amount of

250   time and only within a desired cell lineage. The basic principle underlying this biology

251   could be used with the replicon based strategy outlined here or with existing RNA virus

252   based genetic editors[32]. Taken together, with the rapid advancements of synthetic

253   biology, new CRISPR-Cas systems, and our understanding of virus-host interactions,

254   our progress towards *in vivo* editing may allow us to view genetic-based errors as

255   something that can be seamlessly overwritten.

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METHODS

Cells

All fibroblasts used in this paper were HEK293T cells which were maintained in Dulbecco's

Modified Eagle Medium (Gibco®) supplemented with 1x penicillin-streptavidin solution

(Corning®) and 10% fetal bovine serum (Corning®).

Plasmids

All constructs were synthesized by Twist Biosciences. The sequence will be deposited on NCBI

and the plasmid will be made available on AddGene following publication.

Western blot

Whole cell extract was prepared from live cells lysed in 1% NP-40 lysis buffer. 2µg (pEGFP

plasmid-based experiments) and 70µg (nodamuravirus-based experiments) were analyzed by

SDS-PAGE on a 4-15% acrylamide gradient gel (BIO-RAD®) and gels were subsequently

blotted onto a 0.45µm nitrocellulose membrane (BIO-RAD®) and blocked in 5% milk in TBS for

1h at room temperature. Blots were probed with the following primary antibodies in 5% milk in

TBS-T overnight at 4°C: anti-HA (clone HA-7, MilliporeSigma®), anti-GFP (ab290, Abcam®),

anti-actin (clone Ab-5, Thermo Scientific®) and anti-GAPDH (G9545, MilliporeSigma®). After 4

x 5min washes in 1x TBS-T, blots were probed with HRP-linked secondary antibody for 1h at

room temperature (anti-mouse, NA931V or anti-rabbit, NA934V, GE Healthcare®) and

developed using the Immobilon Western HRP Substrate Kit (MilliporeSigma®).

Small RNA Northern blot

Total RNA was extracted from live cells using TRIzol (Invitrogen). Northern blot was performed

as described in with 20µg total RNA per sample[34]. Probes included the following: B2M-crRNA

283   (5'-GCTGGATAGCCTCCAGGCCA-3') miR-106a (5'-CTACCTGCACTGTAAGCACTTTT-3') and U6

284   (5'-GCCATGCTAATCTTCTCTGTATC-3'). Probes were labeled with ATP-P32 using T4

285   polynucleotide kinase (NEB®) and blot was exposed to a phosphor screen (GE®) and

286   developed on a Typhoon TRIO Storage Phosphorimager (GE®).

287

288   **Flow cytometry**

289   Roughly 7.5×10^5 cells/well were plated on 6-well plates. After attaching overnight, cells were

290   transfected using lipofectamine 2000 (Invitrogen®) and were passaged 1:5 when they reached

291   ~80% confluency for up to ten days. For flow cytometry analysis, cells were trypsinized and

292   washed 2 x stain buffer (FBS) (BD Biosciences®) and stained using the BD Cytofix/Cytoperm

293   Fixation/Permeabilization Kit (BD Biosciences®). The following antibodies and dyes were used:

294   anti-human HLA-A,B,C Pacific Blue (clone W6/32, BioLegend®), anti-HA Alexa Fluor 647 (clone

295   HA.11, BioLegend) and LIVE/DEAD stain Aqua (ThermoFisher®). Fixed cells were analyzed on

296   a 2019 Attune NxT Flow Cytometer. Data processing was done with FlowJo v. 10.6.2.

297

298   **RNA sequencing**

299   Total RNA was extracted using TRIzol (Invitrogen®). 1µg of total RNA per sample was used as

300   starting material for building Illumina libraries for deep sequencing. Libraries were built following

301   Illumina protocols using the TruSeq Stranded mRNA library prep kit (Illumina®). Libraries were

302   enriched for polyadenylated transcripts and sequenced on an Illumina NextSeq 500. Alignment

303   of reads was done using STAR alignment to the human reference genome (hg38) and

304   differential gene expression and statistics were determined by the DEseq2 protocol. Reads

305   mapping to the Nodamuravirus replicon were aligned using Bowtie2.

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308 **FIGURES**

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312 **Fig. 1: Self-inactivation and tissue specificity of Cas12a vector.**

313 **a,** A schematic of how self-inactivation and miRNA-based tissue specificity is incorporated into

314 one single Cas12a vector. GFP and Cas12a are encoded in one open reading frame separated

315 by a P2A site. The 3'-UTR of the same transcript consists of a direct repeat (DR), a spacer with

316 complementarity to a genomic target and a sequence with perfect complementarity to a miRNA

317 referred to as a miRNA-target site (miR-T) and lastly an SV40 polyadenylation signal (pA). **b**,

318 Variations on the construct depicted in **a** with a mutation of nucleotide 18 in the direct repeat

319 from an A to a G (A18G), inversion of nucleotides 16-19 from AAUU to UUAA (UUAA) or a

320 scrambled sequence of the entire direct repeat (scrbl). The last two constructs have two target

321 sites to either a control-miRNA (ctrl-miRT) or miR-106a (miR-106T). Fluorescence images are

322 representative of GFP expression 48 hours after transfection with the constructs indicated. **c**,

323 Western blot of cells transfected for 48 hours with the constructs depicted in **b**, -, no crRNA in

324 3'-UTR, utrf., untransfected cells. **d**, Small RNA Northern blot of total RNA from cells as in **c**,

325 U6, U6 snRNA (loading control). **e**, MHC Class I cell surface expression measured ten days by

326 flow cytometry ten days prost transfection. Data from cells transfected with the constructs

327 overlaid as indicated.

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a

b

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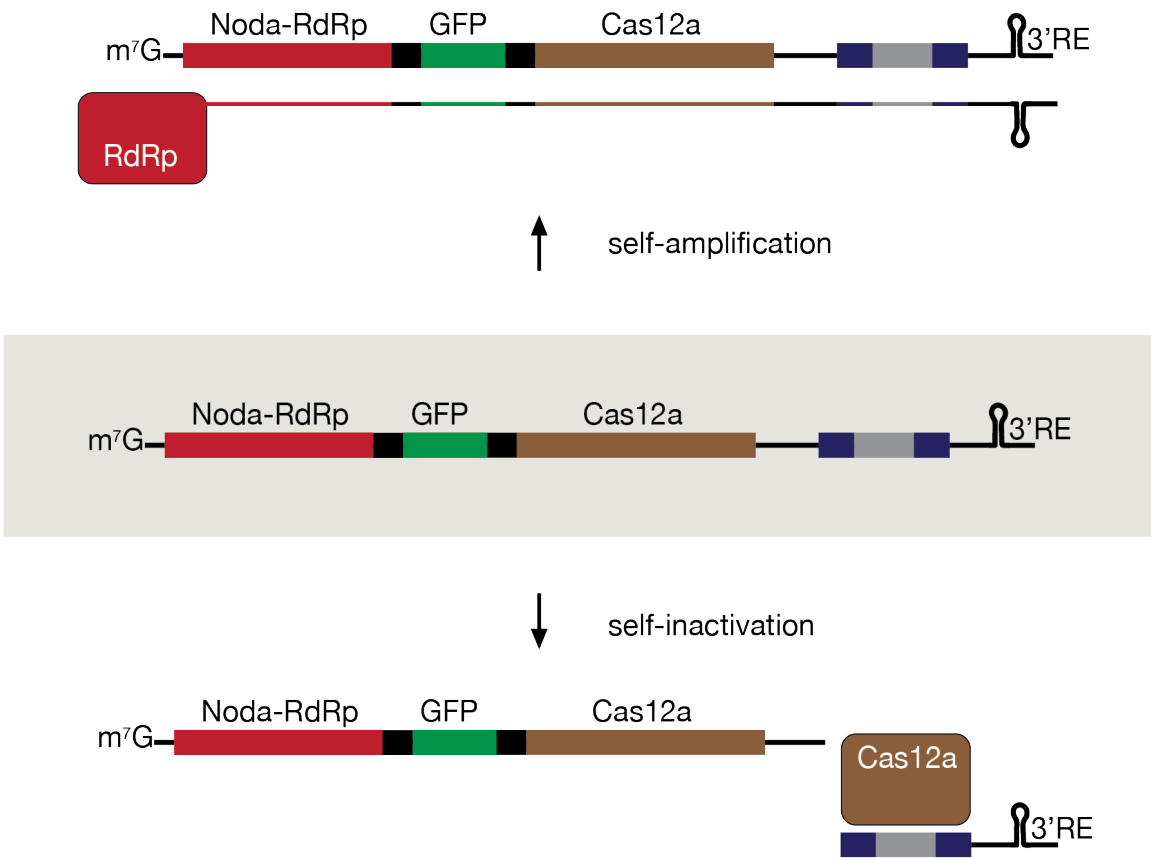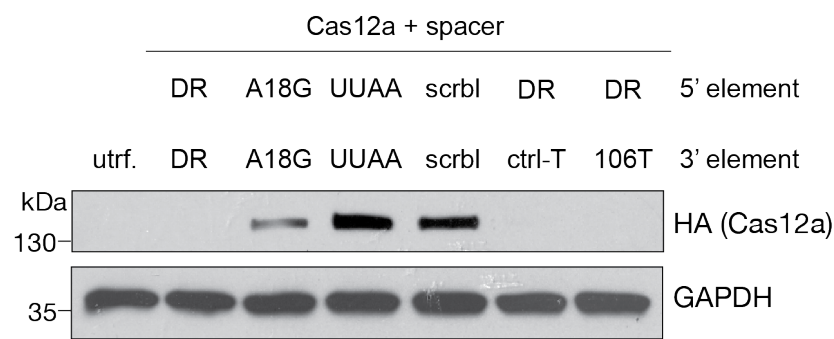
334

335 **Fig. 2: Delivery of Cas12a from a self-inactivating replicon.**

336 **a**, Schematic of a self-inactivating replicon construct. Nodamuravirus RNA dependent RNA

337 polymerase (Noda-RdRp), GFP and Cas12a are encoded in one open reading frame separated

338 by P2A sites. Downstream of the open reading frame, a spacer (grey) flanked by two direct

339 repeats (dark blue) are inserted. **b**, Western blot of cells transfected with the replicon constructs

340 indicated (as in Fig. 1c).

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a

Plasmid vector

b

Replicon vector

c

Replicon vs plasmid

d
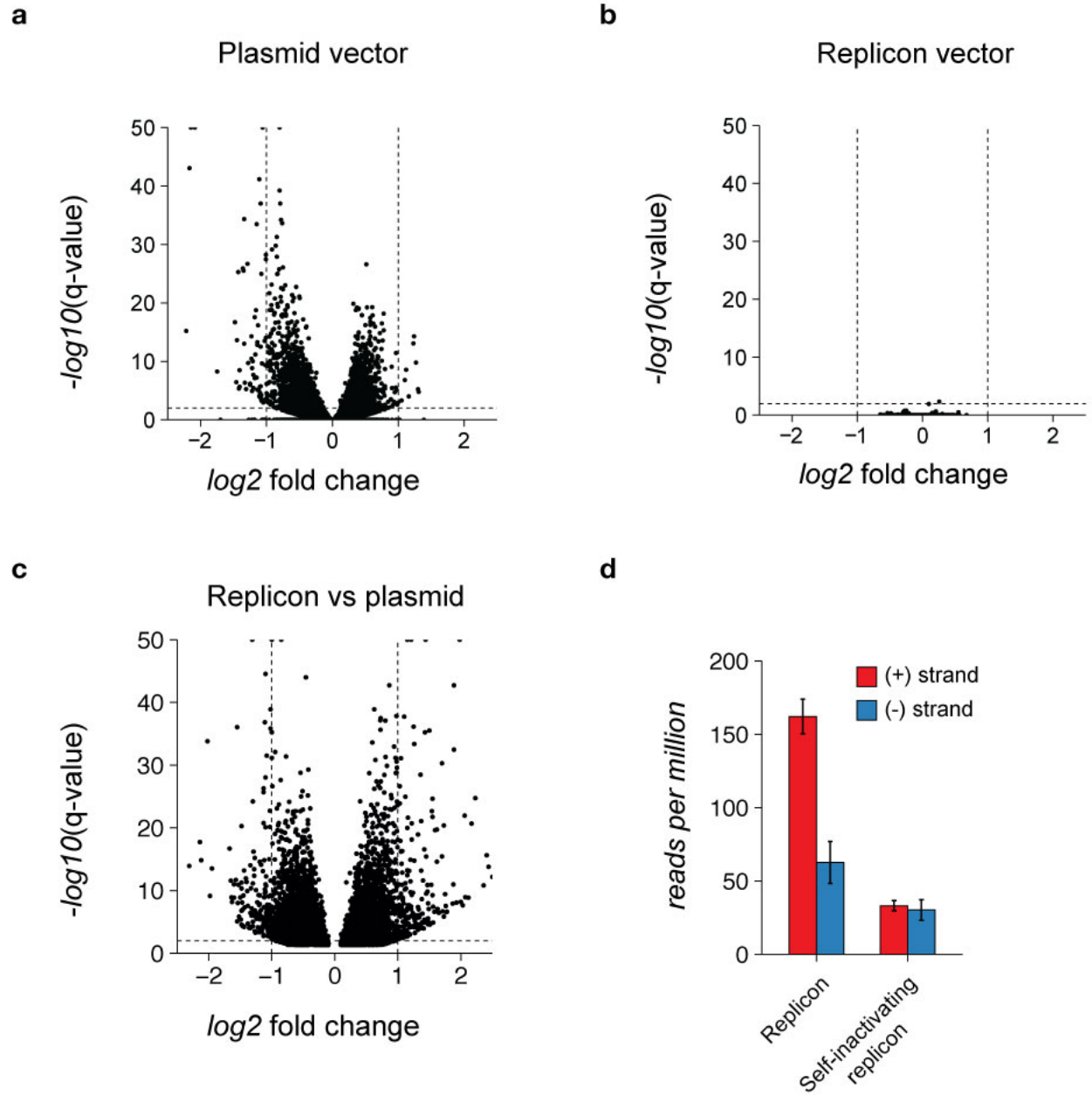
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350 **Fig. 3: Transcriptional response to self-inactivating Cas12a vectors.**

351 **a**, Plot depicting differential gene expression of host genes in cells transfected with a plasmid-

352 based Cas12a construct containing direct repeats in the 3'-UTR compared to cells transfected

353 with a comparable construct without direct repeats. Each dot represents a gene plotted by its

354 log2 fold change between the two conditions and -log10 of the adjusted p-value (q) determined

355 based on triplicate samples. Horizontal line marks a q-value = 0.01 and Vertical lines mark a

356 log2 fold change of -1 and 1. **b**, same as **a**, but comparing replicon-based Cas12a construct

357 containing direct repeats against a comparable construct without direct repeats. **c**, same as **a**,

358 but comparing plasmid-based Cas12a with direct repeats to replicon-based Cas12a with direct

359 repeats. **d**, Stranded read numbers aligning to the replicon as number of reads per million of

360 total reads. Error bars represent standard deviation from three replicates.

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SUPPLEMENTARY MATERIAL

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Table 1. Differentially expressed genes in response to self-inactivating vs. non-processable plasmid-based Cas12a vector. The control group are fibroblasts expressing GFP, Cas12a, and a crRNA lacking direct repeats performed in triplicate. The experimental group are fibroblasts expressing GFP, Cas12a, and a crRNA flanked with direct repeats, also performed in triplicate. Total reads per sample are given. Shown are gene names, mean count per gene, log2fold change in comparing triplicate samples in deSeq2, Standard error and adjusted p-value (q value). Raw data to be deposited on NCBI GEO.

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Table 2. Differentially expressed genes in response to self-inactivating plasmid-based Cas12a versus the corresponding replicon-based construct. The control group are fibroblasts expressing GFP, Cas12a, and a crRNA flanked with direct repeats performed in triplicate. The experimental group are fibroblasts expressing GFP, Cas12a, and a crRNA flanked with direct repeats as delivered by RNA replicon, also performed in triplicate. Total reads per sample are given. Shown are gene names, mean count per gene, log2fold change in comparing triplicate samples in deSeq2, Standard error and adjusted p-value (q value). Raw data to be deposited on NCBI GEO.

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REFERENCES

1   Marraffini, L. A. & Sontheimer, E. J. CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. *Nat Rev Genet* **11**, 181-190, doi:10.1038/nrg2749 (2010).

2   Makarova, K. S. *et al.* An updated evolutionary classification of CRISPR-Cas systems. *Nat Rev Microbiol* **13**, 722-736, doi:10.1038/nrmicro3569 (2015).

3   van der Oost, J., Jore, M. M., Westra, E. R., Lundgren, M. & Brouns, S. J. CRISPR-based adaptive and heritable immunity in prokaryotes. *Trends Biochem Sci* **34**, 401-407, doi:10.1016/j.tibs.2009.05.002 (2009).

4   Fonfara, I., Richter, H., Bratovic, M., Le Rhun, A. & Charpentier, E. The CRISPR-associated DNA-cleaving enzyme Cpf1 also processes precursor CRISPR RNA. *Nature* **532**, 517-521, doi:10.1038/nature17945 (2016).

5   Cho, S. W., Kim, S., Kim, J. M. & Kim, J. S. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nat Biotechnol* **31**, 230-232, doi:10.1038/nbt.2507 (2013).

6   Cong, L. *et al.* Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819-823, doi:10.1126/science.1231143 (2013).

7   Hwang, W. Y. *et al.* Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol* **31**, 227-229, doi:10.1038/nbt.2501 (2013).

8   Mali, P. *et al.* RNA-guided human genome engineering via Cas9. *Science* **339**, 823-826, doi:10.1126/science.1232033 (2013).

9   Zetsche, B. *et al.* Multiplex gene editing by CRISPR-Cpf1 using a single crRNA array. *Nat Biotechnol* **35**, 31-34, doi:10.1038/nbt.3737 (2017).

10   Campa, C. C., Weisbach, N. R., Santinha, A. J., Incarnato, D. & Platt, R. J. Multiplexed genome engineering by Cas12a and CRISPR arrays encoded on single transcripts. *Nat Methods* **16**, 887-893, doi:10.1038/s41592-019-0508-6 (2019).

424 11 Jinek, M. *et al.* A programmable dual-RNA-guided DNA endonuclease in adaptive

425   bacterial immunity. *Science* **337**, 816-821, doi:10.1126/science.1225829 (2012).

426 12 Koo, T., Lee, J. & Kim, J. S. Measuring and Reducing Off-Target Activities of

427   Programmable Nucleases Including CRISPR-Cas9. *Mol Cells* **38**, 475-481,

428   doi:10.14348/molcells.2015.0103 (2015).

429 13 Stadtmauer, E. A. *et al.* CRISPR-engineered T cells in patients with refractory cancer.

430   *Science* **367**, doi:10.1126/science.aba7365 (2020).

431 14 tenOever, B. R. The Evolution of Antiviral Defense Systems. *Cell Host Microbe* **19**, 142-

432   149, doi:10.1016/j.chom.2016.01.006 (2016).

433 15 Mello, C. C. & Conte, D., Jr. Revealing the world of RNA interference. *Nature* **431**, 338-

434   342, doi:10.1038/nature02872 (2004).

435 16 Bartel, D. P. Metazoan MicroRNAs. *Cell* **173**, 20-51, doi:10.1016/j.cell.2018.03.006

436   (2018).

437 17 Aguado, L. C. *et al.* RNase III nucleases from diverse kingdoms serve as antiviral

438   effectors. *Nature* **547**, 114-117, doi:10.1038/nature22990 (2017).

439 18 Aguado, L. C. *et al.* microRNA Function Is Limited to Cytokine Control in the Acute

440   Response to Virus Infection. *Cell Host Microbe* **18**, 714-722,

441   doi:10.1016/j.chom.2015.11.003 (2015).

442 19 tenOever, B. R. RNA viruses and the host microRNA machinery. *Nat Rev Microbiol* **11**,

443   169-180, doi:10.1038/nrmicro2971 (2013).

444 20 Brown, B. D., Venneri, M. A., Zingale, A., Sergi Sergi, L. & Naldini, L. Endogenous

445   microRNA regulation suppresses transgene expression in hematopoietic lineages and

446   enables stable gene transfer. *Nat Med* **12**, 585-591, doi:10.1038/nm1398 (2006).

447 21 Perez, J. T. *et al.* MicroRNA-mediated species-specific attenuation of influenza A virus.

448   *Nat Biotechnol* **27**, 572-576, doi:10.1038/nbt.1542 (2009).

449   22   Langlois, R. A. *et al.* MicroRNA-based strategy to mitigate the risk of gain-of-function

450   influenza studies. *Nat Biotechnol* **31**, 844-847, doi:10.1038/nbt.2666 (2013).

451   23   Langlois, R. A., Varble, A., Chua, M. A., Garcia-Sastre, A. & tenOever, B. R.

452   Hematopoietic-specific targeting of influenza A virus reveals replication requirements for

453   induction of antiviral immune responses. *Proc Natl Acad Sci U S A* **109**, 12117-12122,

454   doi:10.1073/pnas.1206039109 (2012).

455   24   Pham, A. M., Langlois, R. A. & TenOever, B. R. Replication in cells of hematopoietic

456   origin is necessary for Dengue virus dissemination. *PLoS Pathog* **8**, e1002465,

457   doi:10.1371/journal.ppat.1002465 (2012).

458   25   Moller, R. *et al.* miRNA-mediated targeting of human cytomegalovirus reveals biological

459   host and viral targets of IE2. *Proc Natl Acad Sci U S A* **115**, 1069-1074,

460   doi:10.1073/pnas.1719036115 (2018).

461   26   Aguado, L. C. *et al.* Homologous recombination is an intrinsic defense against antiviral

462   RNA interference. *Proc Natl Acad Sci U S A* **115**, E9211-E9219,

463   doi:10.1073/pnas.1810229115 (2018).

464   27   Sharma, P. *et al.* 2A peptides provide distinct solutions to driving stop-carry on

465   translational recoding. *Nucleic Acids Res* **40**, 3143-3151, doi:10.1093/nar/gkr1176

466   (2012).

467   28   Zhong, G., Wang, H., Li, Y., Tran, M. H. & Farzan, M. Cpf1 proteins excise CRISPR

468   RNAs from mRNA transcripts in mammalian cells. *Nat Chem Biol* **13**, 839-841,

469   doi:10.1038/nchembio.2410 (2017).

470   29   Strohkendl, I., Saifuddin, F. A., Rybarski, J. R., Finkelstein, I. J. & Russell, R. Kinetic

471   Basis for DNA Target Specificity of CRISPR-Cas12a. *Mol Cell* **71**, 816-824 e813,

472   doi:10.1016/j.molcel.2018.06.043 (2018).

473   30   Baltimore, D. *et al.* Biotechnology. A prudent path forward for genomic engineering and

474   germline gene modification. *Science* **348**, 36-38, doi:10.1126/science.aab1028 (2015).

475   31   Gitlin, L., Hagai, T., LaBarbera, A., Solovey, M. & Andino, R. Rapid evolution of virus

476   sequences in intrinsically disordered protein regions. *PLoS Pathog* **10**, e1004529,

477   doi:10.1371/journal.ppat.1004529 (2014).

478   32   Park, A. *et al.* Sendai virus, an RNA virus with no risk of genomic integration, delivers

479   CRISPR/Cas9 for efficient gene editing. *Mol Ther Methods Clin Dev* **3**, 16057,

480   doi:10.1038/mtm.2016.57 (2016).

481   33   Collins, F. S., Morgan, M. & Patrinos, A. The Human Genome Project: lessons from

482   large-scale biology. *Science* **300**, 286-290, doi:10.1126/science.1084564 (2003).

483   34   Pall, G. S. & Hamilton, A. J. Improved northern blot method for enhanced detection of

484   small RNA. *Nat Protoc* **3**, 1077-1084, doi:10.1038/nprot.2008.67 (2008).

485