In vitro transcribed guide RNAs trigger an innate immune response via the RIG-I pathway

Short title: *In vitro* transcribed guide RNAs trigger an innate immune response

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Abstract

CRISPR-Cas9 genome editing is revolutionizing fundamental research and has great potential for the treatment of many diseases. While editing of immortalized cell lines has become relatively easy, editing of therapeutically relevant primary cells and tissues can remain challenging. One recent advancement is the delivery of a Cas9 protein and an *in vitro* transcribed (IVT) guide RNA (gRNA) as a precomplexed ribonucleoprotein (RNP). This approach allows editing of primary cells such as T cells and hematopoietic stem cells, but the consequences beyond genome editing of introducing foreign Cas9 RNPs into mammalian cells are not fully understood. Here we show that the IVT gRNAs commonly used by many laboratories for RNP editing trigger a potent innate immune response that can be several thousand times stronger than benchmark immune stimulating ligands. IVT gRNAs are recognized in the cytosol through the RIG-I pathway but not the MDA5 pathway, thereby triggering a type I interferon response. Removal of the 5'-triphosphate from gRNAs ameliorates inflammatory signaling and prevents the loss of viability associated with genome editing in hematopoietic stem cells. The potential for Cas9 RNP editing to induce a potent antiviral response indicates that care must be taken when designing therapeutic strategies to edit primary cells.

18 **Abbreviations:**

19 Cas - CRISPR-associated

20 CIP - calf intestinal alkaline phosphatase

21 CRISPR - clustered, regularly interspaced, short palindromic repeat

22 dCas9 – nuclease-dead Cas9

23 HEK293 - Human embryonic kidney cells 293

24 HEK293T - Human embryonic kidney cells 293 SV40 large T antigen

25 HeLa – Henrietta Lacks cells

26 HSPCs – $CD34^+$ human hematopoietic stem and progenitor cells

27 IFNAR1 - Interferon Alpha And Beta Receptor Subunit 1

28 IFNβ/IFNB1 - Interferon beta

29 ISG15 - Interferon-stimulated gene 15

30 IVT – *in vitro* transcribed

31 KO - knockout

32 MAVS - mitochondrial activator of virus signaling

33 MDA5/IFIH1 - melanoma differentiation-associated gene 5/ Interferon Induced with Helicase C

34 Domain 1

35 PAMP - pathogen-associated molecular pattern

36 RIG-I/DDX58 - retinoic acid-inducible gene I/ DExD-H-box helicase 58

37 gRNA –guide RNA

38 SPRI - solid phase reversible immobilization

39 WT – wild type

40

41 **Introduction**

42 CRISPR (clustered, regularly interspaced, short palindromic repeat)-Cas (CRISPR-associated)
43 genome editing has rapidly become a widely used tool in molecular biology laboratories. Its ease
44 of use and high flexibility allows researchers to modify and edit genomes in cell lines (1), stem
45 cells (2), animals and plants (3,4), and even human embryos (5). At least two components must
46 be successfully delivered into cells during genome editing: the Cas protein, such as Cas9, and a
47 guide RNA (gRNA) to direct the Cas protein to its target site. For *in vitro* cultured cells this can
48 be done by transfecting plasmids encoding gRNA and Cas9 protein. However, transfection of
49 plasmid DNA into sensitive cell types such as primary and stem cells is challenging and inefficient.
50 The introduction of plasmids can also lead to undesired integration of DNA at the cut site (6),
51 increased off-target activity through prolonged expression of the CRISPR-Cas9 components (7),
52 and a delay in editing while the cell expresses gRNA and Cas protein (8).
53
54 The delivery of gRNA and Cas9 protein as a pre-complexed ribonucleoprotein (RNP) sidesteps
55 issues related to plasmid expression and has proved to be a successful strategy to edit human
56 primary cells, including T cells (9,10), hematopoietic stem cells (11–14), and neurons (15). This
57 makes RNP editing a particularly attractive approach for therapeutic applications, but relatively
58 little is known about the non-editing consequences of introducing a foreign gRNA and Cas9
59 protein. Human cells have evolved multiple defense mechanisms to guard against foreign
60 components, and genome editing reagents have the potential to activate these systems. For
61 example, recent data suggests that humans may have a pre-existing adaptive immune response to
62 the Cas9 protein (16). But cellular responses to the gRNAs used to program Cas9 editing have so
63 far not been well explored.

64 Cells respond to infection by RNA-viruses with an innate immune response that protects the host
65 cell from invading foreign genetic material (17). Foreign RNAs are recognized by pathogen-
66 associated molecular pattern (PAMP) binding receptors in the cytosol that include retinoic acid-
67 inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) (18). This
68 triggers a cascade of events mediated by the mitochondrial activator of virus signaling (MAVS)
69 protein resulting in the transcriptional activation of type I interferons and interferon-stimulated
70 genes (ISGs) (19–21).
71
72 PAMPs usually contain exposed 5'-triphosphate ends (18), which may also be present in gRNAs
73 made via T7 *in vitro* transcription (IVT) (22,23). We asked whether IVT gRNAs cause an innate
74 immune response, and here show that RNP genome editing induces upregulation of interferon beta
75 (IFNβ) and interferon-stimulated gene 15 (ISG15) in a variety of human cell types. This activity
76 depends upon RIG-I and MAVS, but is independent of MDA5. The extent of the immune response
77 depends upon the protospacer sequence, but removal of the 5'-triphosphate from gRNAs avoids
78 stimulation of innate immune signaling. The potential for Cas9 RNP editing to induce an antiviral
79 response indicates that care must be taken when designing therapeutic strategies to edit primary
80 cells.
81

82 **Results**

83 To investigate if mammalian cells react to IVT gRNA/Cas9 with an innate immune response, we
84 first performed genome editing in human embryonic kidney 293 (HEK293) cells using Cas9 RNPs.
85 To separate innate immune response from genome editing, we performed these experiments with
86 a non-targeting gRNA that recognizes a sequence within BFP and has no known targets within the

87 human genome (24). Constant amounts of recombinant Cas9 protein were complexed with
88 different amounts of non-targeting IVT gRNA and RNPs were transfected into HEK293 cells using
89 CRISPRMAX lipofection reagent (25). We harvested cells 30h after transfection and measured
90 transcript levels of *IFNB1* and *ISG15* by qRT-PCR (**Figure 1A**). Introduction of gRNAs caused a
91 dramatic increase in both *IFNB1* and *ISG15* levels, and the presence of Cas9 protein did not have
92 an effect on the outcome. Cas9 on its own did not induce *IFNB1* or *ISG15* expression. To our
93 surprise, as little as 1 pmol of gRNA was sufficient to trigger a 30-50-fold increase in the
94 transcription of innate immune genes. We further found that the commonly-administered 100 pmol
95 of a gRNA can induce *IFNB1* by 4,000 fold, which is equal to or even stronger than induction by
96 canonical IFNβ inducers such as viral mRNA from Sendai Virus (26) or a Hepatitis C virus (HCV)
97 PAMP (20,27) (**Figure 1B**).
98
99 RNPs can be delivered into cells via different transfection methods and while lipofection is cost-
100 effective and easy to use, many researchers prefer electroporation for harder-to-transfect cells. We
101 wondered if the transfection method would affect the IFNβ response and compared gRNA
102 transfection via lipofection (Lipofectamine 2000 and RNAiMAX) to nucleofection (Lonza)
103 (**Figure 1C**). Lipofection led to a strong increase in *IFNB1* and *ISG15* transcript levels after as
104 little as 6h post transfection, and the response was sustained for up to 48 hours. Nucleofection also
105 caused an increase in innate immune signaling at early time points, but the response was much
106 milder than in lipofected samples and was greatly diminished by 48 hours.
107
108 Next, we asked if the innate immune response to gRNAs is a common phenomenon across different
109 cell types and compared IFNβ activation in seven commonly used human cell lines of various

110 lineages: HEK293T, HEK293, HeLa, Jurkat, HCT116, HepG2 and K562 (**Figure 2A**). While the
111 magnitude of induction varied between cell lines, all tested cell lines responded to IVT gRNA
112 transfection with activation of *IFNB1* expression. The sole exception was K562 cells, which have
113 a homozygous deletion of the *IFNA* and *IFNB1* genes (28). We also measured transcript levels of
114 two major cytosolic pathogen recognition receptors, RIG-I (*DDX58*) and MDA5 (*IFIH1*), and
115 noticed that all cell lines except K562 upregulated these transcripts in response to introduction of
116 gRNAs. We also confirmed these results on the protein level in HEK293 cells (**Figure 2B**).
117
118 The RIG-I and MDA5 receptors complement each other by recognizing different structures in
119 foreign cytosolic RNAs, but the exact nature of their ligands is not yet fully understood (29,30).
120 To investigate if IVT gRNAs are recognized via RIG-I or MDA5, we generated clonal knockout
121 (KO) cell lines for RIG-I, MDA5, and their downstream interaction partner MAVS in HEK293
122 cells using CRISPR-Cas9. As the expression of both RIG-I and MDA5 are themselves stimulated
123 by IFNβ, we confirmed successful KO after transfection with gRNAs by genomic PCR, Sanger
124 sequencing, and Western Blot (**Supplementary Figure 1A-C**). MAVS KO cells were confirmed
125 by Western Blot (**Supplementary Figure 1D**). Strikingly, activation of *IFNB1 e*xpression after
126 introduction of gRNAs was absent in RIG-I and MAVS KO cells, while MDA5 KO cells did not
127 show a significant decrease in *IFNB1* transcript levels (**Figure 2C**). This indicates that IVT gRNAs
128 are exclusively recognized through RIG-I to trigger a type I interferon response.
129
130 As the structural requirements of RIG-I ligands are still not completely understood, we wondered
131 if different 20 nucleotide protospacers in gRNAs vary in their potency to trigger an innate immune
132 response via RIG-I. We designed 10 additional non-targeting gRNAs that we *in vitro* transcribed

133 and transfected into HEK293 cells. Surprisingly, we found that the cells responded to different
134 protospacers with a wide range of differential *IFNB1* expression. Several gRNAs produced very
135 little innate immune response, and one gRNA (gRNA11) yielded no *IFNB1* activation at all
136 (**Figure 3A**). We speculated that the differential response may be correlated with the purity of the
137 RNA product after IVT or the stability of the secondary structure of the RNA (31). However, we
138 found that there was no obvious correlation between the immune response to certain gRNAs and
139 their purity, predicted protospacer secondary structure, full secondary structure including the
140 constant region, or predicted disruption of the constant region by mis-pairing with the protospacer
141 (**Supplementary Figure 2**). These results indicate that RIG-I recognition patterns of IVT gRNAs
142 are complex and difficult to predict *a priori* based on predicted properties of the variable
143 protospacer.
144
145 One well-established requirement of RIG-I ligands is the presence of a 5'-triphosphate group (32).
146 We asked if preparations that remove the 5' triphosphate might avoid or reduce the innate immune
147 response to IVT gRNAs. We first used a synthetic gRNA that lacks a 5'-triphosphate and verified
148 that this gRNA does not induce *IFNB1* expression when transfected into HEK293 cells (**Figure**
149 **3B**). Synthetic guide RNAs are becoming more commonplace, but are still an order of magnitude
150 more expensive than IVT of gRNAs. This limits their application for high-throughput interrogation
151 of gene function in primary cells. We therefore asked if treatment of IVT gRNA with calf intestinal
152 alkaline phosphatase (CIP) to remove the 5'-triphosphate would reduce *IFNB1* induction. We
153 found that CIP treatment significantly reduced the *IFNB1* response (**Figure 3B**). Using different
154 CIP treatment regimes, we further found that removal of the 5'-triphosphate must to be absolutely
155 complete and should be carried out rigorously to avoid IFN stimulation (**Supplementary Figure**

156 **A-B**). We also compared purification of IVT gRNAs by solid phase reversible immobilization
157 (SPRI) beads to column purification and established that SPRI bead clean-up is not sufficient to
158 completely avoid an immune response even when more CIP is used (**Figure 3B and**
159 **Supplementary Figure 3C**). Taken together, these results indicate that 5'-triphosphate is a
160 necessary requirement for gRNA-induced *IFNB1* activation through RIG-I, but that additional
161 structural properties of the gRNAs also influence the magnitude of the immune response.
162
163 When a cell initiates an antiviral immune response, it also undergoes cellular stress that can affect
164 cell viability (33,34). Hence, we asked if there is a correlation between the IFNβ response and cell
165 viability after transfection with synthetic, IVT or CIP-treated IVT gRNA. Not surprisingly, the
166 viability of the very robust HEK293 cell line was not affected by the antiviral immune response
167 (**Supplementary Figure 3D**). We then turned to primary $CD34^+$ human hematopoietic stem and
168 progenitor cells (HSPCs), which are a much more sensitive cell type. We first nucleofected HSPCs
169 with RNPs targeting the *HBB* gene (11) and compared synthetic and IVT gRNA interferon
170 stimulation and cell viability post transfection. Double-strand breaks have been reported to cause
171 innate immune stimulation and can themselves cause decreases in cell fitness (35,36). Therefore,
172 we performed controls using nuclease-dead Cas9 (dCas9) to form RNPs. We saw a significant
173 decrease in cell viability of HSPCs in both IVT gRNA RNP samples which was associated with
174 an increase in IFN stimulated genes *ISG15* and *RIG-I* (**Figure 3C-D**). We did not see a substantial
175 difference in viability or ISG expression between Cas9 and dCas9 RNPs suggesting that nuclease
176 activity did not affect the immune response. Next, we asked if CIP treatment of gRNAs could
177 reverse the decrease in viability in HSPCs. We nucleofected HSPCs with dCas9 RNPs targeting a

178 non-coding intron of *JAK2* and compared synthetic, IVT and CIP-treated IVT gRNAs. Strikingly,
179 CIP treatment could completely restore the viability in HSPCs (**Figure 3E**).
180

181 ## Discussion

182 We have found that IVT gRNAs used with Cas9 RNPs for many genome editing experiments can
183 trigger a strong innate immune response in many mammalian cell types (**Figure 4**). Lipofection
184 results in a stronger and longer lasting response than nucleofection, possibly because lipofection
185 delivers gRNAs to the cytosol while nucleofection delivers mainly to the nucleus. Using isogenic
186 knockout clones, we found the gRNA-induced response is mediated via the anti-viral RIG-I
187 pathway and results in expression of genes that initiate an antiviral immune response. While
188 introduction of IFN-stimulating gRNAs does not affect viability in HEK293 cells, we found that
189 viability of primary HSPCs is negatively affected by the antiviral immune response.
190

191 These results have several implications. We suggest that the gene signature associated with type I
192 interferon stimulation should be considered when studying the transcriptome of recently edited
193 bulk populations of cells. Furthermore, all mammalian cells can both produce type I interferons
194 and also respond to them through the ubiquitously expressed receptor Interferon Alpha And Beta
195 Receptor Subunit 1 (IFNAR1) (37). Even cells that have not been successfully transfected with
196 RNPs could sense the INFβ produced by neighboring cells and activate downstream antiviral
197 defense mechanisms. This could be an important consideration during *in vivo* genome editing
198 applications as RNP delivery into one set of cells could provoke a wide-spread innate immune
199 response in the surrounding tissues.
200

201 We found that synthetic gRNAs completely circumvent the RIG-I mediated response, offering a
202 valuable path to avoid innate immune signaling during therapeutic editing. However, synthetic
203 gRNAs can become expensive when performing experiments that require testing or using many
204 gRNAs. We found that a cost-effective CIP treatment to remove the 5'-triphosphate before
205 transfection reduces the immune response and increases post-transfection viability in HSPCs.
206 Complete removal of the 5'-triphosphate is essential to restore viability in HSPCs to the same level
207 as observed with synthetic gRNAs. Thus, consideration of a potential innate immune stimulation
208 prior to choice of genome editing reagents, study design, and implementation of controls is critical
209 when performing genome editing using RNPs in mammalian cells.
210 Double-strand breaks have on their own been reported to induce an innate immune response (35).
211 However, we performed controls comparing WT Cas9 to a nuclease-dead Cas9 thereby showing
212 that the gRNA-mediated innate immune response and associated cell death in HSPCs is not caused
213 by double-strand breaks.
214
215 While we were preparing this manuscript for submission, the Kim group reported similar results
216 in HeLa cells and primary human $CD4^+$ T cells (38). They confirmed that the type I interferon
217 response is dependent on the presence of a 5'-triphopsphate group and that CIP treatment can
218 increase viability by avoiding the antiviral response. These results are very much in alignment with
219 our findings and extend the potential problem of innate immune signaling to additional cell types.
220
221 Our study adds extra depth by further outlining the mechanisms by which gRNAs are sensed. We
222 show that gRNA sensing depends upon RIG-I and MAVS, but MDA5 knockout cells are fully
223 capable of inducing IFNβ after IVT gRNA transfection. Hence, gRNA sensing is independent of

224 the MDA5 PAMP receptor. Furthermore, we show that in addition to a 5'-triphosphate, the
225 protospacer sequence is also critical to determine the intensity of the IFNβ response. Not only do
226 different gRNAs induce different innate immune responses, some gRNAs induce no response at
227 all. It has been proposed that 5'-basepaired RNA structures are required to activate antiviral
228 signaling via RIG-I, but we found no correlation between signaling and a variety of predicted RNA
229 properties, including secondary structure (31). Our results therefore suggest that the mechanism of
230 gRNA sensing by the RIG-I pathway is relatively complex, in that it requires 5' triphosphates but
231 that this moiety is not sufficient to induce the response. Future work to delineate the full set of
232 molecular features responsible for gRNA activation of innate immunity might yield accurate
233 predictors of innate immune signaling in general.
234

235 **Materials and methods**

236 *In vitro* transcription of gRNAs

237 gRNA was synthesized by assembly PCR and *in vitro* transcription as previously described (11).
238 Briefly, a T7 RNA polymerase substrate template was assembled by PCR from a variable 58-59
239 nt primer containing T7 promoter, variable gRNA guide sequence, and the first 15 nt of the non-
240 variable region of the gRNA (T7FwdVar primers, 10 nM, Supplementary Table 1; Supplementary
241 Table 2 for gRNA sequences), and an 83 nt primer containing the reverse complement of the
242 invariant region of the gRNA (T7RevLong, 10 nM), along with amplification primers
243 (T7FwdAmp, T7RevAmp, 200 nM each). The two long primers anneal in the first cycle of PCR
244 and are then amplified in subsequent cycles. Phusion high-fidelity DNA polymerase was used for
245 assembly (New England Biolabs). Assembled template was used without purification as a substrate
246 for *in vitro* transcription by T7 RNA polymerase using the HiScribe T7 High Yield RNA Synthesis

247 kit (New England Biolabs) following the manufacturer's instructions. Resulting transcription
248 reactions were treated with DNAse I (New England Biolabs), and RNA was purified by treatment
249 with a 5X volume of homemade solid phase reversible immobilization (SPRI) beads (comparable
250 to Beckman-Coulter AMPure beads) and elution in RNAse-free water.
251
252 CIP treatment of IVT gRNAs
253 When gRNAs were treated with Alkaline Calf intestinal phosphatase (CIP) (New England
254 Biolabs), 20U of CIP (2 µl) were added per 20 µl IVT reaction and samples were incubated at 37C
255 for 3h before proceeding to purification and DNAseI treatment. CIP-treated samples and
256 corresponding no CIP IVT gRNA controls were purified using a Qiagen RNeasy Mini Kit
257 (Qiagen). The detailed protocol and additional notes are available online
258 (dx.doi.org/10.17504/protocols.io.nghdbt6).
259
260 *In vitro* transcription of HCV PAMP and Sendai Virus DI RNA
261 HCV PAMP IVT template (20) was generated by annealing HCV fwd and rev (5 µM each) oligos
262 (Supplementary Table 1). 2 µl of the annealed product was used as DNA template in the
263 subsequent IVT reaction using HiScribe T7 High Yield RNA Synthesis kit (New England
264 Biolabs).
265 The plasmid containing the SeV DI RNA(26) was a gift from Prof. Peter Palese, Icahn School of
266 Medicine at Mount Sinai, New York. Plasmid was digested with HindII/EcoRI before IVT with
267 HiScribe T7 High Yield RNA Synthesis kit (New England Biolabs). The sequence of the IVT DI,
268 including the T7 promoter, hepatitis delta virus ribozyme, and the T7 terminator, is:

269 TAATACGACTCACTATA**ACCAGACAAGAGTTTAAGAGATATGTATCCTTTTAAAT**

270 **TTTCTTGTCTTCTTGTAAGTTTTTCTTACTATTGTCATATGGATAAGTCCAAGAC**

271 **TTCCAGGTACCGCGGAGCTTCGATCGTTCTGCACGATAGGGACTAATTATTACG**

272 **AGCTGTCATATGGCTCGATATCACCCAGTGATCCATCATCAATCACGGTCGTGT**

273 **ATTCATTTTGCCTGGCCCCGAACATCTTGACTGCCCCTAAAATCTTCATCAAAA**

274 **TCTTTATTTCTTTGGTGAGGAATCTATACGTTATACTATGTATAATATCCTCAAA**

275 **CCTGTCTAATAAAGTTTTTGTGATAACCCTCAGGTTCCTGATTTCACGGGATGA**

276 **TAATGAAACTATTCCCAATTGAAGTCTTGCTTCAAACTTCTGGTCAGGGAATGA**

277 **CCCAGTTACCAATCTTGTGGACATAGATAAAGATAGTCTTGGACTTATCCATAT**

278 **GACAATAGTAAGAAAAACTTACAAGAAGACAAGAAAATTTAAAAGGATACATAT**

279 **CTCTTAAACTCTTGTCTGGT**GGCCGGCATGGTCCCAGCCTCCTCGCTGGCGCCGGC

280 TGGGCAACATTCCGAGGGGACCGTCCCCTCGGTAATGGCGAATAGCATAACCCCTT

281 GGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG.

282 The sequence of the SeV DI is highlighted in boldface.

283 Both, HCV PAMP and SeV DI RNA were purified by treatment with a 5X volume of homemade

284 solid phase reversible immobilization (SPRI) beads (comparable to Beckman-Coulter AMPure

285 beads) and elution in RNAse-free water.

286

287 <u>Synthetic gRNAs</u>

288 Chemically synthesized gRNAs, which were purified using high-performance liquid

289 chromatography (HPLC), were purchased from Synthego.

290

291 <u>RNA quality control</u>

292 IVT gRNAs were analyzed using a Bioanalyzer. This was performed by the UC Berkeley
293 Functional Genomics Laboratory (FGL) core facility. gRNAs were denatured for 5 mins at 70C
294 before analysis on bioanalyzer.
295
296 Cas9 protein preparation
297 The Cas9 construct (pMJ915) contained an N-terminal hexahistidine-maltose binding protein
298 (His6-MBP) tag, followed by a peptide sequence containing a tobacco etch virus (TEV) protease
299 cleavage site. The protein was expressed in E. coli strain BL21 Rosetta 2 (DE3) (EMD
300 Biosciences), grown in TB medium at 16°C for 16h following induction with 0.5 mM IPTG. The
301 Cas9 protein was purified by a combination of affinity, ion exchange and size exclusion
302 chromatographic steps. Briefly, cells were lysed in 20 mM HEPES pH 7.5, 1M KCl, 10mM
303 imidazole, 1 mM TCEP, 10% glycerol (supplemented with protease inhibitor cocktail (Roche)) in
304 a homogenizer (Avestin). Clarified lysate was bound to Ni-NTA agarose (Qiagen). The resin was
305 washed extensively with lysis buffer and the bound protein was eluted in 20 mM HEPES pH 7.5,
306 100mM KCl, 300mM imidazole,1 mM TCEP 10% glycerol. The His6-MBP affinity tag was
307 removed by cleavage with TEV protease, while the protein was dialyzed overnight against 20 mM
308 HEPES pH 7.5, 300 mM KCl, 1 mM TCEP, 10% glycerol. The cleaved Cas9 protein was separated
309 from the fusion tag by purification on a 5 ml SP Sepharose HiTrap column (GE Life Sciences),
310 eluting with a linear gradient of 100 mM – 1 M KCl. The protein was further purified by size
311 exclusion chromatography on a Superdex 200 16/60 column in 20 mM HEPES pH 7.5, 150 mM
312 KCl and 1 mM TCEP. Eluted protein was concentrated to 40uM, flash-frozen in liquid nitrogen
313 and stored at -80°C.
314

315 Culture and transfection of immortalized cell lines

316 Cells were obtained from ATCC and verified mycoplasma-free (Mycoalert LT-07, Lonza).
317 HEK293, HEK293T, HCT116, HepG2 and HeLa cells were maintained in DMEM supplemented
318 with 10 % FBS and 100 µg/mL Penicillin-Streptomycin (all Gibco). K562 and Jurkat cells were
319 maintained in RPMI supplemented with 10% FBS and 100 µg/mL Penicillin-Streptomycin.
320 All transfections in cell lines were performed in 12-well cell culture dishes using $2x10^5$ cells per
321 transfection. For lipofection we used Lipofectamine™ CRISPRMAX™-Cas9, Lipofectamine®
322 RNAiMAX or Lipofectamine® 2000 Transfection Reagent (all Invitrogen) in reverse-
323 transfections according to the manufacturer's protocols. Unless stated otherwise, $2x10^5$ cells were
324 transfected with 50 pmol of RNA and harvested 24-30h post transfection for RNA extraction.
325

326 Culture and transfection of primary HSPCs

327 Human CD34+ HSPCs from mobilized peripheral blood (Allcells, Inc) were thawed and cultured
328 in StemSpan SFEM medium (StemCell Technologies) supplemented with StemSpan CC110
329 cocktail (StemCell Technologies) for 48 hours before nucleofection with dCas9 RNP (50pmol of
330 dCas9, 50 pmol of gRNA). $1.5x10^5$ HSPCs were pelleted (100 x g, 10 min) and resuspended in 20
331 µl Lonza P3 solution, mixed with 10ul dCas9 RNP, and nucleofected using ER100 protocol in
332 Lonza 4D nucleofector. Viability of the cells was measured 24 hours post nucleofection using
333 Trypan blue exclusion test. RNA was harvested 16 hours post nucleofection.
334

335 RNA extraction, cDNA synthesis and qRT-PCR

336 Cell cultures were washed with PBS prior to RNA extraction. Total RNA was extracted using
337 RNeasy Miniprep columns (Qiagen) according to the manufacturer's instructions including the

338 on-column DNAseI treatment (Qiagen). 1 μg of total RNA was used for subsequent cDNA
339 synthesis using iScript™ Reverse Transcription Supermix (Biorad). For qRT-PCR reactions, a
340 total of 20 ng of cDNA was used as a template and combined with primers (see Supplementary
341 Table 3) and SsoFast™ EvaGreen® Supermix (Biorad) and amplicons were generated using
342 standard PCR amplification protocols for 40 cycles on a StepOnePlus Real-Time PCR system
343 (Applied Biosystems). Ct values for each target gene were normalized against Ct values obtained
344 for *GAPDH* to account for differences in loading (ΔCt). To determine 'fold activation' of genes
345 ΔCt values for target genes were then normalized against ΔCt values for the same target gene for
346 mock-treated cells (ΔΔCt).
347
348 Generation of knockout cell lines
349 For CRISPR/Cas9 genome editing we used a plasmid encoding both the Cas9 protein and the
350 gRNA. pSpCas9(BB)-2A-GFP (px458) was a gift from Feng Zhang (Addgene plasmid #48138).
351 We designed gRNA sequences using the free CRISPR knockout design online tool from Synthego.
352 Two different gRNA sequences were designed for RIG-I and MDA5, respectively (see
353 Supplementary Table 3).
354 $2x10^5$ HEK293 cells were nucleofected with 2 μg of px458 plasmids containing both targeting
355 gRNAs in a 1:1 ratio using a Lonza 4D nucleofector (Lonza) with the manufacturer's
356 recommended settings. After 48h cells were harvested and subjected to fluorescence-activated cell
357 sorting (FACS). Cells expressing high levels of GFP were single-cell sorted into 96-well plates to
358 establish clonal populations.
359 For the screening process, genomic DNA from clonal populations was extracted using
360 QuickExtract solution (Lucigen). For KO of RIG-I and MDA5 we screened clones by genomic

361 PCR looking for a PCR product that is significantly smaller in size than that of WT HEK293 cells
362 (see Supplementary Table 4 for primers). PCR products were then Sanger sequenced by the UC
363 Berkeley DNA Sequencing facility using the forward primers of the PCR reaction as sequencing
364 primers.
365
366 Western Blot
367 Cells were harvested and washed with PBS. Cells were lysed in 1xRIPA buffer (EMD Millipore)
368 for 10 mins on ice. Samples were spun down at 14000xg for 15 mins and protein lysates were
369 transferred to a new tube. 50 μg of total protein was separated for size by SDS-PAGE and
370 transferred to a nitrocellulose membrane. Blots were blocked in 4% skim milk in 50 mm Tris-
371 HCl (pH 7.4), 150 mm NaCl, and 0.05% Tween 20 (TBST) and then probed for RIG-I,
372 MDA5, MAVS or GAPDH protein using antibodies against RIG-I (D14G6), MDA5 (D74E4),
373 MAVS (D5A9E) or GAPDH (14C10), respectively (all Cell Signaling Technologies). This was
374 followed by incubation with secondary antibody IRDye® 800CW Donkey anti-Rabbit IgG (Li-
375 Cor). Rainbow™ protein standards (GE Healthcare) were loaded in each gel for size estimation.
376 Blots were visualized using a Li-Cor Odyssey Clx (Li-Cor).
377

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380 York, for the Sendai virus DI IVT template DNA. The CRISPR/Cas9 genome-editing plasmid
381 px458 was a gift from Feng Zhang, Broad Institute, Cambridge, MA (Addgene plasmid # 48138).
382

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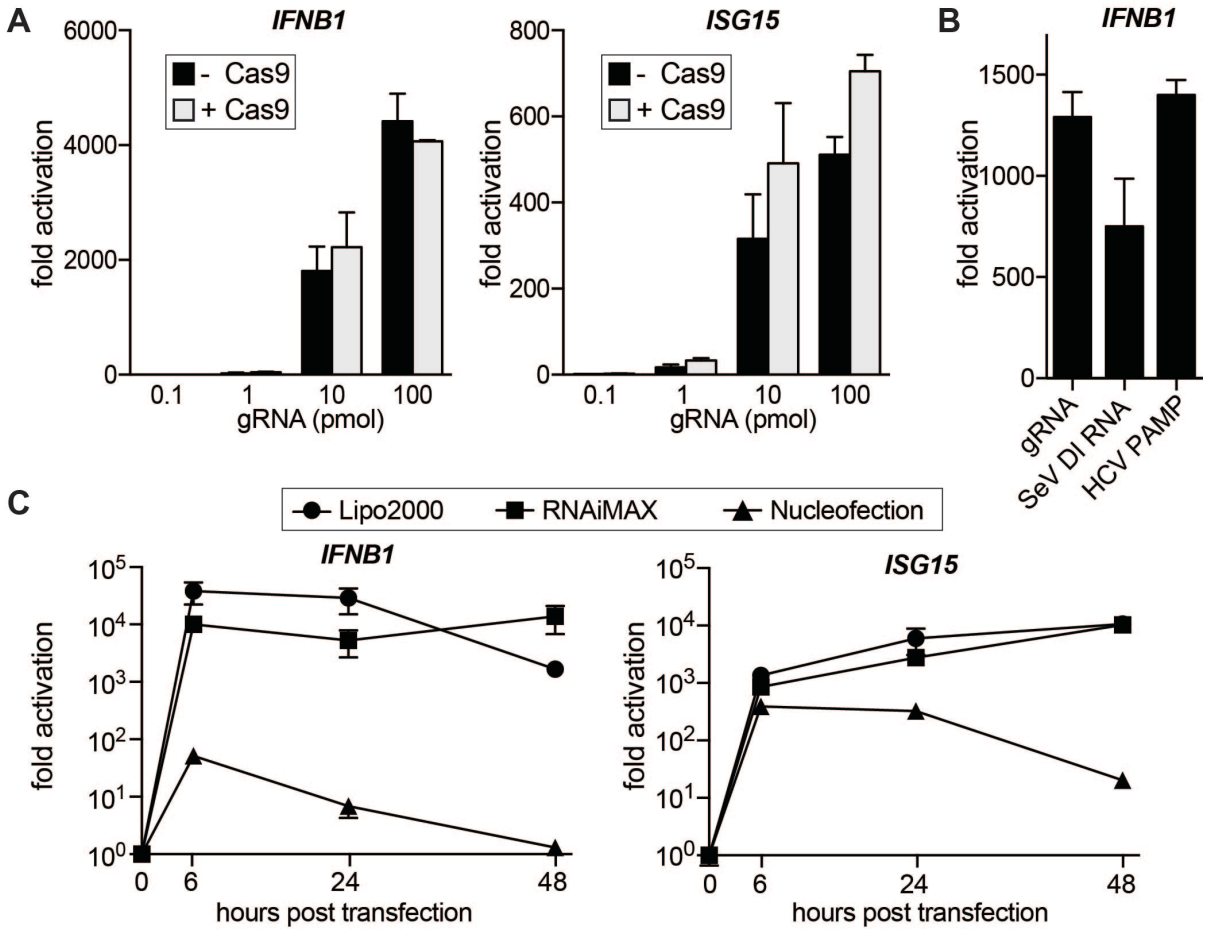
Figure 1

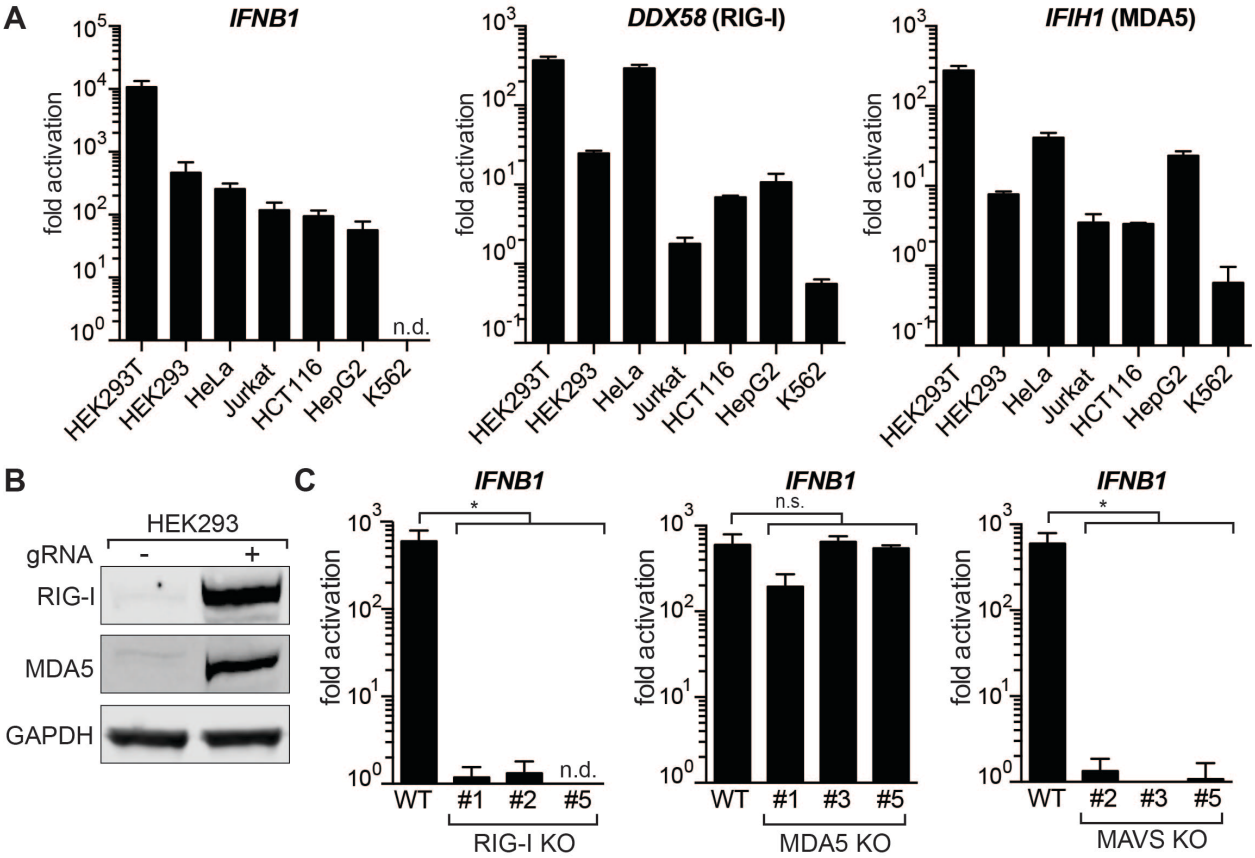


489

490 **Figure 1: Transfection of IVT gRNAs into HEK293 cells triggers a type I interferon response.**
491 (**A**) qRT-PCR analysis of *IFNB1* and *ISG15* transcript levels in HEK293 cells transfected with
492 increasing amounts of gRNA with and without Cas9 protein. In the samples with Cas9, gRNAs
493 were complexed with constant amounts (100 pmol) of Cas9 protein. Cells were harvested for RNA
494 extraction 30h after transfection using CRISPRMAX transfection reagent. Ct values were
495 normalized to Ct values of mock transfected HEK293 cells to determine fold activation. (**B**) qRT-
496 PCR analysis of *IFNB1* transcript levels in HEK293 cells transfected with equimolar amounts (50
497 pmol) of IVT gRNA, Sendai Virus defective interfering (SeV DI) RNA or HCV PAMP,

498 respectively. (**C**) qRT-PCR analysis of *IFNB1* and *ISG15* transcript levels in HEK293 cells over a
499 48h time course after transfection with 50 pmol via lipofection (Lipofectamine2000 or
500 RNAiMAX) or nucleofection, respectively. For all panels average values of three biological
501 replicates +/-SD are shown.
502

Figure 2

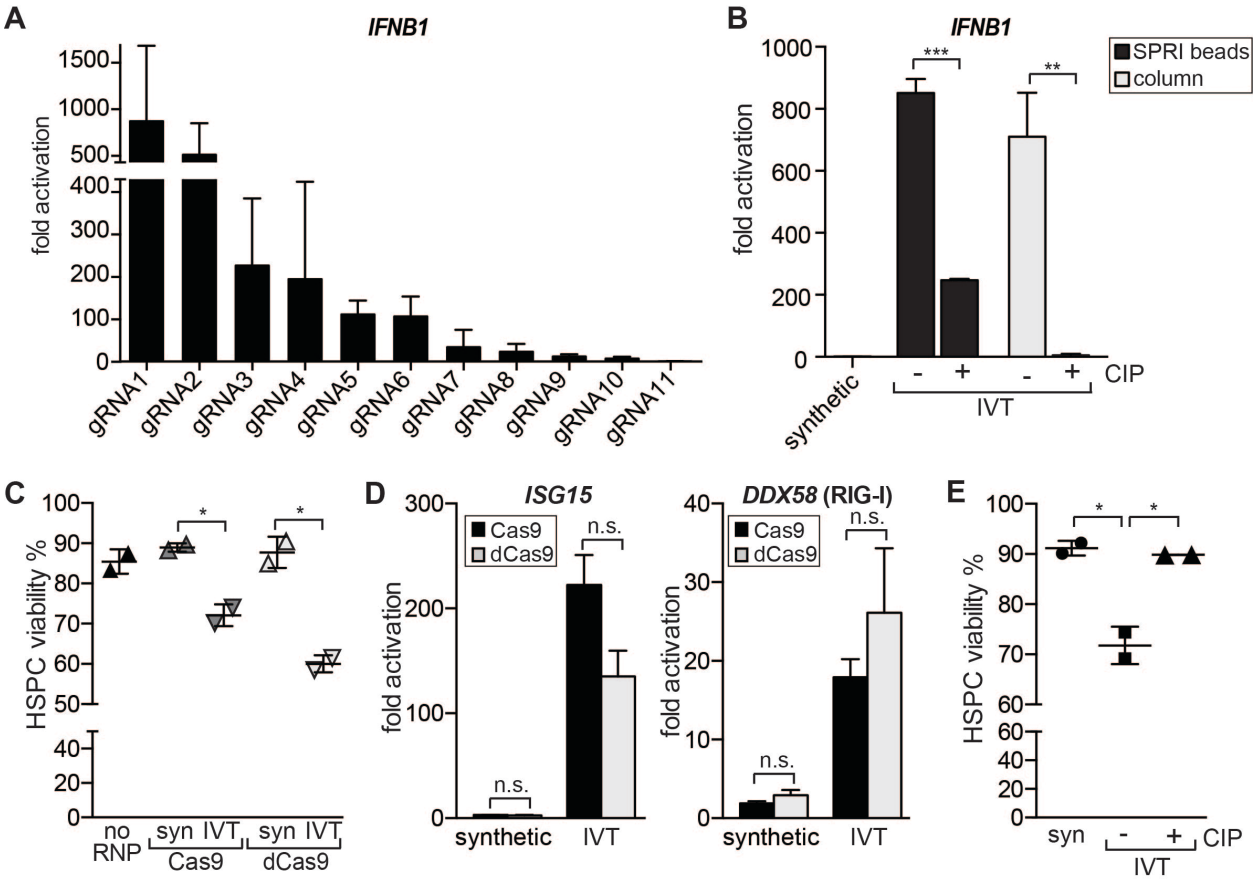


503

504 **Figure 2: IVT gRNAs are recognized via the RIG-I pathway.** (**A**) qRT-PCR analysis of increase
505 in *IFNB1* transcript levels (left) and transcript levels of the two main cytosolic RIG-I-like receptors
506 (*DDX58* and *IFIH1*) after introduction of IVT gRNA. Cell lines were ordered by responsiveness
507 to gRNA-mediated induction of *IFNB1* transcript levels. Cells were harvested for RNA extraction
508 30h after transfection. Ct values were normalized to Ct values of mock transfected cells for each

509 cell line to determine fold activation. *IFNB1* levels for K562 cells were too low to be determined
510 (n.d.). (**B**) Western Blot analysis for RIG-I and MDA5 expression of mock transfected and gRNA
511 transfected HEK293 cells after 48h. (**C**) qRT-PCR analysis of *IFNB1* transcript levels in HEK293
512 RIG-I (left), MDA5 (middle) and MAVS (right) knockout cells. Shown are three biological
513 replicates of three clonal populations of RIG-I, MDA5 or MAVS KO cells, respectively. *IFNB1*
514 levels for RIG-I KO clone #5 were too low to be determined (n.d.). For panels A and C, cells were
515 harvested for RNA extraction 30h after transfection using RNAiMAX transfection reagent.
516 Average values of three biological replicates +/-SD are shown. Statistical significances were
517 calculated by unpaired t-test (*p<0.0001, n.s.: not significant).
518

Figure 3



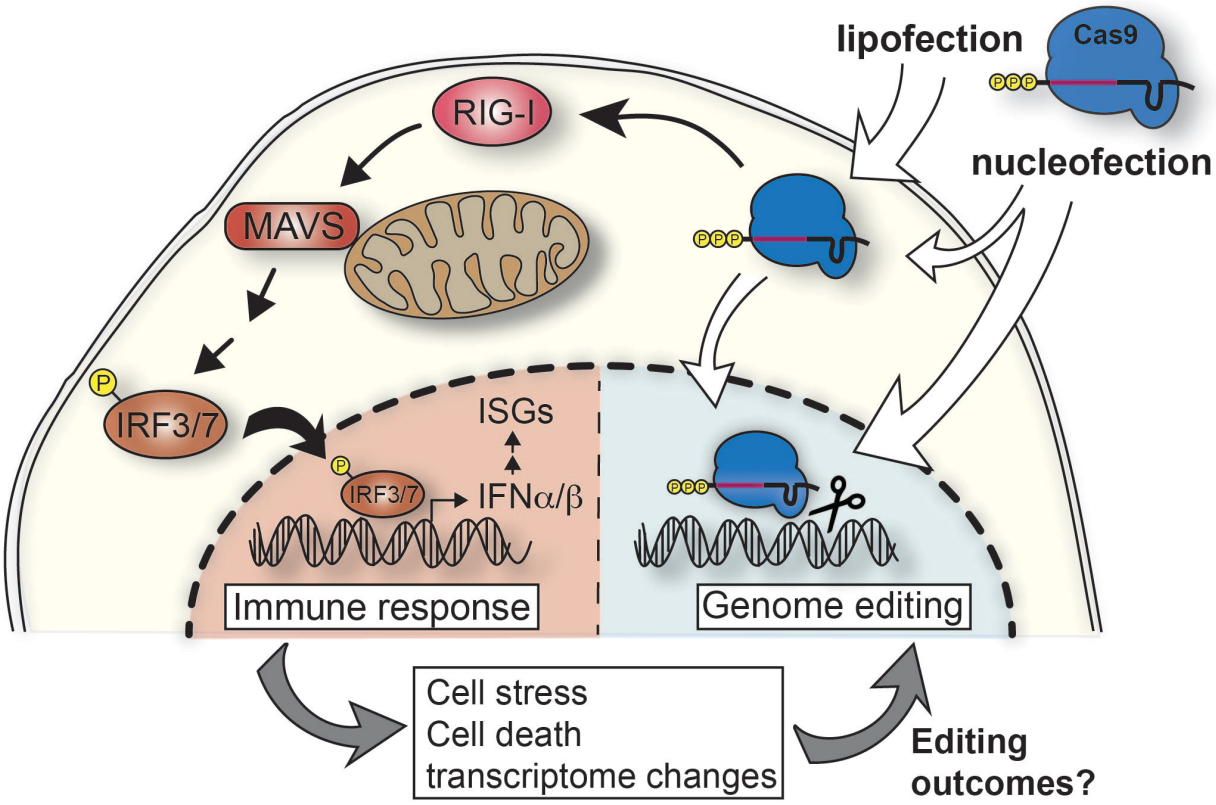
519

520 **Figure 3. Protospacer and 5'-triphosphate determine the intensity of the gRNA-mediated**

521 **IFNβ response.** (**A**) qRT-PCR analysis of *IFNB1* transcript levels in HEK293 cells transfected
522 with equal amounts of gRNAs containing different 20 nucleotide protospacers. gRNAs were
523 ordered by decreasing levels of *IFNB1* activation. gRNA1 refers to the gRNA that has been used
524 in all previous experiments. (**B**) qRT-PCR analysis of *IFNB1* transcript levels in HEK293 cells
525 transfected with synthetic, IVT and Calf intestine phosphatase treated (CIP) IVT gRNAs
526 (gRNA1). After IVT and CIP treatment gRNAs were purified with SPRI beads or spin columns,
527 respectively. Cells were harvested for RNA extraction 30h after transfection with RNAiMAX
528 transfection reagent. Average values of three biological replicates +/-SD are shown. (**C**) Viability
529 of human primary HSPCs 24h post nucleofection with no RNP and Cas9 or dCas9 RNPs. dCas9
530 or Cas9 were complexed with synthetic (syn) or IVT gRNA targeting the *HBB* gene. Viability was
531 determined by Trypan blue exclusion test. (**D**) qRT-PCR analysis of *ISG15* and *DDX58* (RIG-I)
532 transcript levels in human primary HSPCs 16h post nucleofection. dCas9 or Cas9 were complexed
533 with synthetic or IVT gRNA targeting the *HBB* gene, respectively. Ct values were normalized
534 against Ct of mock-nucleofected cells. Average values of two biological replicates +/-SD are
535 shown. (**E**) Viability of human primary HSPCs 16h post transfection with RNPs. RNPs consisted
536 of dCas9 complexed with synthetic, IVT or CIP-treated IVT gRNAs targeting a non-coding intron
537 of *JAK2*. Viability was determined by Trypan blue exclusion test. Statistical significances were
538 calculated by unpaired t-test (*$p<0.05$, **$p<0.01$, ***$p<0.0001$, n.s.: not significant).
539

540

Figure 4



541

542 **Figure 4. Transfection of *in vitro* transcribed gRNAs induces a cytosolic immune response.**
543 Proposed model of IVT gRNA recognition pathways in mammalian cells. IVT gRNAs carry a 5'-
544 triphosphate and when complexed with Cas9 protein and transfected into cells, cytosolic RNPs are
545 recognized by RIG-I triggering a cascade of activation events through the mitochondrial antiviral
546 signaling protein (MAVS). This results in phosphorylation of IRF3/7 and their shuttling into the
547 nucleus to activate expression of type I interferons (IFNα/β). This triggers the expression of
548 interferon-stimulated genes (ISGs). This innate immune response changes the transcriptome of the
549 cell and can cause cell stress and/or death which in turn might affect the editing outcomes.

550