Immunocompromised Cas9 transgenic mice for rapid in vivo assessment of host factors involved in highly pathogenic virus infection

Nicole Collette,1 Pragyesh Dhungel,2,4 Sean J. Lund,2,4 Jennifer L. Schwedler,2,4 Edwin A. Saada,3 Yooli K. Light,3 Anupama Sinha,3 Joseph S. Schoeniger,3 and Oscar A. Negrete2

1Physical and Life Science Directorate, Lawrence Livermore National Laboratory, Livermore, CA, 94550, USA; 2Department of Biotechnology and Bioengineering, Sandia National Laboratories, Livermore, CA 94550, USA; 3Department of Systems Biology, Sandia National Laboratories, Livermore, CA 94550, USA

Targeting host factors for anti-viral development offers several potential advantages over traditional countermeasures that include broad-spectrum activity and prevention of resistance. Characterization of host factors in animal models provides strong evidence of their involvement in disease pathogenesis, but the feasibility of performing high-throughput in vivo analyses on lists of genes is problematic. To begin addressing the challenges of screening candidate host factors in vivo, we combined advances in CRISPR-Cas9 genome editing with an immunocompromised mouse model used to study highly pathogenic viruses. Transgenic mice harboring a constitutively expressed Cas9 allele (Cas9tg/tg) with or without knockout of type I interferon receptors served to optimize in vivo delivery of CRISPR single-guide RNA (sgRNA) using Invivofectamine 3.0, a simple and easy-to-use lipid nanoparticle reagent. Invivofectamine 3.0-mediated liver-specific editing to remove activity of the critical Ebola virus host factor Niemann-Pick disease type C1 in an average of 74% of liver cells protected immunocompromised Cas9tg/tg mice from lethal surrogate Ebola virus infection. We envision that immunocompromised Cas9tg/tg mice combined with straightforward sgRNA in vivo delivery will enable efficient host factor loss-of-function screening in the liver and other organs to rapidly study their effects on viral pathogenesis and help initiate development of broad-spectrum, host-directed therapies against emerging pathogens.

INTRODUCTION

Animal models of viral infection serve a vital role in pre-clinical countermeasure development. Because reagents and genetic strains are widely available in mice, these small rodents typically serve as a starting point for animal model development of viral infection. For pathogens such as Ebola virus (EBOV), use of immunocompromised mice is necessary for productive pathogenic infection with wild-type or primary virus isolates.1,2 The type I interferon knockout (Ifnar1−/−) mouse model, which lacks expression of the interferon (IFN)-α/β receptors, is immunocompromised and, thus, more susceptible to infection by many highly pathogenic viruses, including filoviruses, flaviviruses, alphaviruses, bunyaviruses, and henipaviruses.3,4 Because the Ifnar1−/− mouse is susceptible to a range of emerging viruses of public health concern, this model can serve as a powerful platform for early in vivo screening of medical countermeasures.

Traditional therapeutic agents directly targeting the pathogen of interest face issues of rapid emergence of resistance and lack of broad-spectrum activity against multiple pathogens or variants. Host-directed therapeutic agents, on the other hand, which target the host cellular proteins required for infection, offer alternative strategies against emerging viruses with the advantage of broad-range utility in targeting common cellular pathways used by multiple viruses. It follows that regulation of such host genes will be less susceptible to incurring resistance.5 Characterization of host factors in animal models provides strong evidence of their involvement in human disease pathogenesis, but it has been proven difficult to perform in vivo analyses on comprehensive lists of target genes.6,7 With the advent of genome editing technologies via CRISPR-mediated engineering,6,7 in vivo host factor target validation may become more efficient and high throughput than previously possible if optimized CRISPR delivery platforms are developed for broad applicability and can be formulated with speed and ease.9,10

We set out to optimize the simple and easy-to-use lipid nanoparticle (LNP) reagent Invivofectamine 3.0 (INVFT3.0) for CRISPR applications in mice. INVFT3.0 is a proprietary LNP reagent originally designed for liver-targeted delivery of small interfering RNA (siRNA) in mice via systemic administration12. Given the relative similarities in mass sizes between CRISPR single-guide RNA (sgRNA) (32 kDa) and siRNA (15 kDa), we reasoned that sgRNA delivery into Cas9tg/tg mice using INVFT3.0 for rapid characterization of host factors in animal models was feasible. As a proof-of-concept study, sgRNAs were designed to efficiently target the critical EBOV...
host factor gene Niemann-Pick disease type C1 (Npc1)\textsuperscript{13} to generate a loss-of-function (LOF) allele. Npc1 is an endo/lysosomal membrane protein involved in intracellular cholesterol trafficking, and its luminal domain C is an essential endosomal receptor for Ebola and Marburg filoviruses.\textsuperscript{14} Moreover, Npc1 is a critical host factor in general for the filoviruses because bat-derived filovirus strains also utilize this receptor to gain entry into cells.\textsuperscript{13,15,16} These findings suggest that Npc1 might be an interesting broad-spectrum therapeutic target of filovirus infection.

To further understand the role of Npc1 in EBOV disease progression, we utilized Cas9\textsuperscript{tg/tg} mice and those crossed with knockout of type I IFN receptors (Cas9\textsuperscript{tg/tg}; Ifnar1\textsuperscript{−/−/−}) to optimize in vivo delivery of sgRNA targeting mouse Npc1 using INVFT3.0. Additionally, a replication-competent pseudovirus based on vesicular stomatitis virus (VSV) encoding the Ebola glycoprotein (VSV-EBOV) that mimics authentic EBOV cell entry\textsuperscript{16} was used for development of an infection model in Cas9\textsuperscript{tg/tg}; Ifnar1\textsuperscript{−/−/−} mice. We report the parameters required for efficient in vivo genome editing in immuno-compromised Cas9\textsuperscript{tg/tg} mice as being completely protective against lethal VSV-EBOV challenge.

**RESULTS**

**Screening Npc1 sgRNAs in mouse cell lines identifies a target sequence for in vitro evaluation**

To achieve efficient in vivo editing using CRISPR genome engineering, sgRNAs were first screened in vitro. Three sgRNA sequences targeting the mouse Npc1 locus were designed using publicly available algorithms (Figure S1A) and labeled in Figure 1 as sgRNA-A, sgRNA-B, and sgRNA-C. These three top-ranking sgRNA designs were synthesized and assembled as CRISPR ribonucleoprotein (RNP) complexes for transfection into mouse-derived LA4 (lung epithelial), Hepa1-6 (hepatoma), and IC-21 (macrophage) cells. As shown in Figure 1A, 2 of 3 sgRNA designs efficiently edited Npc1 4 days after transfection with sgRNA-C, resulting in the highest percentage of insertions or deletions (indels) in all three cell types (77% LA4, 42% Hepa1-6, and 75% IC-21) compared with the other two sequences.

Figure 1. Screening Npc1-targeting sgRNAs in mouse cell lines for in vitro efficacy

(A) Three sgRNAs targeting mouse Npc1 were evaluated in three mouse cell lines. Percent indels were calculated based on duplicate RNP transfection per sgRNA and per cell type. Data are depicted as mean ± standard deviation. (B) Cell lysates from LA4 RNP-transfected cells were subjected to western blot analysis using NPC1 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies. (C) RNP-transfected LA4 cells were infected with VSV-EBOV-GFP at 1 MOI, analyzed at 18 hpi for percent infection and normalized as percent inhibition (red text) (see also Figure S1). Representative infection levels are shown as images of nucleus staining (DAPI, blue) and VSV-EBOV-GFP (green) separately or merged. (D) Similarly, RNP-transfected LA4 cells were analyzed for cholesterol accumulation using filipin and PI nucleus staining; representative images are shown. Percent filipin-positive cells was analyzed using cell cytometry. Scale bars, 300 μM.
image-based assays. Western blotting demonstrated that sgRNA-A and sgRNA-C-mediated Npc1 LOF reduced protein to undetectable levels compared with controls (Figure 1B). Additionally, VSVEBOV expressing green fluorescent protein (GFP) (VSVEBOV-GFP) was used to infect RNP-transfected LA4 cells, and again sgRNA-A and sgRNA-C were highly potent because they reduced the number of infected GFP-expressing cells by approximately 98% when normalized to the control (no sgRNA) condition (Figures 1C and S1B). Last, knockout of the cholesterol transporter function of Npc1 resulted in intracellular cholesterol accumulation that was visualized through filipin staining. Filipin is an antibiotic isolated from Streptomyces filipinensis that is used for diagnosis of Niemann-Pick type C disease and binds to unesterified cholesterol, resulting in a fluorescence shift. Treatment with RNPs containing either sgRNA-A or sgRNA-C resulted in 67–68% cholesterol accumulation in LA4 cells compared to 11% in the control transfection condition as observed via filipin staining (Figure 1D). Interestingly, sgRNA-A and sgRNA-C Npc1 targeting sequences mapped to exon 11 and were spaced only 3 nt apart, whereas sgRNA-B mapped to exon 10 (Figure S1B), suggesting that exon 11 may be more accessible for CRISPR-Cas9 genome editing than exon 10 (Figure S1C). Based on the sum of the in vitro efficacy data, sgRNA-C was selected for in vivo characterization because of its high level of editing in all cell types tested, which resulted in efficient Npc1 LOF indel formation, protein reduction, phenotypic loss of cholesterol transport function, and elimination of the EBOV receptor that protected cells from infection by VSVEBOV-GFP.

Ultra-modified sgRNAs are highly efficient at editing the liver of Cas9 transgenic mice using INVF3.0

To start, mouse Npc1 sgRNA-C selected from in vitro screening experiments (now referred to as Npc1 sgRNA) was encapsulated into INVF3.0 LNPs using a protocol slightly modified from that described for siRNA (Materials and methods). In parallel, we also composed INVF3.0 LNPs with a published sgRNA sequence targeting the endogenous proprotein convertase subtilisin/kexin type 9 (Pcsk9), which is also expressed in liver cells, and knockout leads to lipid and cholesterol accumulation in the liver. As an additional control, we targeted GFP because Cas99\%t\%e mice express a Cas9-GFP fusion transcript. Instead of using the 2.4 mg/mL starting siRNA concentration recommended by the manufacturer for loading INVF3.0 LNPs, 1 mg/mL or 2 mg/mL sgRNA loading concentrations were used. LNP formulations and PBS mock controls were administered to Cas9 mice via intravenous (i.v.) dosing, and editing at the Npc1, Pcsk9, or GFP locus of the mouse liver was analyzed on day 7 after dosing (4 mice per group) (B). End- versus ultra-modified sgRNA chemical compositions. (C) Performance of ultra-modified Npc1 sgRNA-loaded INVF3.0 LNPs was evaluated by comparing i.v. and i.p. injections at a dose of 0.75 mg/kg (sgRNA wt/mouse wt). (D) Direct editing efficiency comparison of end- or ultra-modified sgRNAs targeting Npc1 was performed via i.p. administration at a dose of 1.5 mg/kg. (E) Percent indels is reported based on decomposition of chromatographs from Sanger sequencing using Synthego ICE, and a comparison with indel sequences and percentages based on amplicon sequencing using NGs is shown for a single sample. Data are depicted as mean ± standard deviation. Not significant (ns), p > 0.1; ** p < 0.001.
enhanced modifications and reformulated at a 1.2 mg/mL loading concentration using INVF3.0 for comparison of i.v. versus intraperitoneal (i.p.) systemic delivery routes. Using i.p. administration, on average, 21% ± 5.3% editing was achieved in the liver, and these results were comparable with i.v. injection (17% ± 7.2%) when groups were dosed equally at 0.75 mg/kg (sgRNA weight (wt)/mouse wt) (Figure 2C). To increase editing efficiencies in the liver, we decided to increase the dose volume as opposed to loading higher concentrations of sgRNA in a fixed dose volume because liver editing using sgRNA loading concentrations of 1 mg/mL or 2 mg/mL were comparable (Figure 2A). Because i.p. administration can tolerate higher volumes of dosing solutions at more repetitive frequencies, we reasoned that this route offered the most flexibility to optimize in vivo editing efficacy. In addition to the higher dose potential of the i.p. route, the efficacy of standard end-modified versus ultra-modified sgRNAs was compared directly. Cas9tg/tg mice dosed i.p. at 1.5 mg/kg with sgRNA-INVFT3.0 formulations increased the average editing percentage in the liver from 21% ± 4.8% using end-modified sgRNA to 51% ± 5.2% using ultra-modified sgRNA (Figure 2D).

Although the LOF indels reported here and throughout the manuscript are based on Sanger sequencing using Synthego Inference of CRISPR Edits (ICE) (Materials and methods), the frequencies compared with amplicon sequencing using next-generation sequencing (NGS) techniques highlighted a slight underestimation because ultra-modified sgRNA-generated indels scored by Sanger sequencing reported 53% for one data point, and NGS indicated 62% indel formation for the same sample (Figure 2E). These differences were also detected from cell line-edited samples (Figure S1D). Because of the speed and reasonable reliability to detect high-frequency indels provided by decomposition of Sanger sequencing, we continued to use this method for the remainder of the study with acceptance of potential under-representation of the actual editing events (Figures 2E and S1D). INVF3.0 provided an uncomplicated method for rapidly assessing ultra-modified sgRNA efficiencies in vivo with reproducible indel frequencies of greater than 50% using a single dose in Cas9tg/tg mice. Because these reagents and mice are commercially readily available, in vitro studies can transition to pre-clinical testing in an expedited manner.

**Immunocompromised Ifnar1 knockout mice and those crossed with Cas9tg/tg mice serve as lethal models for VSV-EBOV**

Various mouse models have been developed for EBOV, and common models use mouse-adapted EBOV (MA-EBOV) strains. Mouse adaptation is required for infection of WT immunocompetent mice because these mice are resistant to WT or primary isolate EBOV infection. MA-EBOV causes uniform mortality between days 7–9 with an infection characterized by extensive viremia and disease pathogenesis of the endoreticular organs of the liver and spleen. Although adapted for lethal disease in mice, MA-EBOV strains still require biosafety level 4 (BSL-4) containment for handling. Replication-competent VSV-EBOV is a BSL-2 virus that mimics viral entry and fusion of authentic EBOV and has been useful for in vitro studies that include discovery and characterization of EBOV countermeasures. VSV-EBOV is also a vaccine that provides complete protection to lethal EBOV challenge in immunocompetent mice. In immunocompromised mice such as those with STAT1 deficiency, VSV-EBOV causes lethal disease. Because the Ifnar1 knockout mouse is a common model for many viruses that do not cause disease in immunocompetent mice, including WT EBOV strains, and is now available more readily from commercial vendors, we began VSV-EBOV mouse model development under animal biosafety level 2 (ABSL-2) containment by verifying the lethal dose in Ifnar1 knockout (KO) mice.

Ifnar1−/− mice were challenged with VSV-EBOV lacking the GFP reporter through i.p. administration in a six-point, 10-fold dilution series starting with 10^7 plaque-forming units (PFUs) (Figure 3A). All challenge doses resulted in uniform lethality, with mice succumbing to disease within 2–3 days of infection for the higher doses and 3–4 days for the lower doses. To characterize the tissue tropism of VSV-EBOV infection in Ifnar1−/− mice using a 100 PFU dose, groups of infected mice were euthanized 24 h post-infection (hpi), 48 hpi, or 72 hpi and the liver, spleen, and serum were analyzed for virus titers (Figure 3B). Onset of infection in these individuals was rapid and reached 10^6–10^7 PFU per gram of tissue or per milliliter of serum by 48 hpi. The kinetics and titers of virus dissemination in Ifnar1−/− mice closely resembled the tropism of MA-EBOV in WT mice, suggesting that Npc1-dependent entry in mice dictates the tissue targets. Considering the parallel of VSV-EBOV and MA-EBOV animal models, we continued to develop this model with the goal of enabling rapid in vivo characterization of host factor involvement in the pathogenesis of EBOV by crossing Cas9 transgenic mice with Ifnar1−/− mice.

When a cohort of double-homozygous Cas9tg/tg; Ifnar1−/− mice was available, they were infected i.p. with VSV-EBOV using 100 PFUs to assess the survival profile compared with Ifnar1−/− mice (Figure 3C). Introduction of the Cas9 transgene allele into Ifnar1−/− mice did not alter their survival pattern compared with Ifnar1−/− mice because lethal disease occurred similarly between days 3–4 after infection with VSV-EBOV. At the endpoint of this study, liver and spleen tissues were dissected for histological analysis and assessment of the presence of viral antigen (Figures 3D and 3E). Severe tissue necrosis was apparent in the liver of VSV-EBOV-infected mice by day 3, and viral replication in liver and spleen tissues was also abundant, as indicated by immunohistochemistry staining of the EBOV glycoprotein. Therefore, Cas9tg/tg; Ifnar1−/− mice can serve as a versatile ABSL-2 model for rapid characterization of genetic factors affecting entry of EBOV in vivo.
editing thresholds, Cas9tg/tg; Ifnar1−/− mice were given a multi-dose treatment of 0.75 mg/kg and high (1.5 mg/kg) concentrations, which were compared with single-dose regimens (Figure 4A). Multi-dose treatments consisted of two doses given 7 days apart, and liver editing percentages were analyzed synchronously between single- and double-dosed mice 14 days after the first dose. Cumulative editing occurred under low- and high-dose conditions, and editing increased on average from 28.5% ± 12.3% to 39.8% ± 13.6% in the 0.75 mg/kg treatment groups and from 50.8% ± 16% to 73.5% ± 16% in the 1.5 mg/kg dose groups when comparing single- versus double-dose treatments, respectively. Interestingly, variability in editing percentages under the high double dose condition was minimal and less than half of all other conditions tested. This result suggested that we achieved consistency and reproducibility by maximizing the performance of the formulation. The LOF indel frequencies of high-dose-treated mice were then analyzed in additional tissues, and these studies revealed liver-specific editing of sgRNAs delivered using INVFT3.0 because the spleen, kidneys, or lungs were not edited (Figure 4B). I.P. delivery coupled with the LNP sizes associated specifically with sgRNA cargo could influence the liver-only editing effect.

To validate functional reduction of Npc1 protein expression in sgRNA LNP-dosed mice, liver of mice with approximately 0%, 25%, or 50% editing in the liver were subjected to western blot analysis (Figure 4C). These western blots indicated that, with 50% editing at the Npc1 locus, we observed relatively undetectable levels of Npc1 protein in liver homogenates of Cas9tg/tg; Ifnar1−/− mice. Last, histological analysis from tissues of high-double-dosed Npc1-sgRNA-treated mice revealed no apparent gross anatomical changes compared with controls within 14 days after the first dose (Figure 4D). Multi-dosing sgRNA delivery with INVFT3.0 provided high levels of liver-specific LOF editing at the Npc1 locus in immunocompromised Cas9 transgenic mice.

Liver-specific editing of Npc1 in Cas9tg/tg; Ifnar1−/− mice provides complete protection against lethal VSV-EBOV challenge

Previous studies of EBOV infection in mice carrying Npc1 homozygous or heterozygous mutations have demonstrated a strong role of systemic Npc1 expression as a determinant of EBOV pathogenesis because Npc1−/− mice were completely protected from lethal EBOV infection, and 90% of Npc1+/− mice were also protected.24 However, the role of particular tissues in EBOV disease progression is not fully understood. These types of studies can be assessed using conditional KO mutant mice or now more rapidly using CRISPR technology. Using our optimized liver-specific editing in mice using INVFT3.0, Npc1-edited Cas9tg/tg; Ifnar1−/− mice that received double-dose sgRNA treatments using I.P. delivery were infected I.P. with VSV-EBOV 7 days after the second sgRNA dose (Figure 5A).
As controls, mice dosed with PBS and non-targeting sgRNAs formulated using INVFT3.0 were infected similarly with VSV-EBOV. As expected, VSV-EBOV infection in both control groups led to uniform lethality by day 3 after infection, but, interestingly, Npc1 sgRNA-treated mice were completely protected from lethal VSV-EBOV challenge. According to daily weight measurements of Npc1-edited mice, most mice initially lost weight between days 5–8 after infection before recovering and gaining weight (Figure 5B). These data indicated that, although liver-specific editing protected mice from severe disease, it was unable to completely prevent symptomatic infection, perhaps because of early infection outside of the liver. Virus replication (Figure 5C) and Npc1 editing levels (Figure 5D) in the liver and spleen were examined at the end of the study and compared with control mice dosed with non-targeting sgRNA that succumbed to disease. Viral RNA copies in the tissues of control mice, on average, were $3.7 \times 10^4$ in the liver and $4.9 \times 10^3$ in the spleen, whereas viral RNA copies in Npc1-edited mice were 11.2 in the liver and 3.5 in the spleen. The final Npc1 editing levels in Cas9$^{9^9}$; Ifnar1$^{-/-}$ mice on day 28 after the initial sgRNA dose was $62\% \pm 6.4\%$, suggesting that editing was stable and maintained during the infection study. These results suggest that safe prophylactic therapeutic agents targeting Npc1 in the liver could provide efficient protection against EBOV infection.

**DISCUSSION**

In this study, we demonstrated facile methods that enable highly efficient genome editing in the liver of Cas9$^{9^9}$ mice and those crossed with IFNAR1 KO mice. Although these techniques are useful for a variety of applications involving diseases affecting the liver, we highlight their utility for studying host factors involved in highly pathogenic virus infection. By targeting Npc1 for LOF in the liver using INVFT3.0-based CRISPR sgRNA delivery, mice survived lethal infection in a surrogate BSL-2 EBOV model that mimics the tissue tropism of the BSL-4 MA-EBOV model.21 These results extend the current understanding of NPC1 function in EBOV pathogenesis. Systemic KO and knockdown of Npc1 obtained through homozygous or heterozygous mutations, respectively, have been shown to protect against lethal EBOV challenge to levels of 90% or greater.24 However, our results indicate that the liver may serve as the most critical organ involved in EBOV pathogenesis in mouse models because liver-specific partial KO of Npc1 resulted in complete protection against replication-competent Ebola pseudovirus lethality. Duplicating these studies using WT or MA EBOV in Cas9$^{9^9}$; Ifnar1$^{-/-}$ or Cas9$^{9^9}$ mice, respectively, would ultimately confirm these findings, but the data as shown provide evidence for continued investigation of prophylactic treatments targeted to the liver by traditional or novel means against EBOV.

Because the parameters are now optimized for rapid genome editing in the liver of Cas9$^{9^9}$ and Cas9$^{9^9}$; Ifnar1$^{-/-}$ mice, other host factor genes can be screened rapidly in vivo using these commercially available reagents. To continue screening host factors in vivo using the methods and models described here, additional factors would be limited to those that facilitate virus entry because of the limitations of a VSB-based surrogate virus infection model. A number of host genes involved in EBOV attachment and fusion have been described over the years,13,25,26 but more recently, CRISPR KO screening against WT EBOV conducted under BSL-4 containment has identified novel entry factors that await in vivo validation.27 The Cas9$^{9^9}$; Ifnar1$^{-/-}$ model for genome editing of host factors could easily extend to other viruses of pandemic potential.4 For instance, Rift Valley fever virus (RVFV) is a category A high-priority pathogen listed as a biodefense threat alongside EBOV that causes mainly hepatic disease in animal models.28 WT RVFV studies are conducted under BSL-3 containment, but studies with the vaccine strain MP-12 are performed under BSL-2 containment.
containment.29 RVFV-MP-12 causes lethal disease in Ifnar1−/− mice,30 therefore, INVFT3.0-mediated, liver-specific genome editing could be used for in vivo screening of host factors involved in RVFV infection and pathogenesis. Beyond EBOV and RVFV, IFNAR KO mice have been useful in animal model studies involving a diverse set of filoviruses, bunyaviruses, flaviviruses, alphaviruses, and henipaviruses, facilitating use of Cas9tg/tg; Ifnar1−/− mice for rapid characterization of host factors for many threats with pandemic potential.2 

Last, although INVFT3.0 was originally developed for efficient delivery of siRNA to the liver of mice, other studies have expanded their versatility by using localized delivery methods to the brain, retina, and other regions.31–33 Given the similarities between sgRNA and siRNA delivery to the liver using INVFT3.0, it is reasonable to conclude that the optimized sgRNA delivery formulations reported here could also edit tissues outside of the liver for further characterization of non-hepatic tissue involvement in viral pathogenesis. Cas9tg/tg; Ifnar1−/− mice can serve as an ABSL-2 model for rapid in vivo characterization of genetic factors affecting entry of EBOV and, by extension, can serve as a more general platform for characterization of host factors in disease pathogenesis and therapy development for many other emerging viruses.

MATERIALS AND METHODS

Cells, viruses, and reagents

All cell lines were maintained in culture medium supplemented with 10% fetal bovine serum (FBS) (15% FBS for LA4 cells), 100 g/mL penicillin, and 100 U/mL streptomycin (Thermo Fisher Scientific, Waltham, MA) at 37°C under 5% CO2. LA4 (mouse lung epithelial) cells were cultured in Ham’s F12K medium, Hepa1-6 (mouse hepatoma) cells were cultured in Dulbecco’s modified Eagle’s medium, IC-21 (mouse macrophage) cells were cultured in RPMI-1640 medium, and Vero (African green monkey kidney) cells were cultured in minimum essential medium alpha. The recombinant VSV expressing the Ebola glycoprotein (EBOV-GP) gene was derived from a full-length cDNA clone of VSV Indiana serotype 1, in which the VSV-G envelope protein has been replaced with EBOV-GP (VSV-EBOV). The EBOV-GP gene (GenBank: L11365) was cloned from a plasmid available from BEI Resources (Manassas, VA; catalog number NR-19814). VSV-EBOV-GFP was derived from the cDNA clone of VSV-EBOV, where the VSV-P gene contained an N-terminal fusion to GFP, and this reporter virus was only used for in vitro experiments. VSV-EBOV and VSV-EBOV-GFP were propagated in Vero cells, and virus titers were quantified by standard agarose overlay plaque assays. Antibodies used for western blot analysis included rabbit monoclonal anti-Gapdh (Cell Signaling Technology, Danvers, MA) according to the manufacturer’s instructions.

In vitro Npc1 sgRNA screening

sgRNAs targeting mouse Npc1 were selected using publicly available design tools, and top-ranking sequences were cross-referenced to each other (Figure S1A). Three sequences were identified and synthesized as end-modified sgRNAs (Synthego, Redwood City, CA) (Table S1). sgRNAs were complexed with SpyCas9 protein (Integrated DNA Technologies, Coralville, IA) and transfected into LA4, Hepa1-6, or IC-21 cells using CRISPRmax (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s instructions. Transfected cells were subsequently subjected to phenotypic and indel editing analysis 4 days or longer after transfection.
**Indel analysis**

DNA from *Npc1* LOF-edited samples derived from cell cultures or mouse tissues were isolated and purified using the Blood and Tissue Genomic Mini-prep Kit (Epoch Life Science, Sugar Land, TX). PCR products encompassing the mouse *Npc1* editing site were amplified using primers listed in Table S1, column purified, and sent for Sanger sequencing (Genewiz, South San Francisco, CA). Chromatograph files from control and edited samples were analyzed by the sequence decomposition software Synthego ICE, which provided rapid and reliable assessment of indel profiles and frequencies from CRISPR editing experiments. Amplicon sequencing was also performed on a subset of *Npc1*-edited samples using NGS techniques. PCR products were amplified in two rounds, adding adaptor sequences and barcodes to the ends of the amplicons. Pooled PCR reactions were column purified and additionally gel purified. PCR product concentration and quality were assessed using the Qubit reaction. PCR products were amplified in two rounds, adding adaptor sequences and barcodes to the ends of the amplicons. Pooled PCR reactions were column purified and additionally gel purified. PCR product concentration and quality were assessed using the Qubit reaction. Amplicon sequencing was also performed on a subset of *Npc1*-edited samples using NGS techniques. PCR products were amplified in two rounds, adding adaptor sequences and barcodes to the ends of the amplicons. Pooled PCR reactions were column purified and additionally gel purified. PCR product concentration and quality were assessed using the Qubit reaction.

**Cholesterol accumulation through filipin staining**

LA4 cells with or without indels at the *Npc1* locus were fixed with 3% paraformaldehyde (PFA) for 1 h at room temperature (RT). After quenching free PFA with 1.5 mg glycine/mL in PBS for 10 min at RT, cells were stained with 0.05 mg/mL filipin (Sigma-Aldrich, St. Louis, MO; catalog number SAE0088) in PBS/10% FBS for 2 h at RT. Following the PBS wash, the cells were counterstained with the nuclear dye propidium iodide (PI) for 10 min at RT. For imaging and cell cytometry analysis, the CellInsight CX7 microscope platform was used with the standard excitation/emission wavelength for filipin and PI, respectively. Using the built-in cell cytometry analysis feature, the number of filipin-stained cells (surrogate for accumulated cholesterol) and total number of cells by counting PI-stained nuclei were determined. The percentage of filipin stained cells was determined by dividing the number of filipin stained cells by the total number of cells.

**Cas9<sup>flg</sup>, Ifnar1<sup>−/−</sup> and Cas9<sup>flg</sup>, Ifnar1<sup>−/−</sup> mouse models**

All animal work was conducted in accordance with protocols approved by the Lawrence Livermore National Laboratory (LLNL) Institution Animal Care and Use Committee. *Cas9<sup>flg</sup>* mice (Rosa26-Cas9 knockin on the C57BL/6J genetic backgroud, B6.J129(Cg)-Gt(ROSA)26Sor<sup>1</sup>(CAG-cas9<sup>sgr</sup>−EGFP)Feh1<sup>l</sup>; The Jackson Laboratory, Farmington, CT; catalog number 026179) and *Ifnar1<sup>−/−</sup>* mice (global *Ifnar1* KO on the C57BL/6J genetic backgroud, B6(Cg)-Ifnar1<sup>1.2Kas</sup>1; The Jackson Laboratory, Farmington, CT; catalog number 028288) were bred separately at LLNL and also mated to generate a double-homozygous mutant *Cas9<sup>flg</sup>*, *Ifnar1<sup>−/−</sup>* mouse strain. All experiments were performed with 6- to 10-week-old mice.

**In vivo sgRNA delivery using INVFT3.0**

CRISPR sgRNAs were encapsulated into INVFT3.0 LNPs originally designed for efficient in vivo delivery of siRNA. The manufacturer’s protocol (Thermo Fisher Scientific, Waltham, MA) was modified slightly for sgRNA loading and delivery. Initially, standard end-modified sgRNAs purchased from Synthego (Redwood City, CA) were diluted to concentrations of 1 mg/mL or 2 mg/mL in distilled water (dH2O), mixed with complexation buffer (CB) from the kit, and subsequently combined with INVFT3.0 at a volume ratio of 1:1:2 (sgRNA:CB:INVFT3.0). The solution was heated to 50°C for 30 min on a heat block, diluted 5-fold with PBS, and then administered i.v. via tail vein injection using 200 µL of sgRNA LNPs. For all other in vivo editing experiments, INVFT3.0 LNPs were formulated using an initial loading concentration of 1.2 mg/mL of end-modified or ultra-modified sgRNAs (Synthego, Redwood City, CA) (Figure 2B) using similar volume ratios and protocols as before, but the final dilution with PBS was 4-fold instead of 5-fold. LNP administration typically occurred within 1–2 h after formation and through i.p. injection at doses of 0.75 mg/kg (~200 µL LNPs per 20-g mouse) or 1.5 mg/kg (~400 µL LNPs per 20-g mouse). At the end of the study, mice were euthanized, and liver or liver, spleen, kidneys, and lungs were dissected from 3–4 animals per group.

**Mouse model of VSV-EBOV infection**

*Ifnar1<sup>−/−</sup>* mice were tested for their susceptibility to lethal disease by surrogate EBOV infection using VSV-EBOV. *Ifnar1<sup>−/−</sup>* mice were challenged with VSV-EBOV through i.p. administration in a six-point, 10-fold dilution series starting with 10<sup>7</sup> PFUs. All mice were maintained in positive airflow barrier housing under specific pathogen-free conditions. Animals were moved into negative airflow ABSL-2 containment housing 24 h prior to VSV-EBOV infection. Mice were observed at least daily and twice daily when symptomatic, with body condition scores and animal weights measured. Animals that reached the humane endpoint were euthanized. For time course experiments, four mice from each group (24 hpi, 48 hpi, or 72 hpi) were euthanized and dissected for liver, spleen, and serum. Organs were weighed and then homogenized using disposable tissue grinders, and tissue lysate was assessed for viral load using a standard plaque assay. The identified lethal dose of 100 PFUs of VSV-EBOV in IFNAR KO mice was then verified in *Cas9<sup>flg</sup>*, *Ifnar1<sup>−/−</sup>* mice. At the endpoint of the *Cas9<sup>flg</sup>*, *Ifnar1<sup>−/−</sup>* mouse study, tissues (liver and spleen) were dissected for histological analysis and assessment of the presence of viral antigen.

**Histology and immunohistochemistry**

Mouse tissues derived from liver, spleens, kidneys, and lungs were dissected and fixed in 4% PFA overnight. Dissected tissues were then transitioned into paraffin wax using an ASP3000 tissue processor (Leica, Buffalo Grove, IL). Tissue samples were then embedded in paraffin blocks using a Histocore Arcadia H embedder (Leica, Buffalo Grove, IL). Samples were cut to generate 5-µm sections and mounted on slides. Hematoxylin and eosin staining was performed manually in house. Stained slides were imaged using a Leica ICC50E camera and microscope at 40×, 100×, or 400× magnification. For
immunohistochemistry, tissues mounted on charged slides were deparaffinized, rehydrated through alcohol steps, and then treated with citrate buffer under high pressure (Cuisinart Pressure Cooker) for antigen retrieval. Following antigen retrieval, the slides were subjected to 3% hydrogen peroxide to inactivate endogenous peroxidases and blocked with 5% goat serum and 0.1% Triton X-100. The slides were incubated with a primary rabbit anti-EOBV-GP antibody (1:100) (IBT Bioservices, Rockville, MD) overnight at 4°C. The primary antibody was removed, samples were washed 5 times with PBS, and secondary goat anti-rabbit horseradish peroxidase (HRP) antibody (1:500) (Thermo Fisher Scientific, Waltham, MA) was then added. DAB substrate (3,3′-diaminobenzidine) reagent (SK-4100, Vector Laboratories, Burlingame, CA) was added to samples for 10 min for detection of HRP activity, and then the samples were washed with water. Mayer’s hematoxylin (Sigma-Aldrich, St. Louis, MO) was used to counterstain for 8 min with subsequent water washes and stepwise dehydration with ethanol, and samples were finally coverslipped using Permount mounting medium. Stained slides were imaged on a Leica ICC50E camera and microscope at 40×, 100×, or 400× magnification.

**Measurement of VSV-L gene copies**
To extract RNA, mouse tissues were homogenized in RX tissue lysis buffer (GenCatch Total RNA Miniprep Kit, Epoch Life Science, Sugar Land, TX) using a handheld pestle motor. The resulting homogenate was clarified by centrifugation, and the RNA from the supernatant was isolated and purified using the GenCatch Total RNA Miniprep Kit (Epoch Life Science, Sugar Land, TX). Following extraction of RNA, cDNA was synthesized using the Superscript III First Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA). Quantitative PCR reactions were set up in triplicate using Primetime Gene Expression Master Mix (IDT, Coralville, IA), 100 ng of cDNA, and a custom VSV-L primer and probe set (Table S1). Reactions were then performed on a Bio-Rad (Hercules, CA) CFX96 Touch real-time system, and data analysis for expression of VSV-L was carried out using a standard curve of VSV-L DNA.

**Statistical analysis**
All results where individual values were averaged were depicted as mean ± standard deviation. To determine significance, two-tailed t tests for the experiments in Figure 2 were performed. In Figure 5, for viral RNA copy number statistics, graphs were plotted in GraphPad Prism 9, and p values were calculated in Prism 9 using a Mann-Whitney test. p < 0.05 was considered significant and p < 0.01 very significant.

**SUPPLEMENTAL INFORMATION**
Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2021.09.012.

**ACKNOWLEDGMENTS**
This work was supported by the Laboratory Directed Research and Development Program at Sandia National Laboratories (SNL) and the DARPA Safe Genes Program under contract HR0011-17-2-0043. SNL is a multi-mission laboratory managed and operated by National Technology & Engineering Solutions of Sandia, LLC, a wholly owned subsidiary of Honeywell International Inc., for the U.S. Department of Energy’s National Nuclear Security Administration under contract DE-NA0003525. This paper describes objective technical results and analyses. Any subjective views or opinions that might be expressed in the paper do not necessarily represent the views of the U.S. Department of Energy or the United States Government. All work performed at Lawrence Livermore National Laboratory is performed under the auspices (LLNL-JRNL-821248) of the U.S. Department of Energy under contract DE-AC52-07NA27344. The authors gratefully acknowledge Richard Mosssos for Cx7 imaging training, Christine Thatcher for histology training, and Kimberly Butler for manuscript review.

**AUTHOR CONTRIBUTIONS**
N.C., P.D., S.J.L., E.A.S., J.L.S., A.S., and O.A.N. conducted experiments. N.C., P.D., S.J.L., E.A.S., J.L.S., and O.A.N. designed experiments and analyzed data. Y.L. implemented computer code and supporting algorithms. N.C. and O.N. wrote the original draft, and all authors provided review and editing. J.S.S. and O.N. provided project supervision and supported funding acquisition.

**DECLARATION OF INTERESTS**
The authors declare no competing interests.

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