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Development and Delivery of Safe CRISPR-based Anti-Viral Countermeasures Targeting Host and Viral Genomes

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Background

- CRISPR/Cas9 genome editing technology has created a new modality for treating viral infections indirectly by targeting critical host factors[1]
- Although the Cas9 endonuclease can target DNA viruses directly for degradation, many viruses of biodefense concern have RNA genomes that prevent direct targeting approaches by current CRISPR technology
- By developing programmable CRISPR systems that target RNA instead of genomic DNA, deleterious off-target concerns may be alleviated
- Additionally, to realize the full potential of CRISPR-based countermeasures, significant improvements in delivery are needed to reduce the risks associated with viral vectors
- Lipid-coated mesoporous silica nanoparticle (LC-MSN) technology can be adapted for large cargo delivery and provide advantages of both MSNs and liposomes, including controlled release, targeting specificity, colloidal stability, and biocompatibility[2]

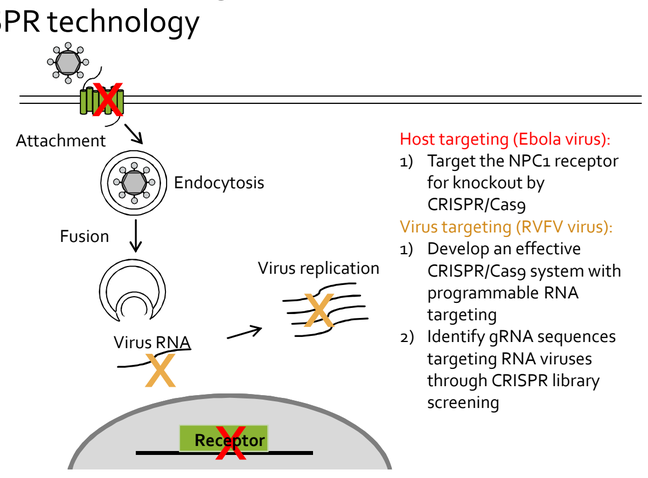

Figure 1. Approaches to CRISPR-based anti-viral countermeasures targeting host and virus

Objective: Develop safe CRISPR-based anti-viral countermeasures targeting host and viral genomes, packaged using nanoparticle technology for efficient and specific *in vivo* delivery

[1]Soppe JA. et al. *Trends in Microbiology* 2017; [2]Butler K. et al. *Small* 2016

Discovery and Characterization of an RNA-targeting Cas9

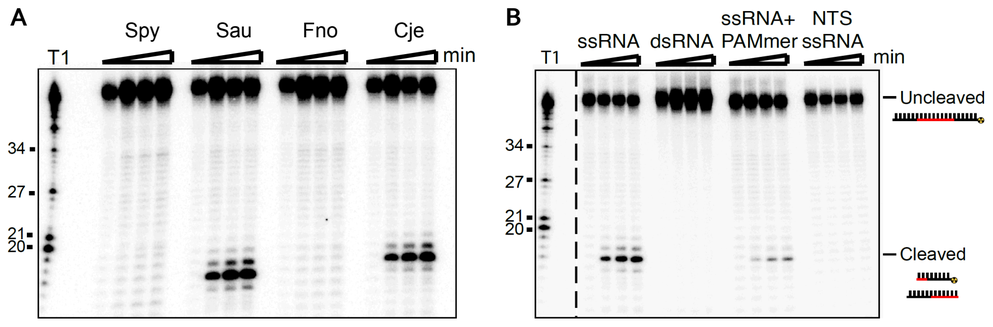

Figure 2. RNA-targeting Cas9 (R-Cas9) cleaves ssRNA without a PAMmer. [3] (A) *In vitro* cleavage of ssRNA by Cas9-sgRNA RNP complexes of several SpyCas9 homologs. (B) *In vitro* cleavage assay of various RNA substrates (PAMmer=DNA oligo).

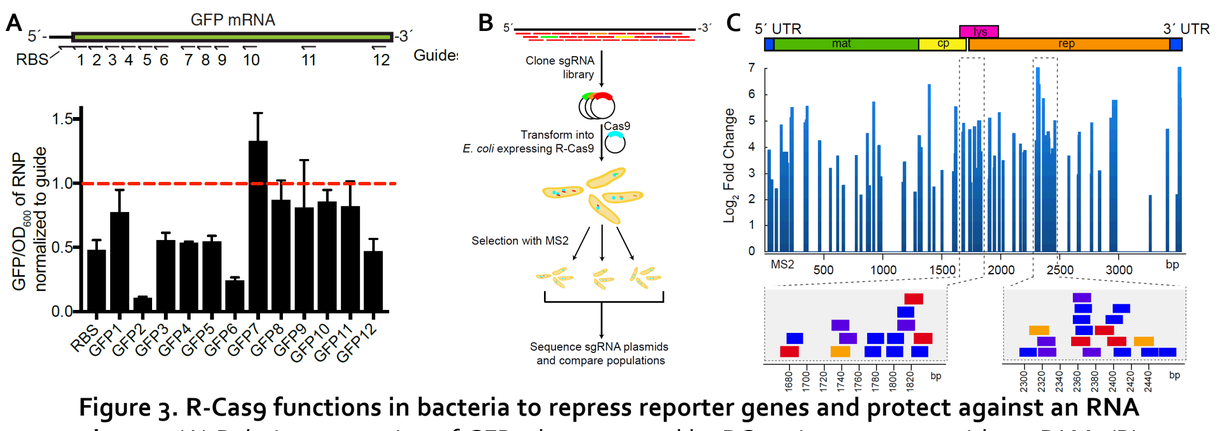

Figure 3. R-Cas9 functions in bacteria to repress reporter genes and protect against an RNA phage. [3] (A) Relative expression of GFP when targeted by RCas9 in sequences without PAMs (B) Overview of an MS2 phage CRISPR tiling library screen. (C) Log2 fold changes of guides with an FDR-corected p-value <0.05 mapped to the MS2 genome.

A subset of Cas9 enzymes recognize and cleave ssRNA substrates which can be exploited for programmable RNA targeting applications

[3]Strutt S, Torrez R, Kaya E, Negrete O, Doudna J; ***eLife*** 2018;7:e32724

CRISPR/Cas9 library screening for host factor identification or direct antiviral activity

Methods: The GeCKOv2 CRISPR knockout library or a tiling library of guide RNAs targeting the Rift Valley fever virus (RVFV) RNA genome was introduced into cells stably expressing Cas9 and then infected with RVFV strain MP12. Surviving cells were collected and sequenced to identify enriched gRNA sequences compared to uninfected controls.

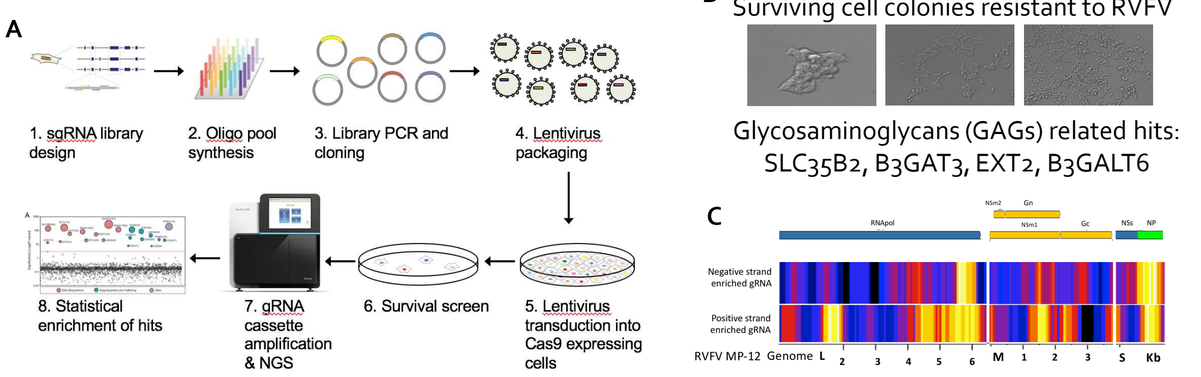

Figure 4. CRISPR library screening for identification of host or anti-viral gRNAs. (A) Discovery of host and viral genetic targets for CRISPR-based anti-viral development involves screens with a pipeline illustrated above. (B) GeCKO-RVFV host factor screening in 293Ts expressing SpyCas9 identified genes involved in GAG synthesis. (C) Anti-RVFV library screening in SaCas9 expressing 293Ts identifies hot spots of gRNA targeting.

Cas9 library screening against RVFV identifies GAG-related host factors and potent guide RNA sequences for direct anti-viral countermeasure development

Host-directed CRISPR-based countermeasures

Methods: HEK293T cells were transfected with Cas9/gRNA (RNP) complexes targeting the NPC1 (Ebola receptor) or a control genome sequence. At day 4 post-transfection, editing efficiency was measured by T7E1 and the edited cells were infected with VSV-EBOVGP-GFP. Resistant cells were allowed to grow to create an NPC1 knockout cell line. These edited cells were then mixed with wild type 293T cells to obtain varying knockout ratios.

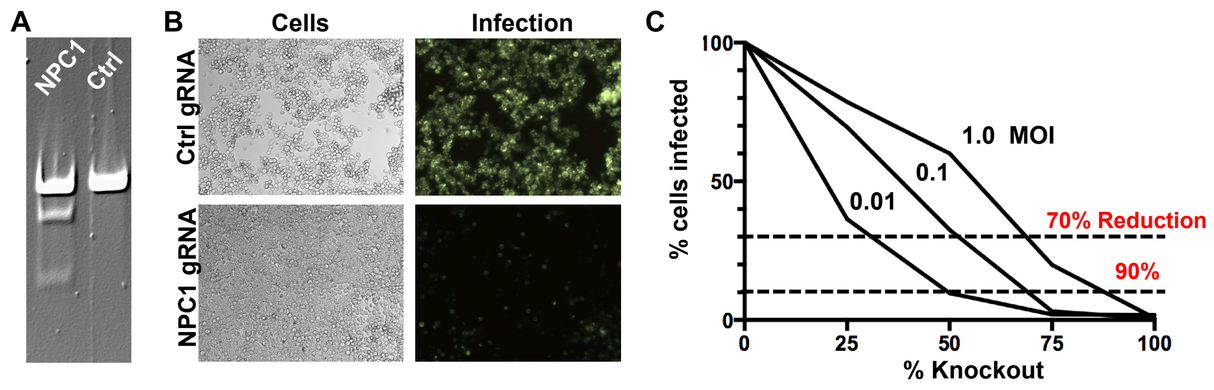

Figure 5. Targeting NPC1 using CRISPR RNPs create cells resistant to surrogate Ebola virus. (A) T7E1 genome editing analysis on cells transfected with CRISPR RNPs targeting NPC1 or a control locus. (B) Cells edited with NPC1 or control CRISPR RNPs were infected with VSV-EBOVGP-GFP and imaged 3dpi. (C) Analysis of editing thresholds necessary to reduce surrogate Ebola virus infection. Results indicate at a low initial dose of infection (0.01 MOI), 25-50% editing is required to inhibit virus infection at levels of 70-90%.

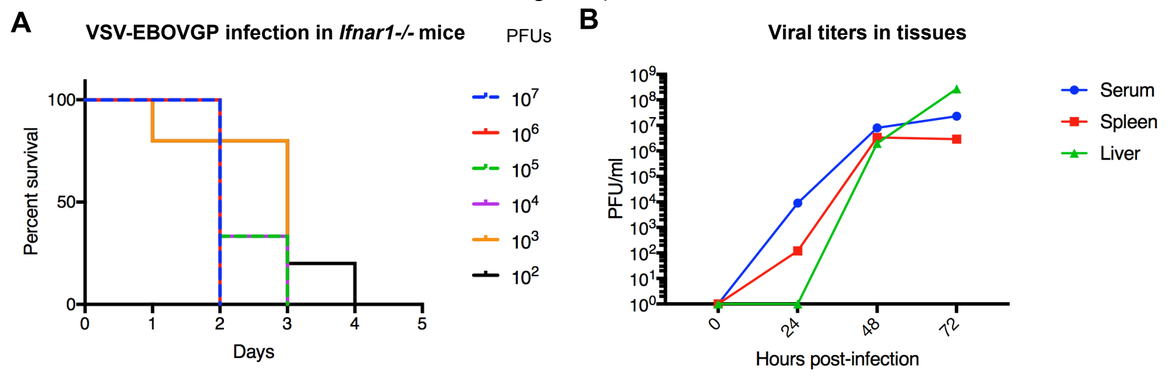

Figure 6. Establishing an ABSL-2 mouse model for testing host-directed CRISPR countermeasures targeting Ebola virus entry. (A) Groups of six C57BL/6 mice deficient for the interferon alpha receptor gene (*Ifnar1* -/-) were infected with VSV-EBOVGP through intraperitoneal injection (IP) at indicated doses. (B) Viral dissemination in *Ifnar1* -/- mice infected IP with 10 PFUs of VSV-EBOVGP.

CRISPR countermeasures targeting a host factor gene involved in Ebola virus infection are effective and can be tested *in vivo* in less than BSL-4 containment

nanoCRISPR delivery technology

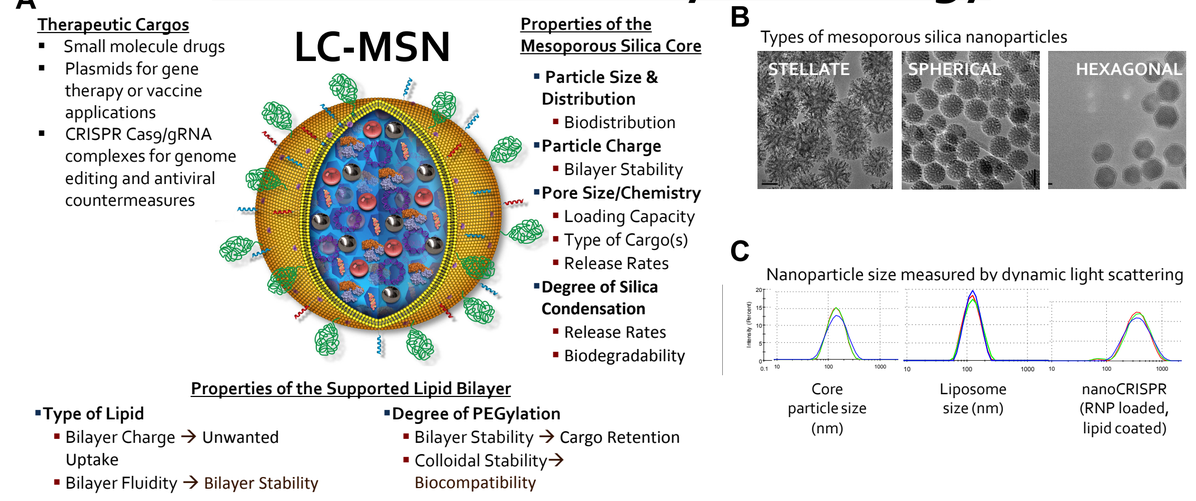

Figure 7. Lipid-coated mesoporous silica nanoparticle (LC-MSN) technology for *in vivo* delivery of Cas9/gRNA ribonucleoprotein complexes ("nanoCRISPR"). (A) Advantages of LC-MSN technology for therapeutic cargo delivery. (B) Various types of mesoporous silica nanoparticle cores with varying pore sizes. (C) The assembly of a nanoCRISPR formulation as measured by dynamic light scattering after fusion with a cationic lipid bilayer.

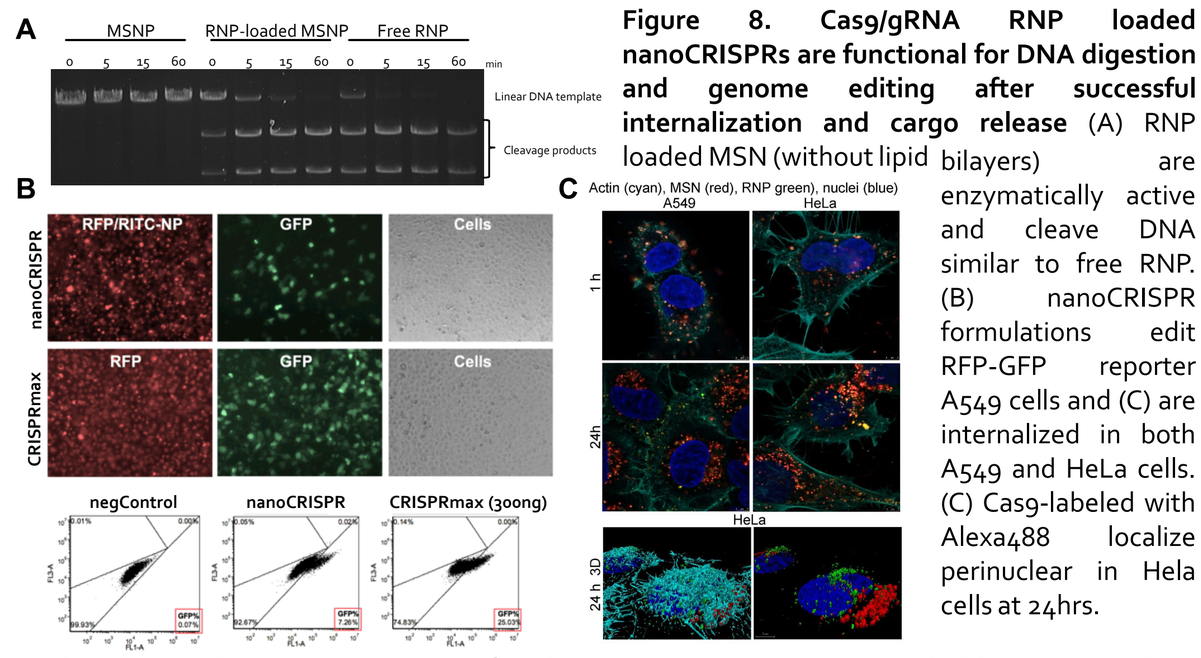

Figure 8. Cas9/gRNA RNP loaded nanoCRISPRs are functional for DNA digestion and genome editing after successful internalization and cargo release (A) RNP loaded MSN (without lipid bilayers) are enzymatically active and cleave DNA similar to free RNP. (B) nanoCRISPR formulations edit RFP-GFP reporter A549 cells and (C) are internalized in both A549 and HeLa cells. (C) Cas9-labeled with Alexa488 localize perinuclear in Hela cells at 24hrs.

LC-MSN technology adapted for Cas9/gRNA cargos (nanoCRISPR) provides a novel approach to non-viral CRISPR delivery

Conclusion

In this work, we highlight the use CRISPR/Cas9 technology targeting human pathogenic viruses as a potential new anti-viral strategy. CRISPR-based approaches can modify host genes involved in viral infection or target viral RNA directly using newly discovered functions in SpyCas9 homologs. As a host-directed therapeutic, we have focused on NPC1, the entry receptor for Ebola virus. By utilizing Cas9 directed knockouts of NPC1, we blocked Ebola infection in cell culture. As a direct defense against RNA viruses, we performed a tiling library screen using RNA-targeting Cas9 against RVFV and identified several guide RNAs that protect cells from infection. Additionally, we demonstrated the adaptation of lipid-coated mesoporous silica nanoparticle technology for cellular delivery of Cas9/gRNA RNP in an attempt to overcome a major hurdle in the clinical translation of CRISPR-based countermeasures.

Acknowledgments

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