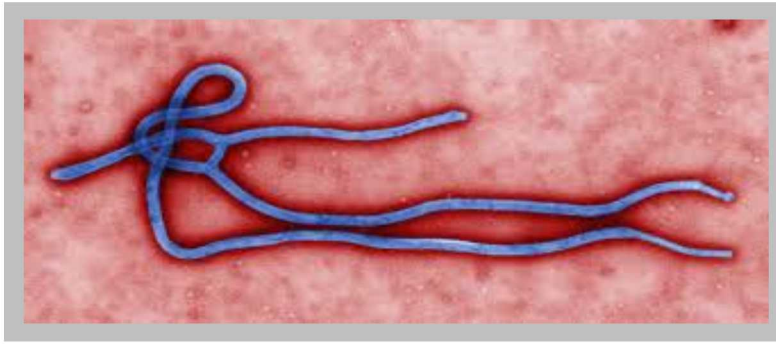
SAND2019-3698C

DARPA PREPARE Kick-off Meeting
April 3-5, 2019
New York City, NY



Next-Generation CRISPR and anti-CRISPR Tools and Delivery Systems For Safely Engineering the Genome and Epigenome

Joe Schoeniger, M.D, Ph.D
Oscar Negrete, Ph.D.

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U.S. DEPARTMENT OF ENERGY
NNSA National Nuclear Security Administration

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DEFENSE ADVANCED RESEARCH PROJECTS AGENCY

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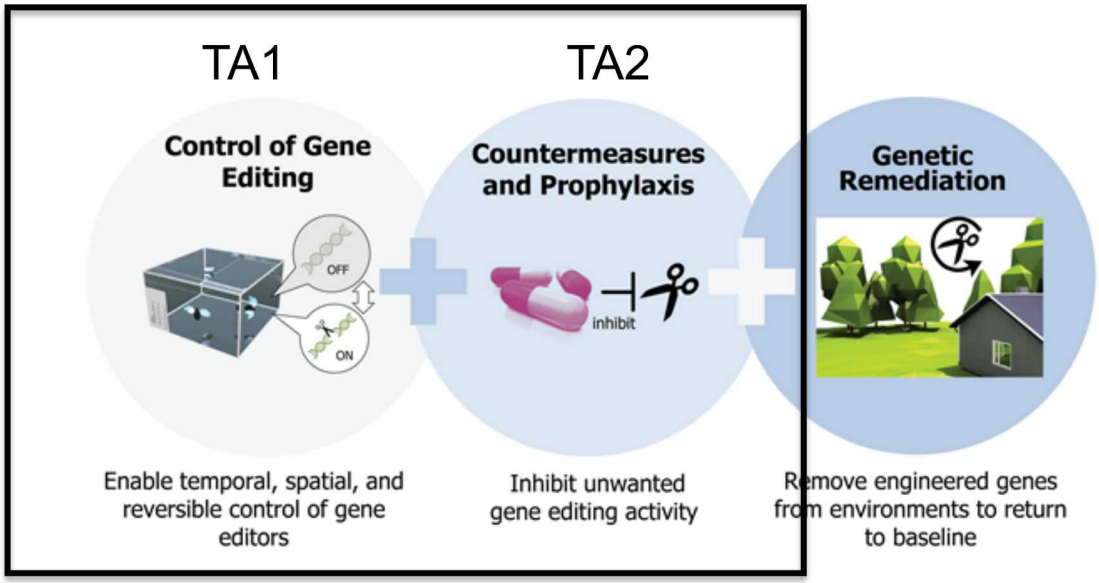
Safe genes program



TEAM

PI: Jennifer Doudna (UCB)
SNL PI: Joe Schoeniger (SNL)
Co-PIs: Jonathan Weissman (UCSF)
Luke Gilbert (UCSF)
Joe Bondy-Denomy (UCSF)

Technical Area 1 (TA1)

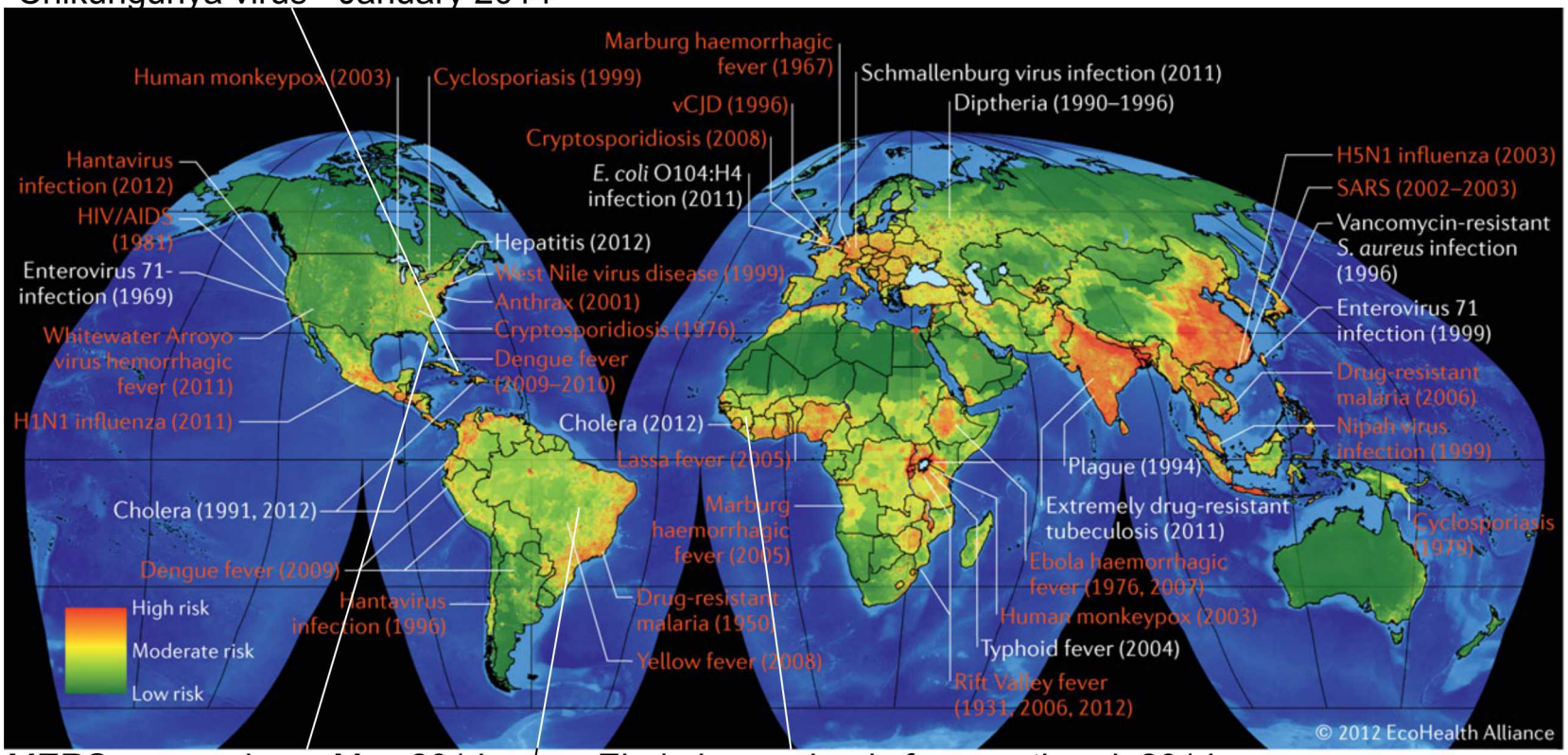
- Develop gene editors targeting the epigenome and transcriptome (UCSF, UCB)
- Target viral pathogens of biodefense concern with editors (SNL)
- Reformulate *in vivo* delivery platforms for new editor systems (SNL)
- Test delivery and preliminary anti-viral effectiveness in animal models (UCSF, SNL/ LLNL)

Technical Area 2 (TA2)

- Identify broad spectrum anti-crispr (Acr) proteins (UCSF, UCB)
- Characterize *in vitro* efficacy of Acrs in cell reporters (SNL)
- Reformulate *in vivo* delivery platforms for Acrs (SNL)
- Test delivery and obtain *in vivo* efficacy data for Acrs inhibitors in CRISPR reporter mice (UCSF, SNL)

Emerging infectious diseases:
The need for rapid countermeasure development

Chikungunya virus - January 2014



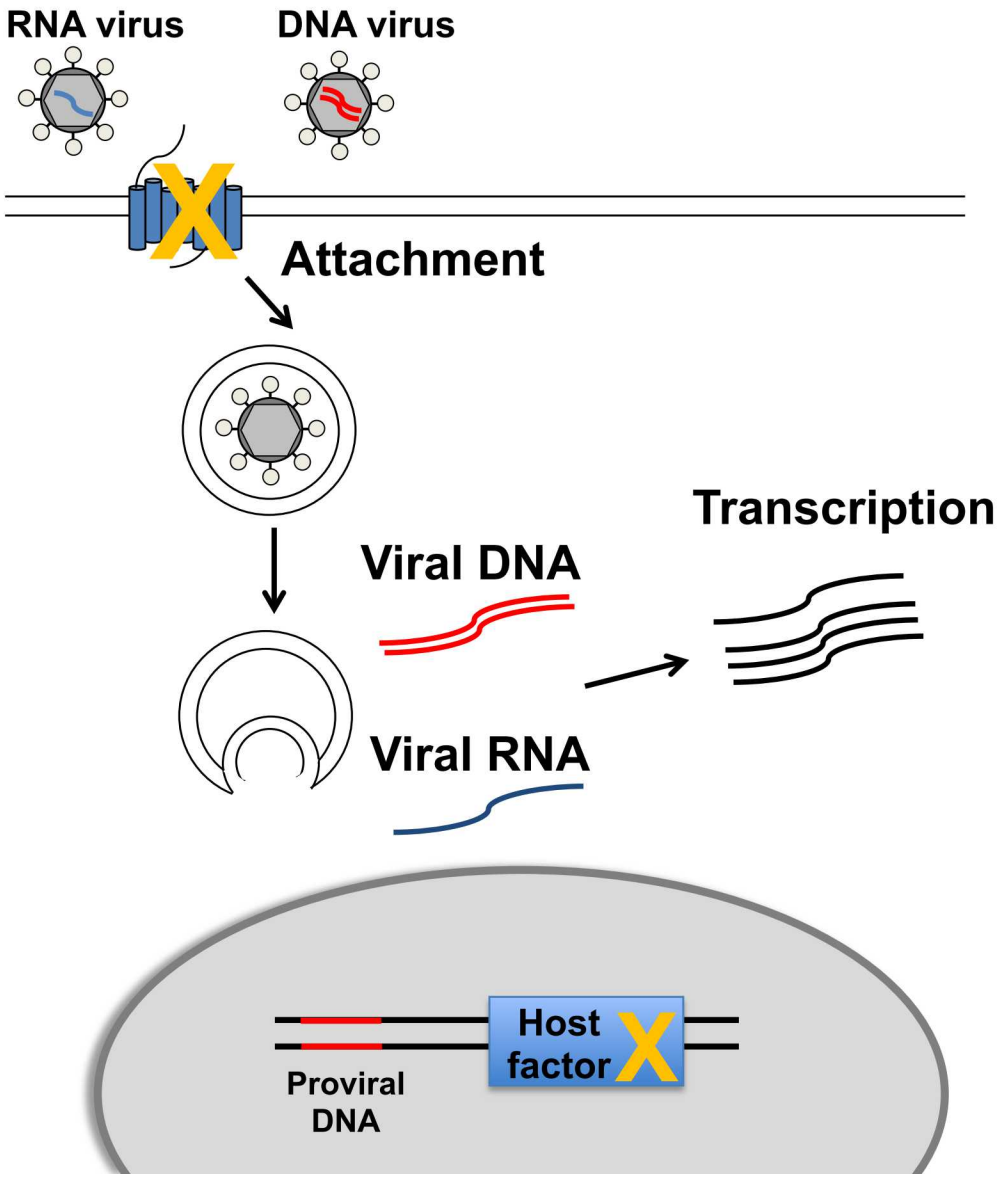
MERS coronavirus - May 2014

Ebola hemorrhagic fever outbreak 2014

Nature Reviews | Microbiology
W. Ian Lipkin

Zika virus - 2015

Antiviral Countermeasures using CRISPR-Based Methods



GOAL: Target host and viral genomes for anti-viral development

APPROACH: Optimize CRISPR/Cas tools for safely targeting DNA or RNA

DNA targeting

Controlled gene disruption:
Ligand inducible Cas9

Long term gene silencing:
Epigenetic dCas fusion

RNA targeting

Sa/CjCas9

Cas13a/d

Targeting viral genomes of biodefense concern

NIAID Biodefense Category A, B, C Priority Pathogens

Category A

Smallpox related-viruses

Viral Hemorrhagic fevers
- -Arenaviruses
- -Bunyaviruses
  - - **RVFV**
- -Filoviruses
  - - **Ebola**
  - - Marburg

Category B

Mosquito-borne viruses
- -Alphaviruses
  - - **VEEV**
  - - EEEV
  - - EEEV
  - - CHIKV
- -**Flaviviruses**
  - - JE
  - - YFV
  - - SLEV
  - - Zika virus

Category C

Nipah and Hendra viruses

SARS, MERS-CoV

Influenza virus

Genomes from viruses security concern are mainly

single stranded RNA

Viral models and host factors for genome targeting

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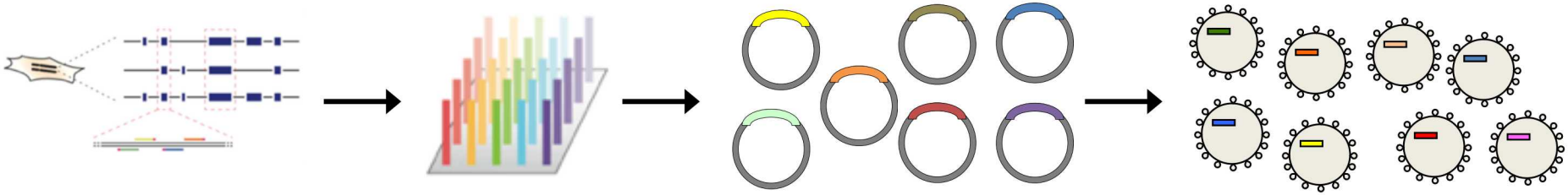
Virus family	Virus model	Host factor	Discovery method and broad spectrum implications
Filovirus	Ebola surrogate (VSV-EBOV)	Niemann-Pick C1 cholesterol transporter (NPC1)	Haploid mutagenesis screen (Carette et al, Nature, 2011), important for all strains of Ebola and Marburg
Flavivirus	Zika, (Dengue)	Signal peptidase (SPCS1)	CRISPR genomewide KO screen, (Zhang et al, Nature, 2016), SPCS1 KO in cells is effective against, West Nile, Dengue, Zika, Yellow Fever, Japanese encephalitis and HCV.
Alphavirus	VEEV TC83	N/A	N/A
Bunyavirus	RVFV MP12	B4GALT7 and other glycosaminoglycan (GAG) synthesis enzymes (e.g. B3GAT3)	Haploid mutagenesis screen, (Riblett et al, J.Virology, 2016), GAGs are important attachment factors for many viruses including Dengue, HIV, some alphaviruses

Host factor identification using CRISPR screening



1. sgRNA library design

2. Oligo pool synthesis

3. Library PCR and cloning

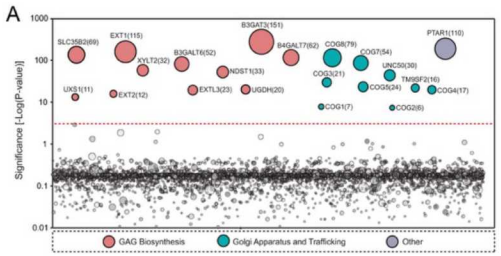
4. Lentivirus packaging

5. Lentivirus transduction into Cas9 expressing cells

Virus Infection

6. Survival screen

7. gRNA cassette amplification & NGS

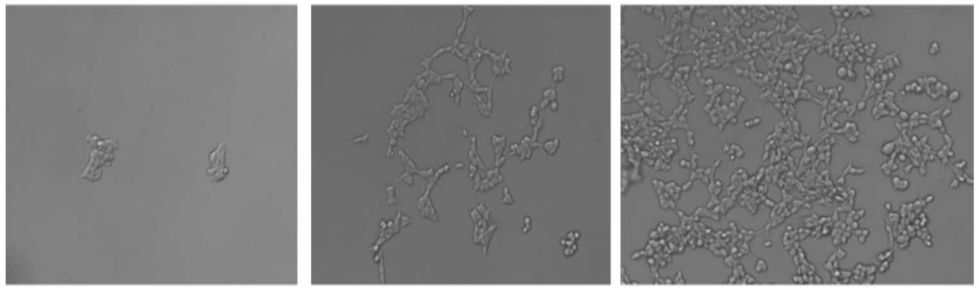


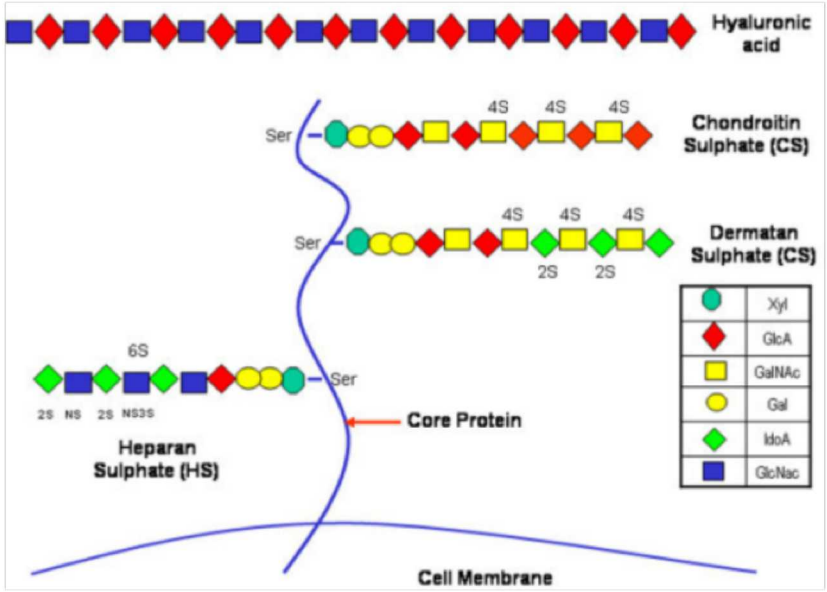
8. Statistical enrichment of hits

CRISPR KO screening for RVFV-host interactions

- Surviving cell colonies resistant to RVFV after performing CRISPR KO screening

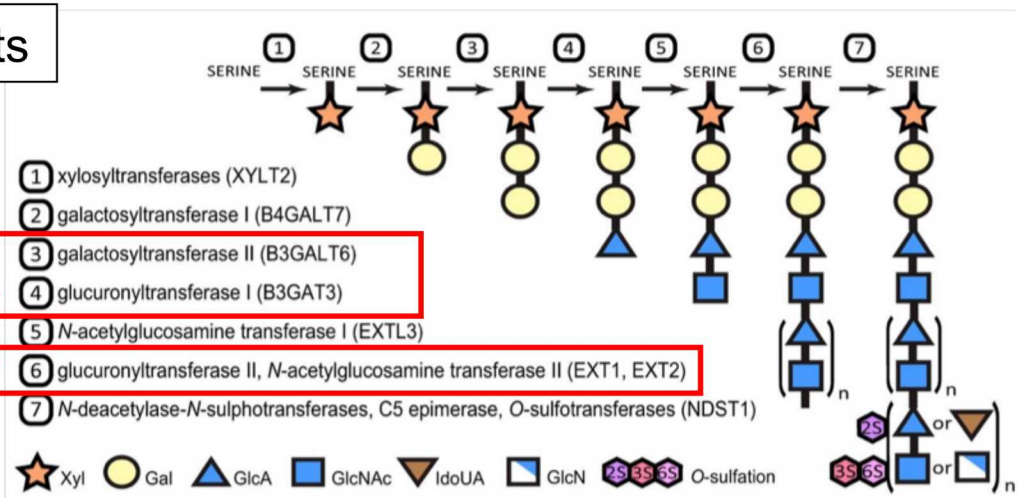
~10 ~100 ~1000



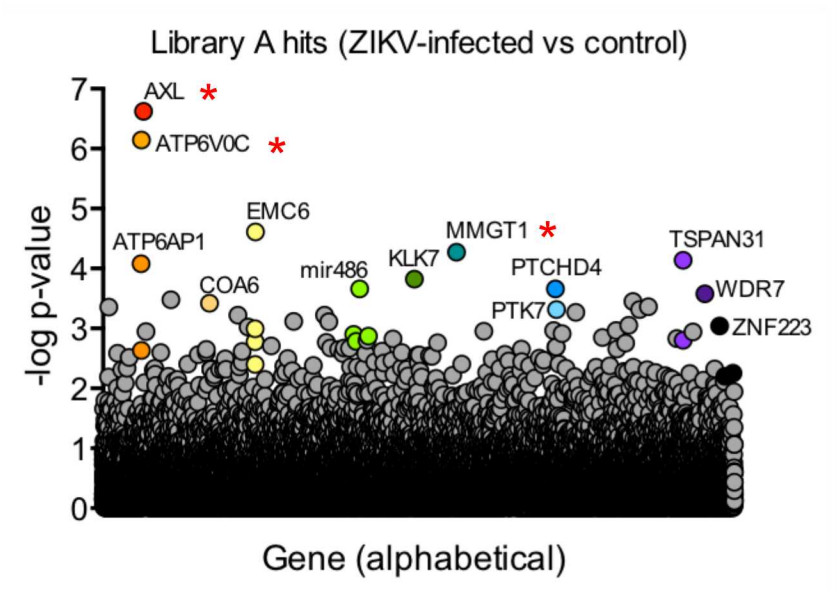


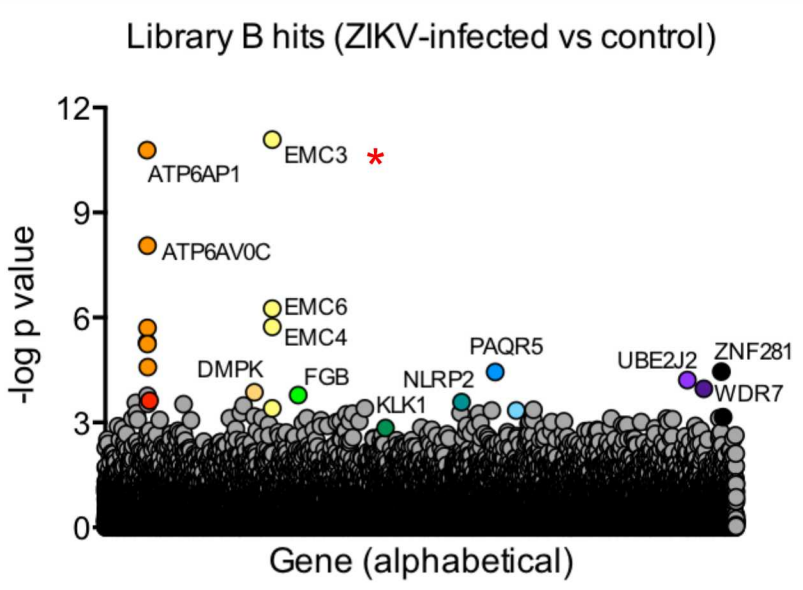
Glycosaminoglycans (GAGs) related hits

Top hits are enzymes involved in glycan elongation



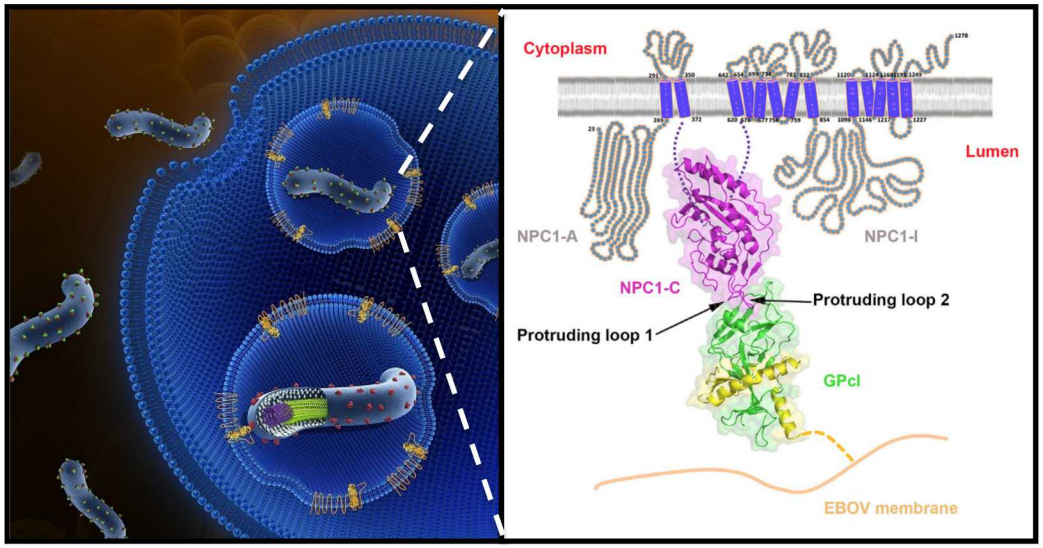
CRISPR KO screening for novel host factor candidates of Zika infection



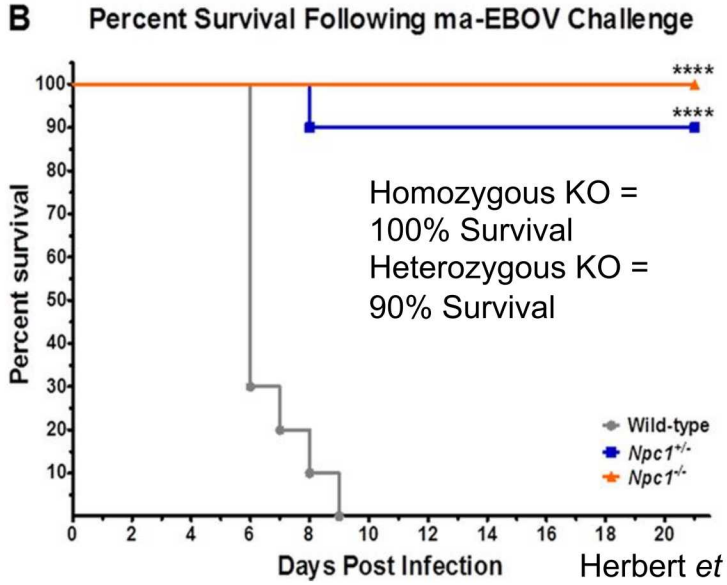


Gene	Protein type	Function	Relevant to viruses?	Location
AXL	receptor tyrosine kinase (extracellular, plasma membrane)	signaling, transferase activity	entry receptor for neuronal stem cells	plasma membrane
EMC3	Component of the ER membrane protein complex (EMC)	transmembrane protein folding	required for both ZIKV and DENV	ER
ATP6V0C	vacuolar ATPase	organelle acidification	important for flaviviruses	vacuoles, lysosome
MMGT1	membrane magnesium transporter 1	mediates magnesium transport	found in ZIKV screen	ER, golgi

Targeting the Ebola receptor for CRISPR-based anti-viral countermeasure development

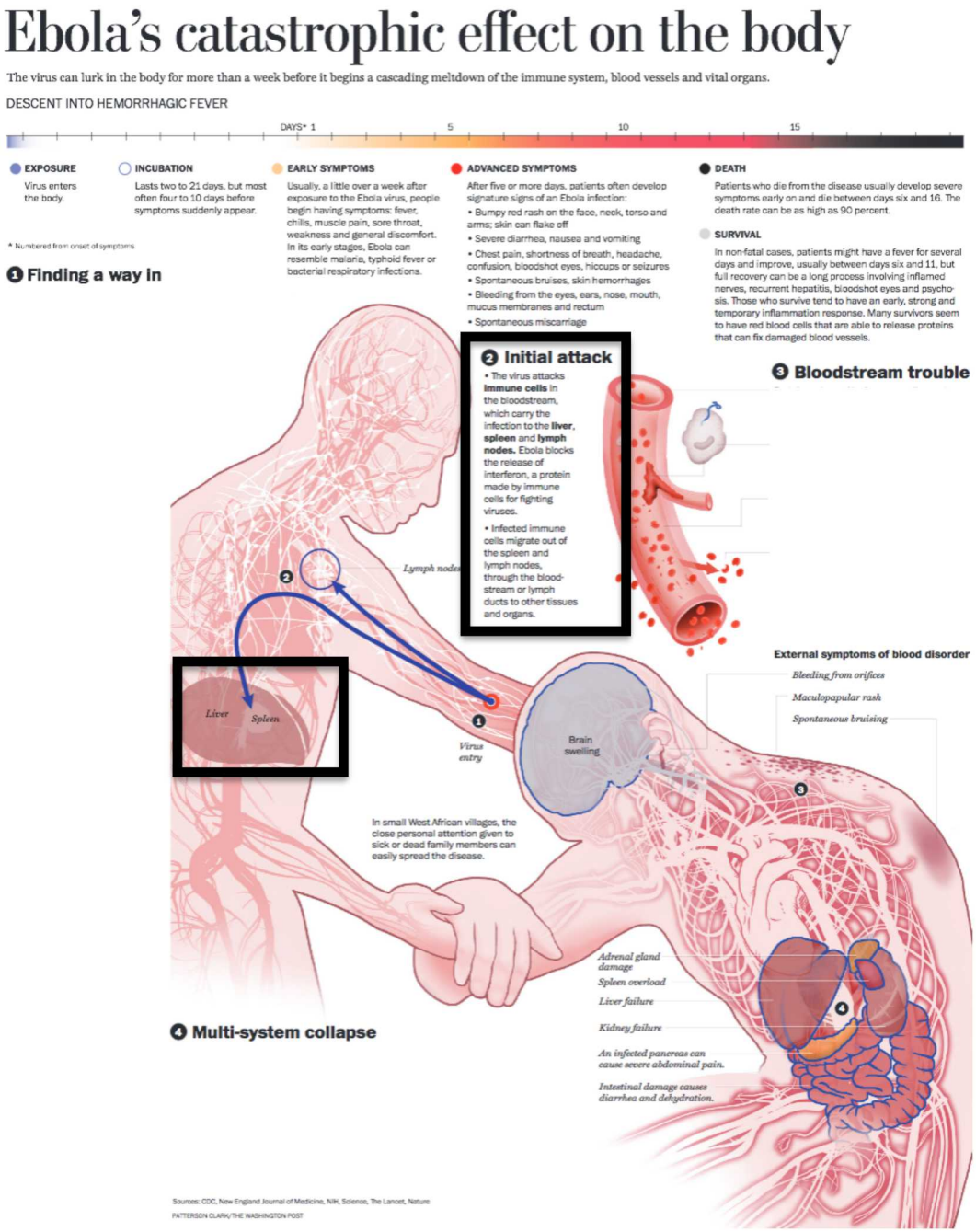


Wang *et al*, *Cell* 2016



Herbert *et al*, *mBio* 2015

- Niemann-Pick C1 cholesterol transporter (NPC1) is a large transmembrane protein found in late endosomes/lysosomes of cells and mediates the transport of luminal cholesterol across the endosomal/lysosomal membrane for dispersal to other cellular compartments.
- NPC1 is required for the entry of filovirus infection that includes all strains of Ebola and Marburg viruses
- Mice with a homozygous (-/-) deletion in NPC1 are completely protected from lethal Ebola virus challenge, while heterozygous NPC1 (+/-) mice protected at 90%.



- Ebola disease begins with amplification of the virus in the liver and spleen after initial infection of immune cells leading to high titer viremia and spread to secondary tissues

- Hypothesis: **Targeting the entry receptor NPC1 gene** using CRIPSR-based methods **with delivery to the liver and spleen** will reduce virus amplification and spread allowing for the immune response to effectively combat infection

Ebola countermeasures using host-directed CRISPR targeting

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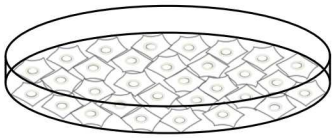
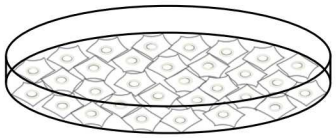
In vitro

Human cells



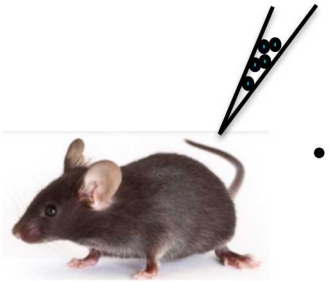
- Proof of concept in easy to transfect cells (e.g. 293Ts)
- KO line generation

Mouse cells – Disease relevant types



- Identify gRNA target sequences for SpyCas9 and SaCas9

In vivo



Ifnar1 -/-

VSV-Ebola (BSL-2) model

- Identify lethal dose in immunocompromised mice
- Measure viral kinetics and tissue tropism

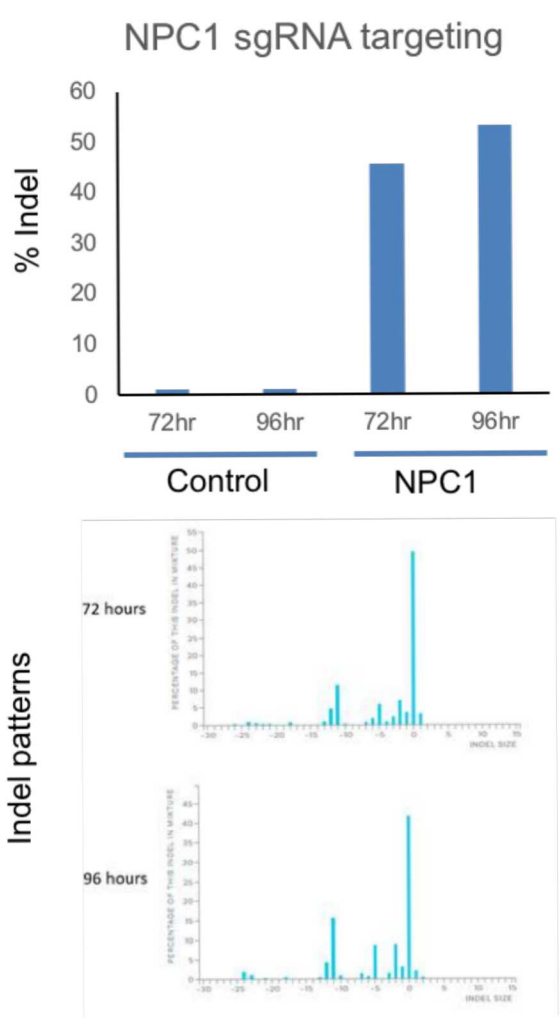
Target tissue editing

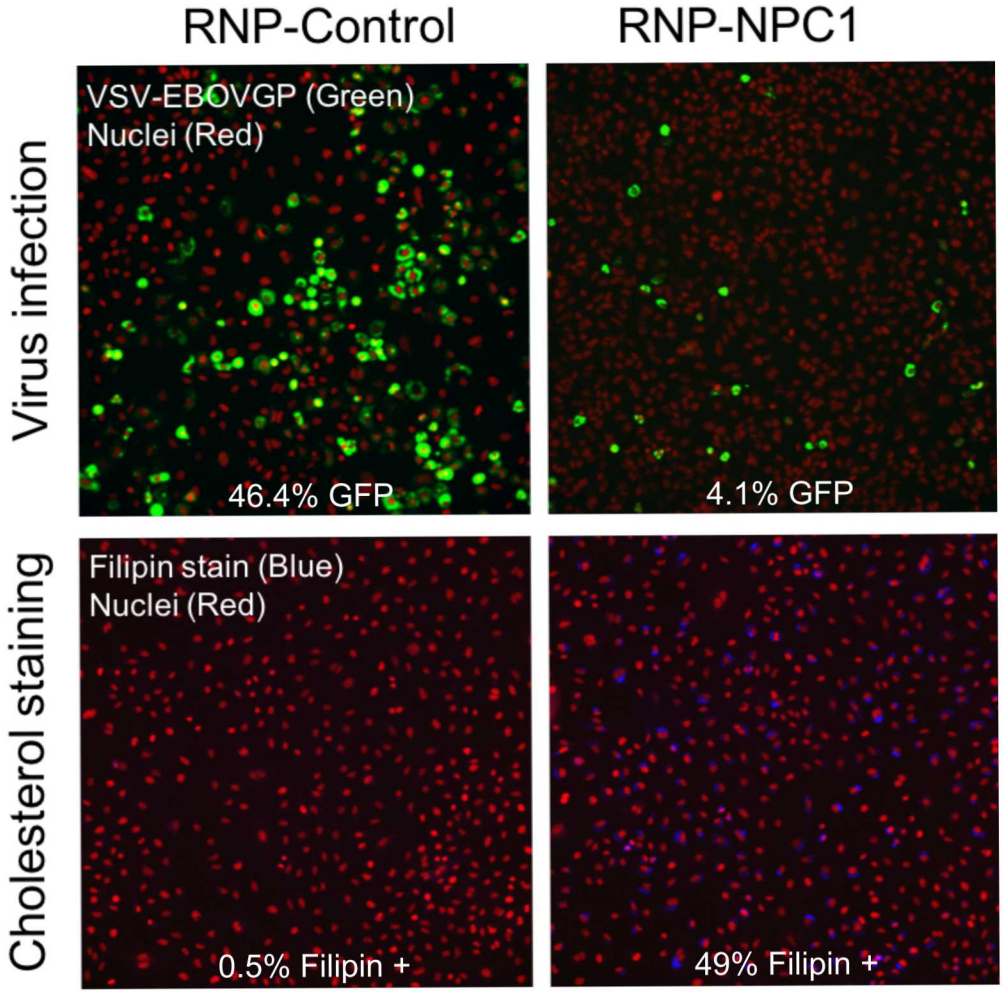
- First, use commercially available delivery systems (e.g. AAV)
- Measure editing in target tissues (e.g. liver, spleen) and optimize AAV dose with length of time

Efficacy studies

- Use best gRNAs from AAV studies to measure protection against VSV-Ebola
- Use NanoCRISPRs for protection studies

Cas9/gRNA RNP complexes targeting NPC1 reduce surrogate Ebola virus infection





Cas9/gRNA RNP complexes targeting NPC1 reduce surrogate Marburg virus infection

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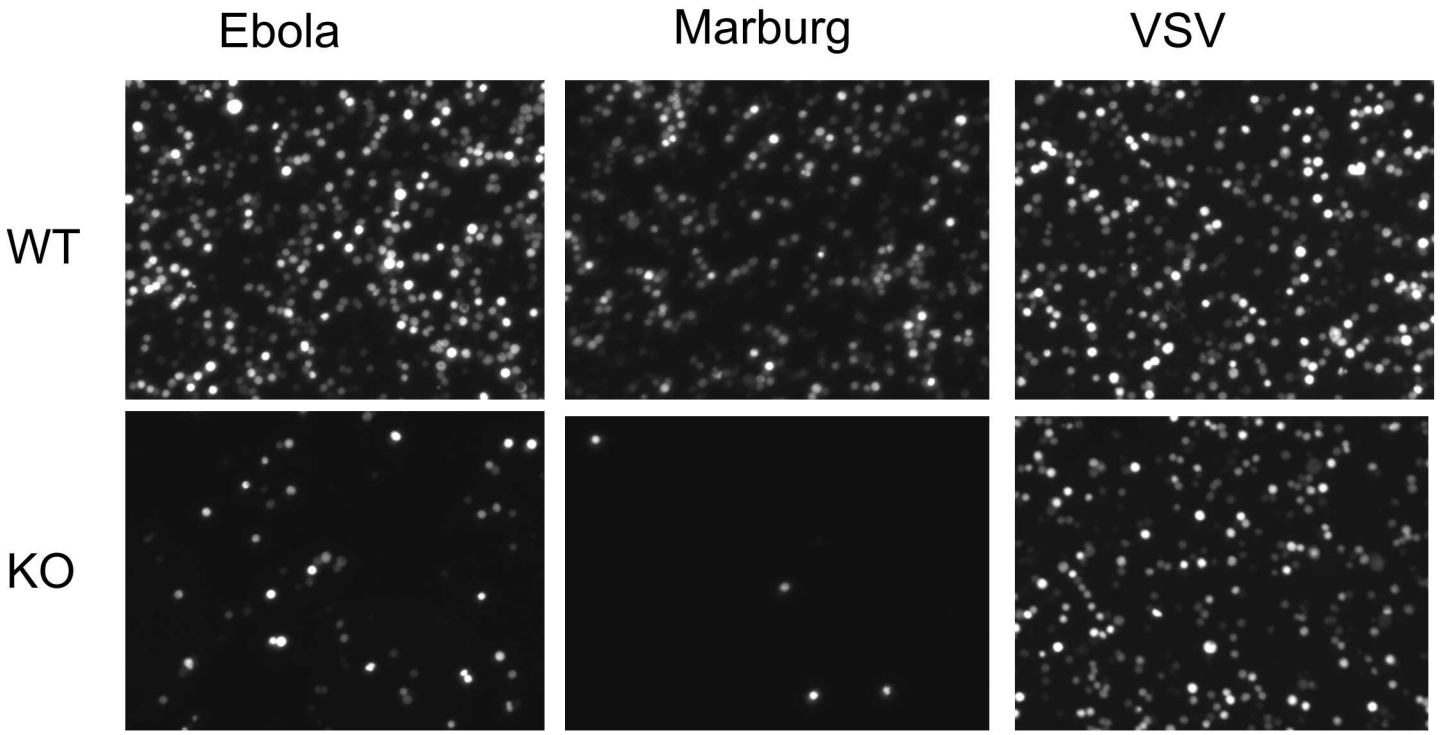
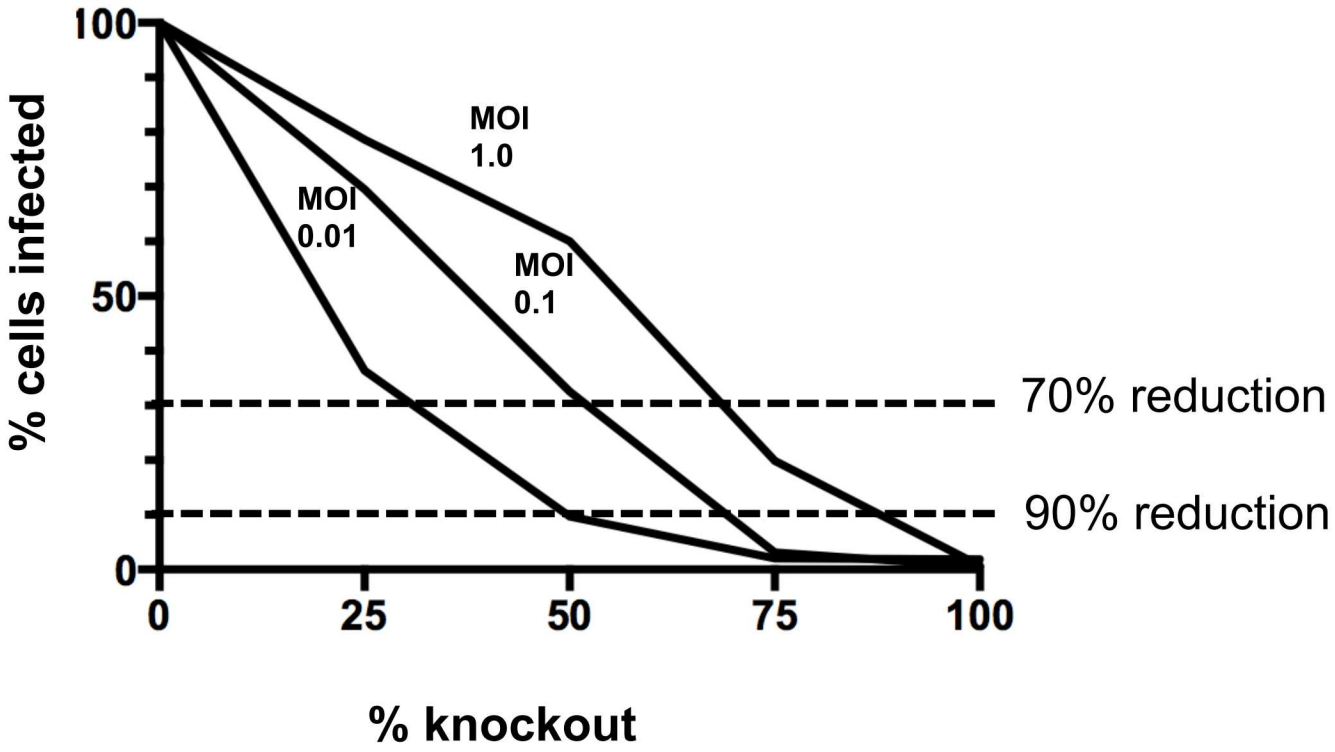
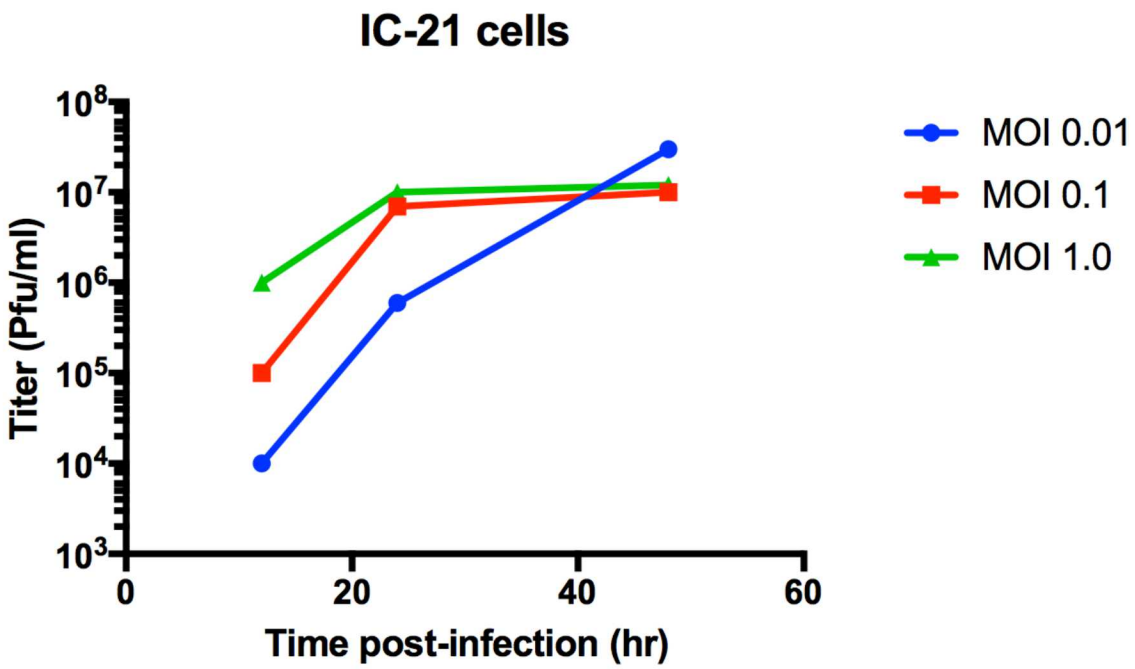


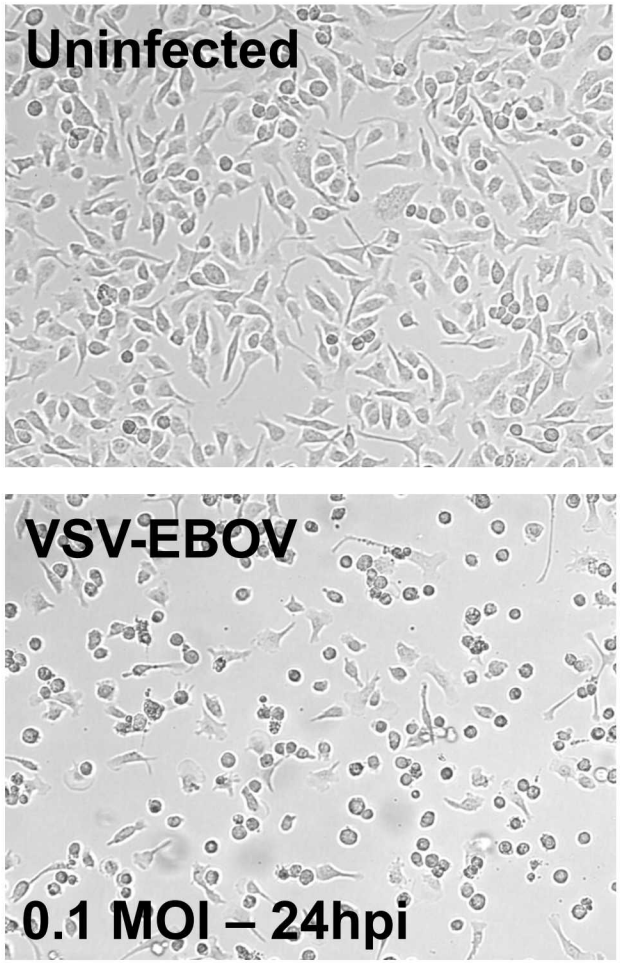
Evaluation of editing efficiencies required for a reduction of virus infection >90%



- Results suggest editing of at least 50% will result in approximately 90% reduction in infection.

IC-21 mouse macrophages are readily infected by VSV-EBOV





**Raw247 and P388D1 mouse macrophages did not infect well

SpyCas9 gRNAs targeting mouse NPC1 inhibit surrogate Ebola infection

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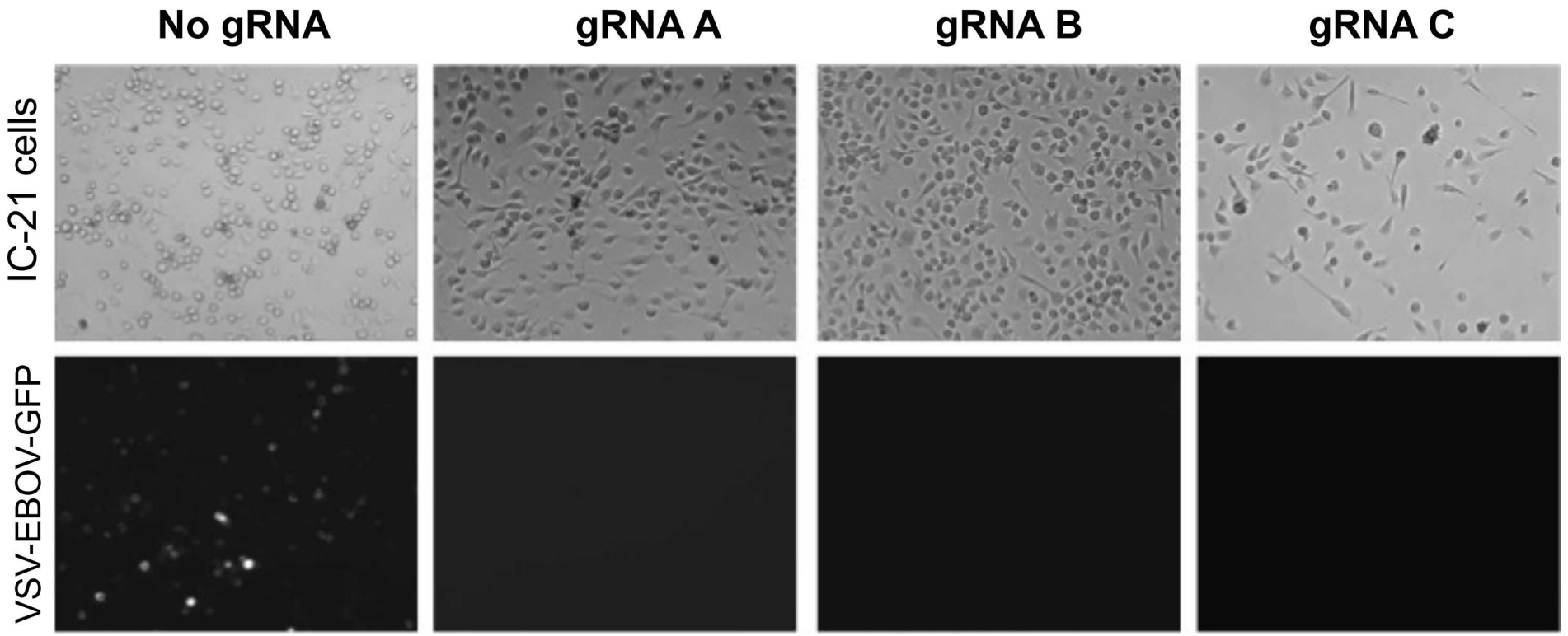

- SpyCas9/gRNA RNPs were transfected in IC-21 via CRISPRmax, passaged for 1 week, infected with VSV-EBOV to select for resistance cells. Then, re-infected with VSV-EBOV-GFP

Ebola countermeasures using host-directed CRISPR targeting

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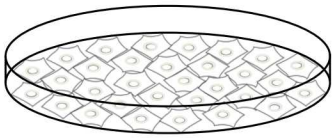
In vitro

Human cells



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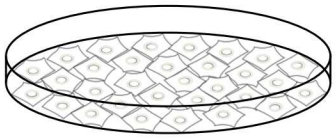

- Identify gRNA target sequences for SpyCas9 and SaCas9

In vivo

***Ifnar1* -/-**
Cas9+/+

VSV-Ebola (BSL-2) model

- Identify lethal dose in immunocompromised mice
- Measure viral kinetics and tissue tropism

Target tissue editing

- First, use commercially available delivery systems (e.g. invivofectamine)
- Measure editing in target tissues (e.g. liver, spleen) and optimize dose with length of time

Efficacy studies

- Use best gRNAs fror in vivo studies to measure protection against VSV-Ebola
- Use NanoCRISPRs for protection studies

Mouse models of Ebola virus mimic the tissue tropism observed in human disease

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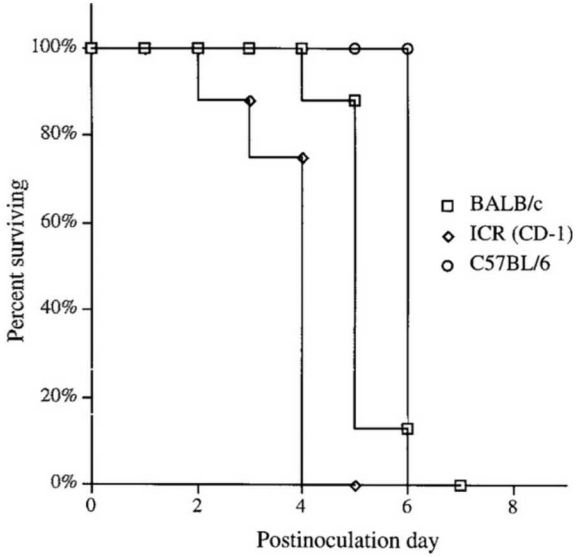
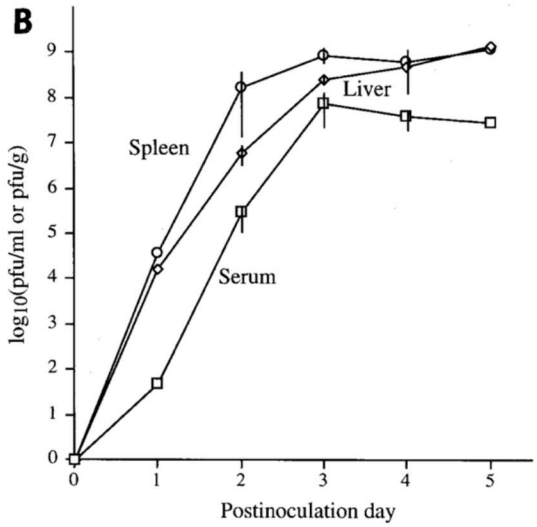
Serially passage EBOV in progressively older suckling mice produced a mouse adapted virus strain that was lethal in several immunocompetent mice

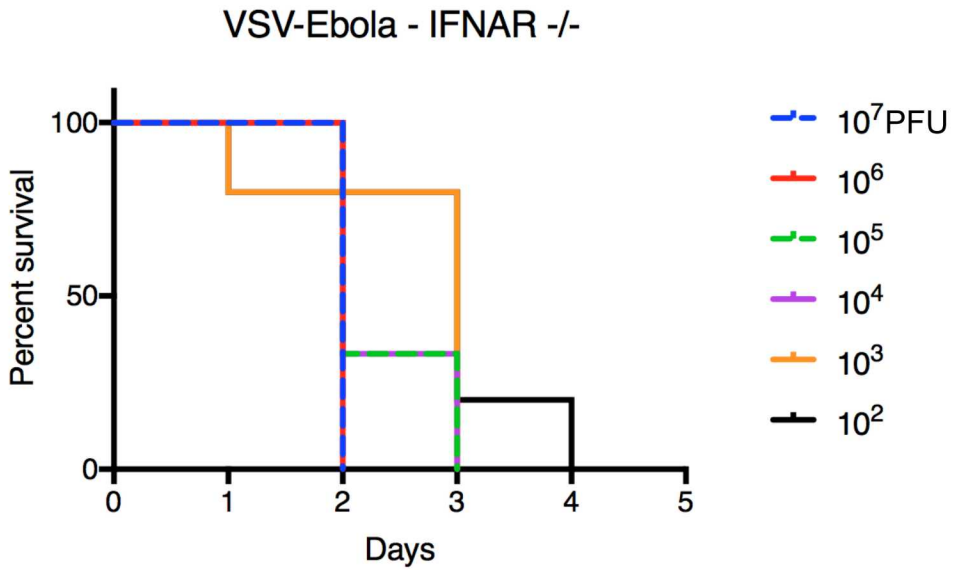


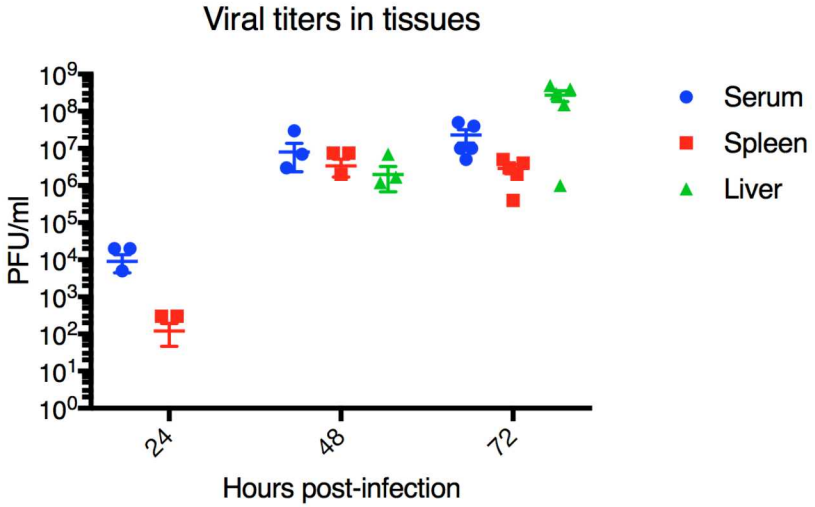


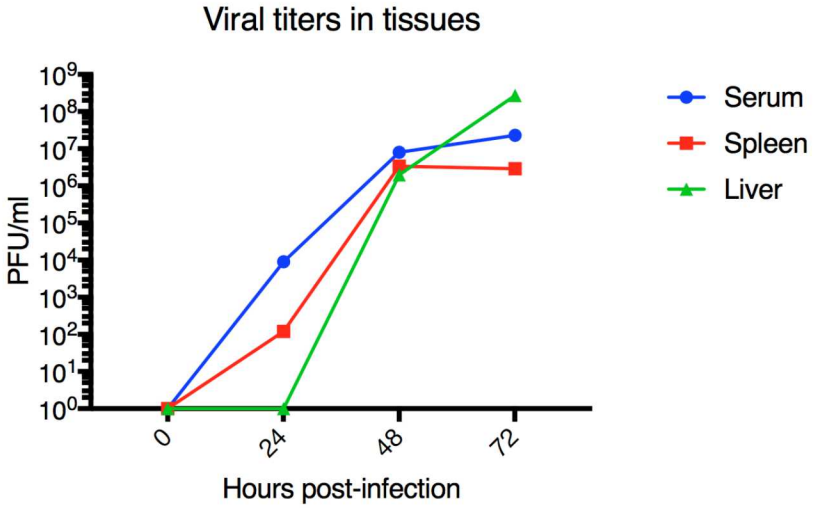
- Highly lethal in several strains of mice by challenging with 100PFU by IP
- In this model, the virus spreads rapidly reaching high titers in the spleen, liver and serum by day 3 post-inoculation
- This model requires **BSL-4** containment

(J of Infectious Diseases, 1999, Bray et al)

ABSL-2 animal model of surrogate Ebola virus in immunocompromised mice (IFNAR -/-) mimics ABSL-4 model

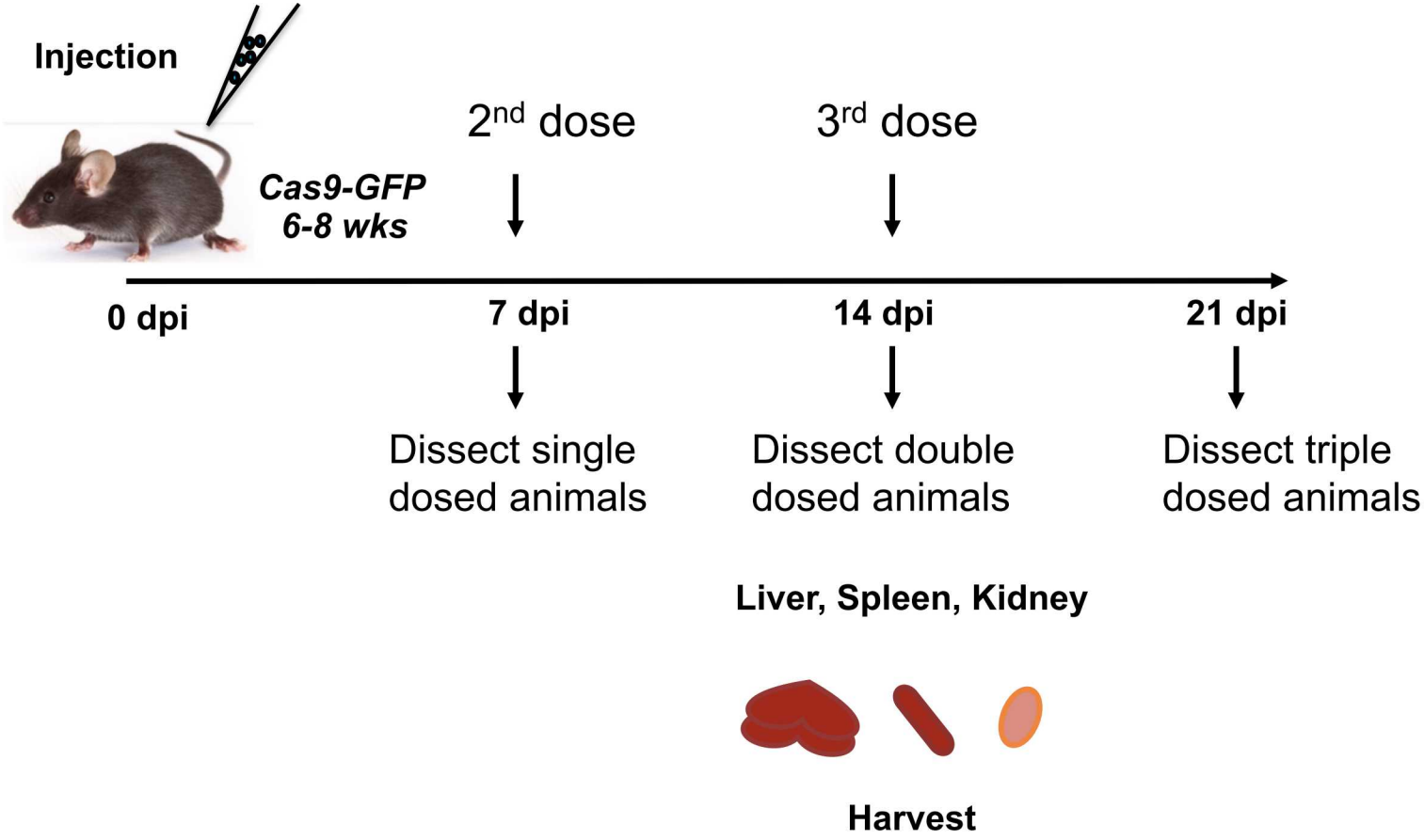




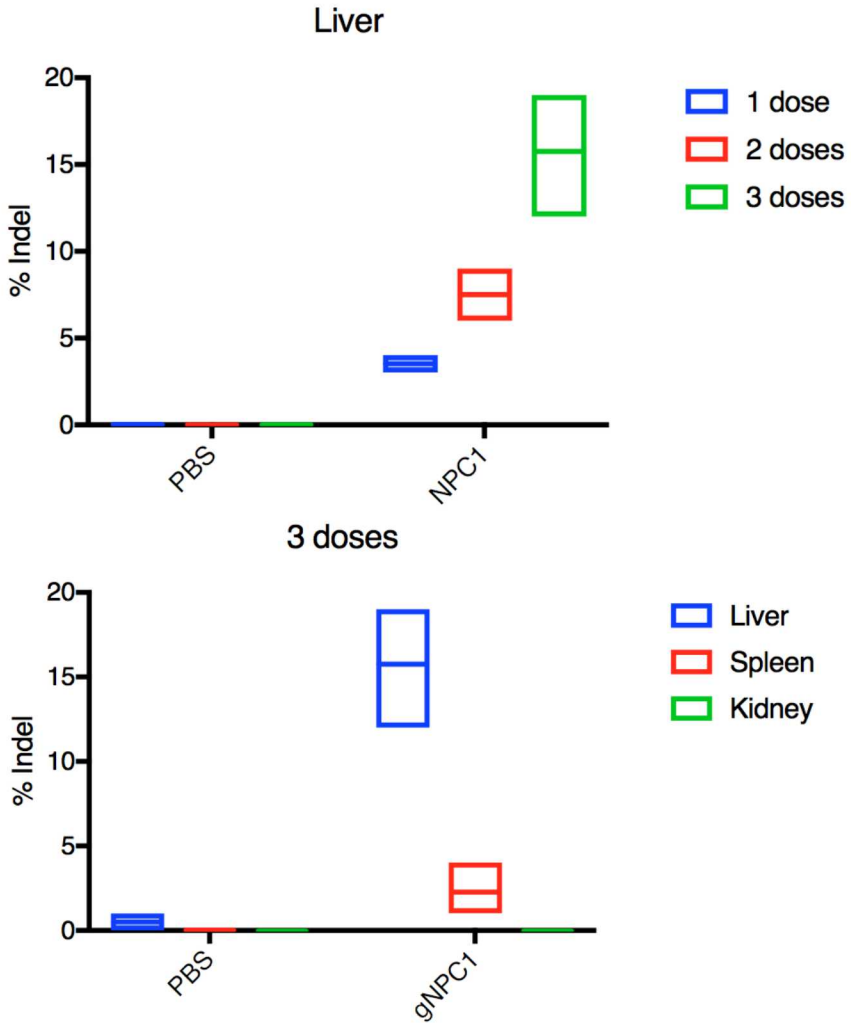


- C57Bl/6 mice deficient in the IFN-alpha receptor produce uniform lethality with 100PFU of VSV-EBOVGP challenged IP
- The tropism of this virus and spread emulates the mouse adapted Ebola strain model

Cumulative *in vivo* editing: Deliver guide RNAs into SpyCas9-GFP transgenic mice using a lipid nanoparticle reagent (invivofectamine)



Cumulative editing was achieved through multiple gRNA dosing and tissue distribution was mainly localized to the liver



A Single Administration of CRISPR/Cas9 Lipid Nanoparticles Achieves Robust and Persistent *In Vivo* Genome Editing

Jonathan D. Finn,[1] Amy Rhoden Smith,[1] Mihir C. Patel,[1] Lucinda Shaw,[1] Madeleine R. Youniss,[1] Jane van Heteren,[1] Tanner Dirstine,[1] Corey Ciullo,[1] Reynald Lescarbeau,[1] Jessica Seitzer,[1] Ruchi R. Shah,[1] Aalok Shah,[1] Dandan Ling,[1] Jacqueline Growe,[1] Melissa Pink,[1] Ellen Rohde,[1] Kristy M. Wood,[1] William E. Salomon,[1] William F. Harrington,[1] Christian Dombrowski,[1] Walter R. Strapps,[1] Yong Chang,[1] and David V. Morrissey[1,2,*]
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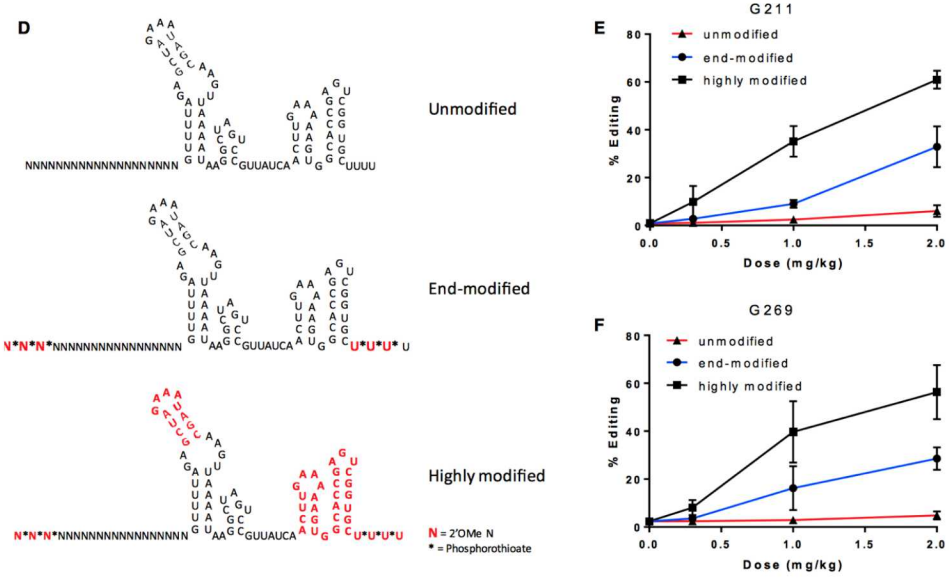
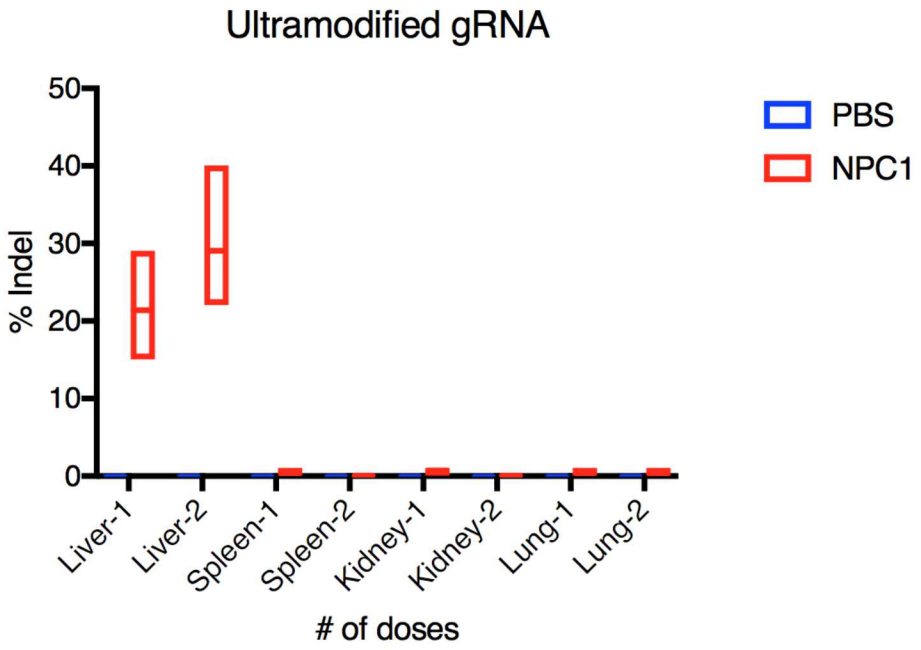
*Correspondence: davidm@intelliatx.com
https://doi.org/10.1016/j.celrep.2018.02.014

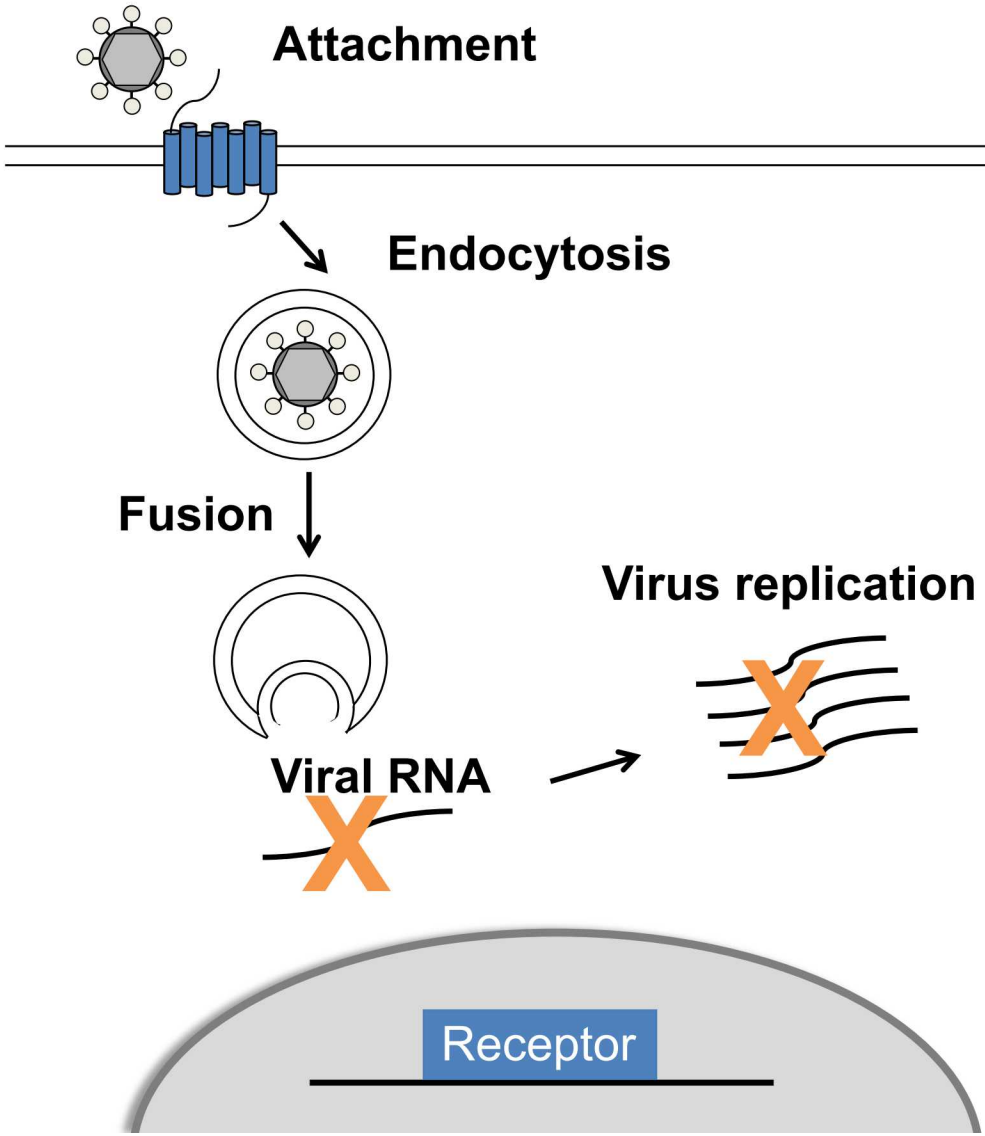


In vivo delivery of ultramodified sgRNAs result in high efficiency editing



- Avg. 20% editing with a single dose (1.5mg/kg)
- Avg. 30% editing with a two dose regimen
- One animal in the two dose treatment group had as high as 40% NPC1 gene KO in the liver

Antiviral Countermeasures: viral-directed approaches

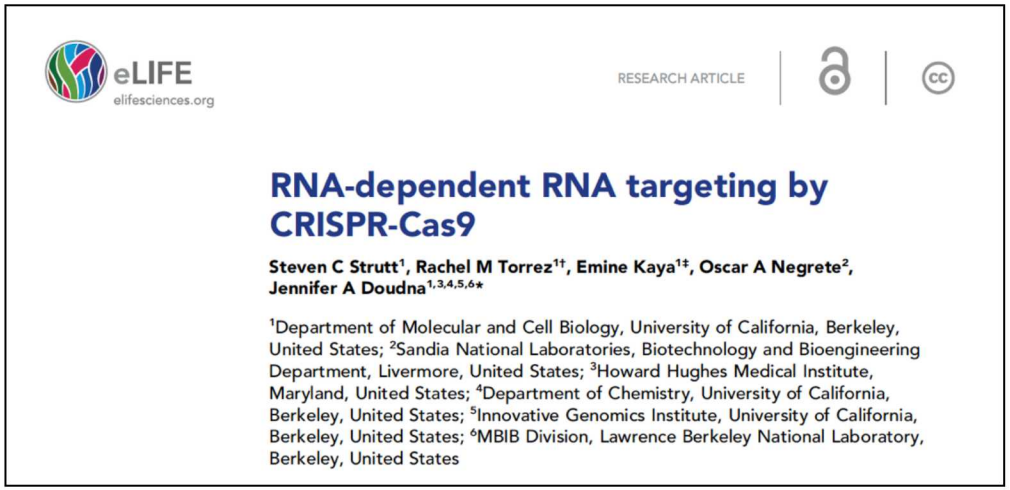


Virus targeting (VEEV):

Characterize RCas9 activity for ssRNA targeting

Identify most potent antiviral target sequences through CRISPR screening

RNA targeting by Cas9 enzymes



eLIFE elifesciences.org RESEARCH ARTICLE

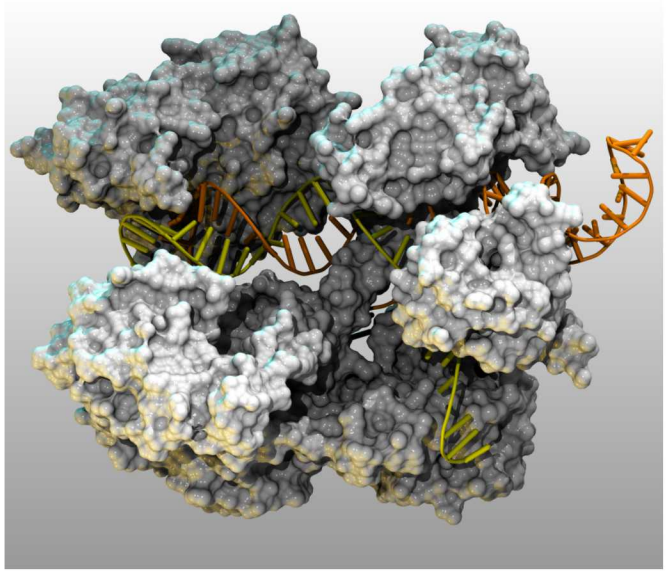
RNA-dependent RNA targeting by CRISPR-Cas9

Steven C Strutt[1], Rachel M Torrez[1†], Emine Kaya[1‡], Oscar A Negrete[2], Jennifer A Doudna[1,3,4,5,6*]

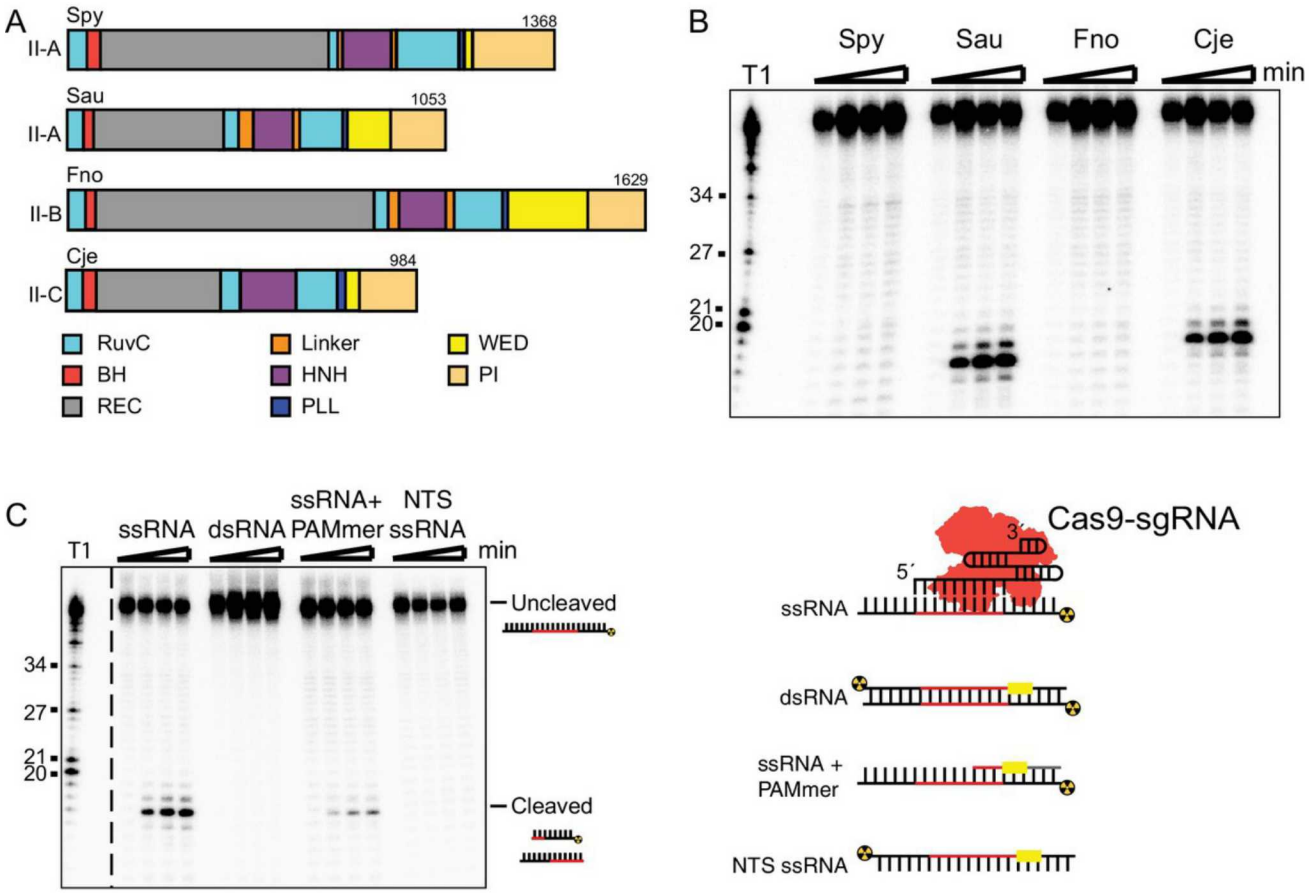
[1]Department of Molecular and Cell Biology, University of California, Berkeley, United States; [2]Sandia National Laboratories, Biotechnology and Bioengineering Department, Livermore, United States; [3]Howard Hughes Medical Institute, Maryland, United States; [4]Department of Chemistry, University of California, Berkeley, United States; [5]Innovative Genomics Institute, University of California, Berkeley, United States; [6]MBIB Division, Lawrence Berkeley National Laboratory, Berkeley, United States

Publication in *eLife* –UC Berkeley and Sandia

- Cas9 can target and cut DNA, but until recently, it was not clear whether this protein could also efficiently target RNA
- Here, we show that Cas9 enzymes from *Staphylococcus aureus* and *Campylobacter jejuni* recognize and cleave single-stranded RNA (ssRNA) in a programmable and site-specific manner
- RNA targeting activity can be exploited to reduce infection by RNA phage and reduce gene expression in bacteria cells
- This discovery provides new and exciting tools for modulating RNA that can be potentially used to combat RNA viruses of biodefense concern.



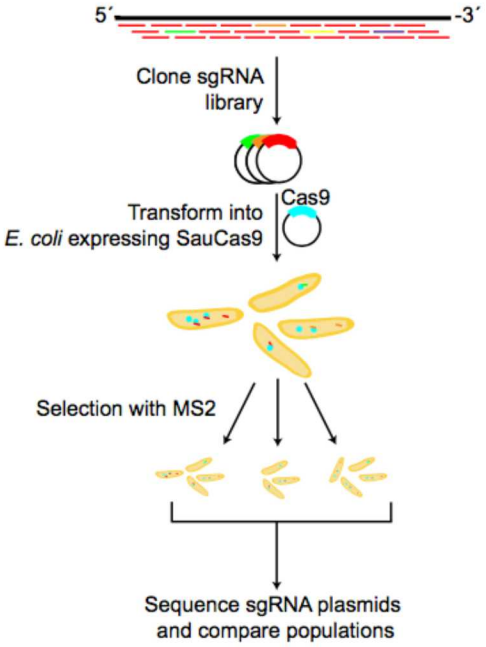
Cas9 enzymes with ssRNA targeting activity

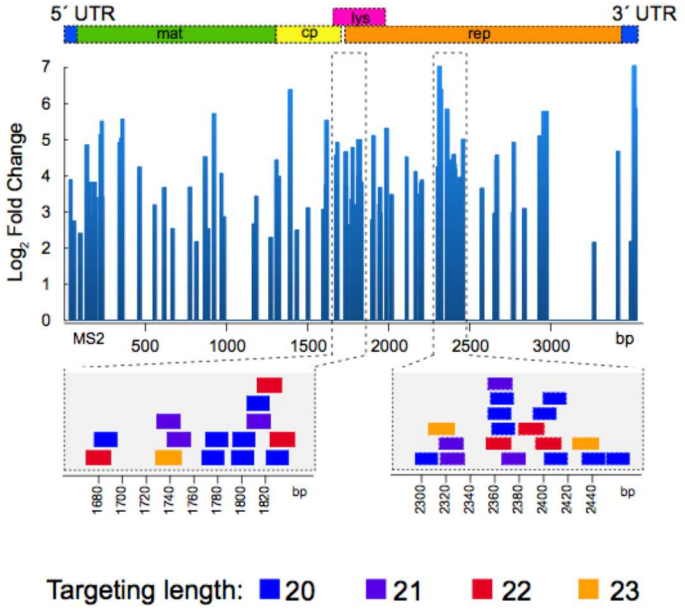


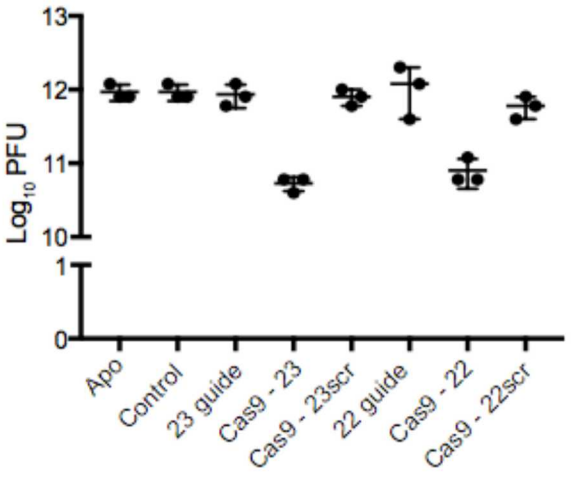
- *Staphylococcus aureus* Cas9 (SaCas9) can recognize and cleave single-stranded RNA by an RNA-guided mechanism that is independent of a protospacer-adjacent motif sequence in the target RNA.

Strutt S. et al ***eLife*** (2018)7:e32724***e***

RNA targeted library screening against MS2 phage



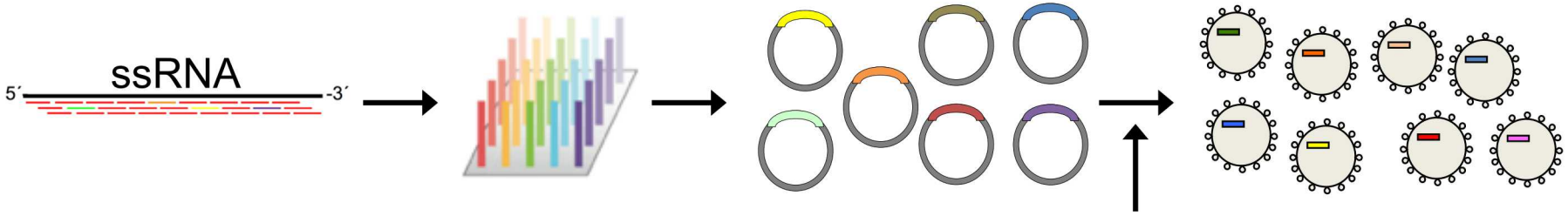




- SaCas9 RNA cleavage is programmable and site-specific, and can be exploited to reduce infection by single-stranded RNA phage.

Strutt S. et al ***eLife*** (2018)7:e32724***e***

gRNA selection for antiviral RNA targeting CRISPR systems requires library screening for enhanced survival of infected cells



1. Tiling sgRNA library design

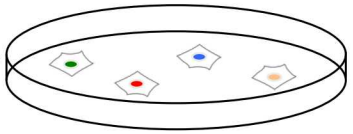
2. Oligo pool synthesis

3. Library PCR and cloning

4. Lentivirus packaging

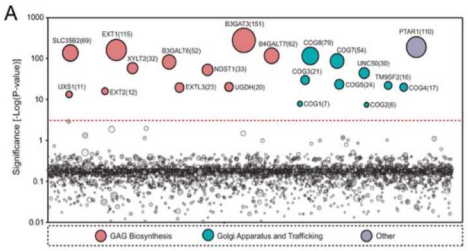
Coverage analysis via NGS in next slides

5. Lentivirus transduction into **SaCas9/CjCas9** expressing cells



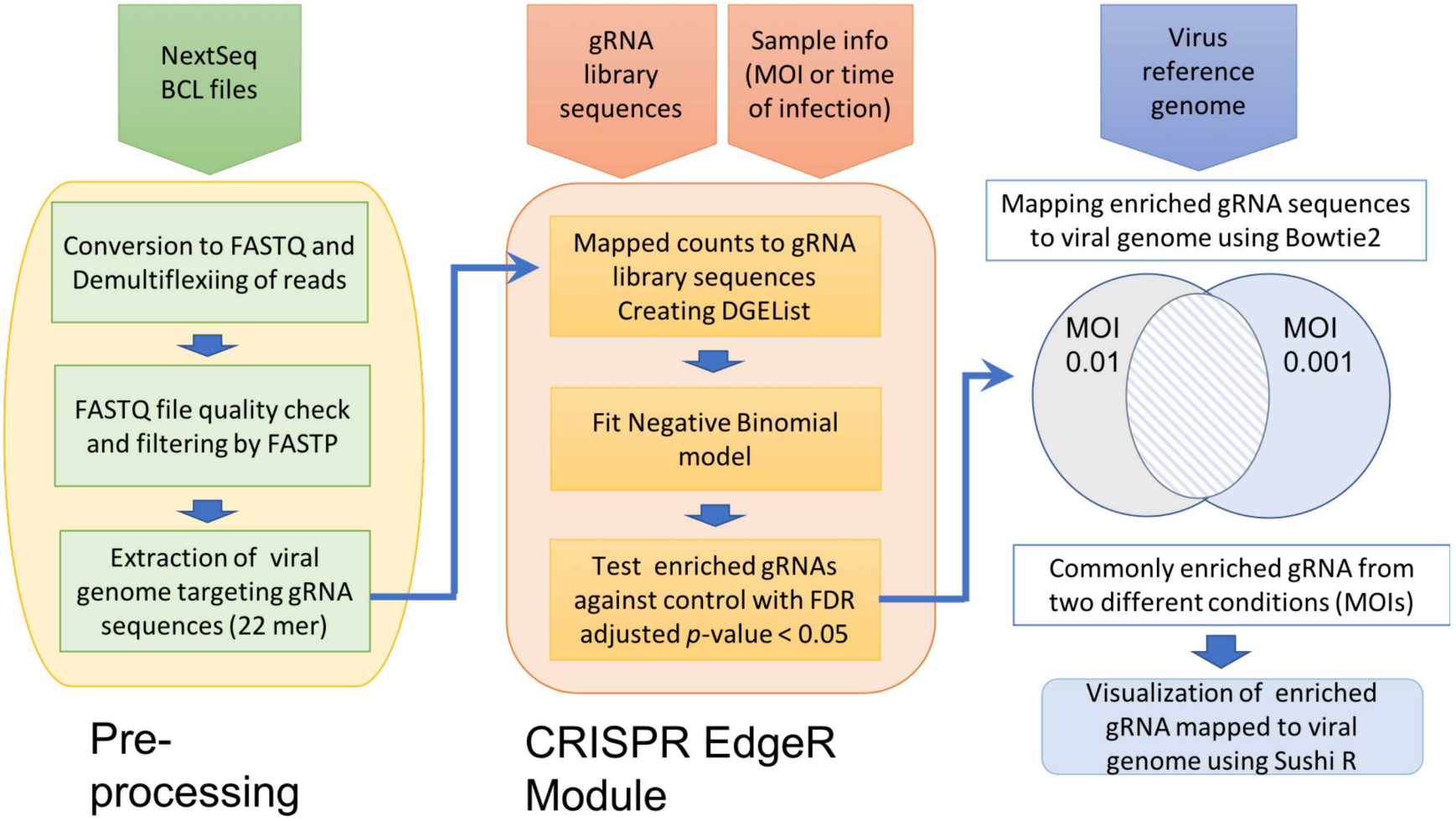
6. Survival screen

7. gRNA cassette amplification & NGS

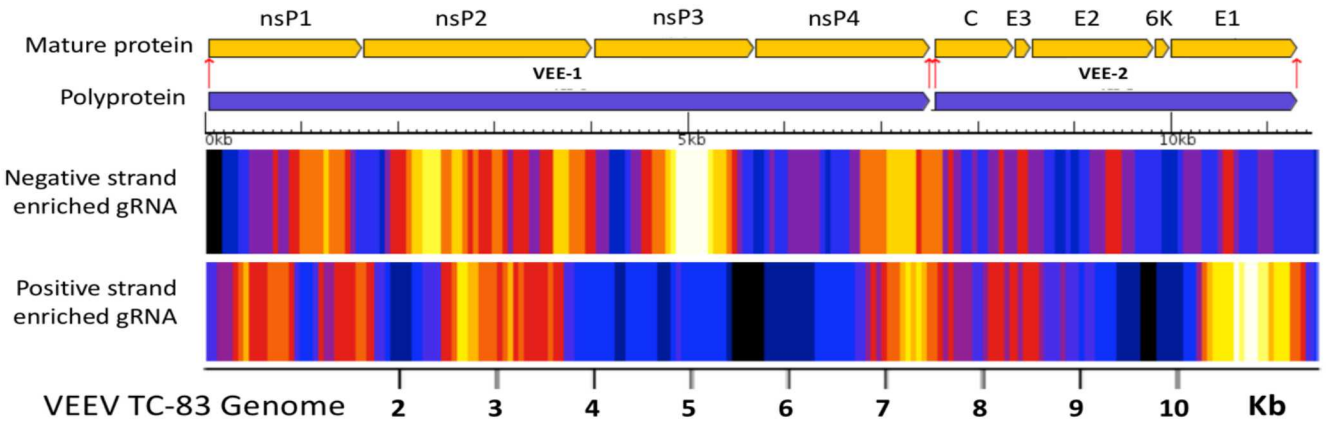


8. Statistical enrichment of hits

Bioinformatics screening data processing



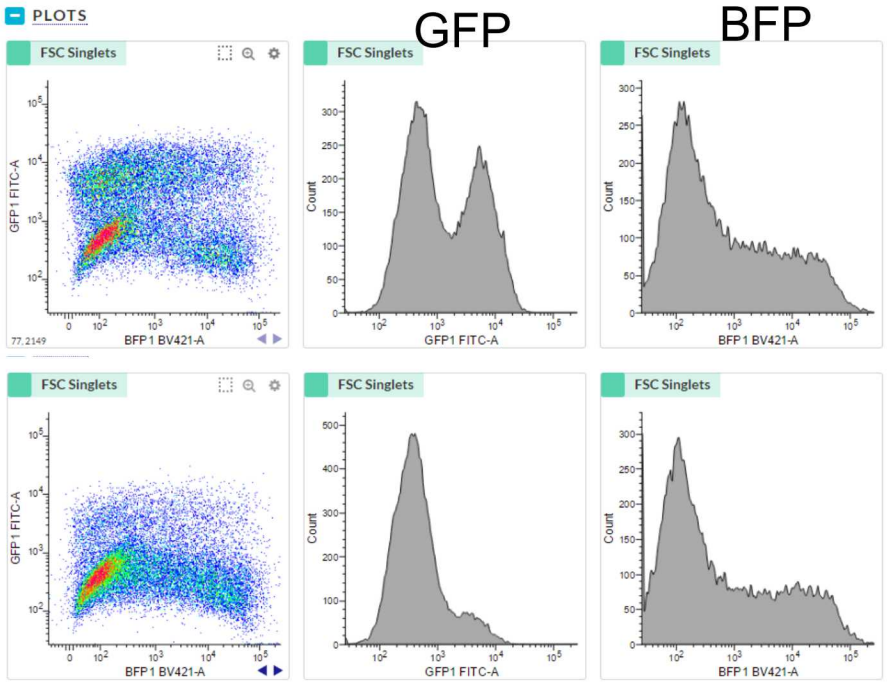
R-Cas9 CRISPR screen against VEEV



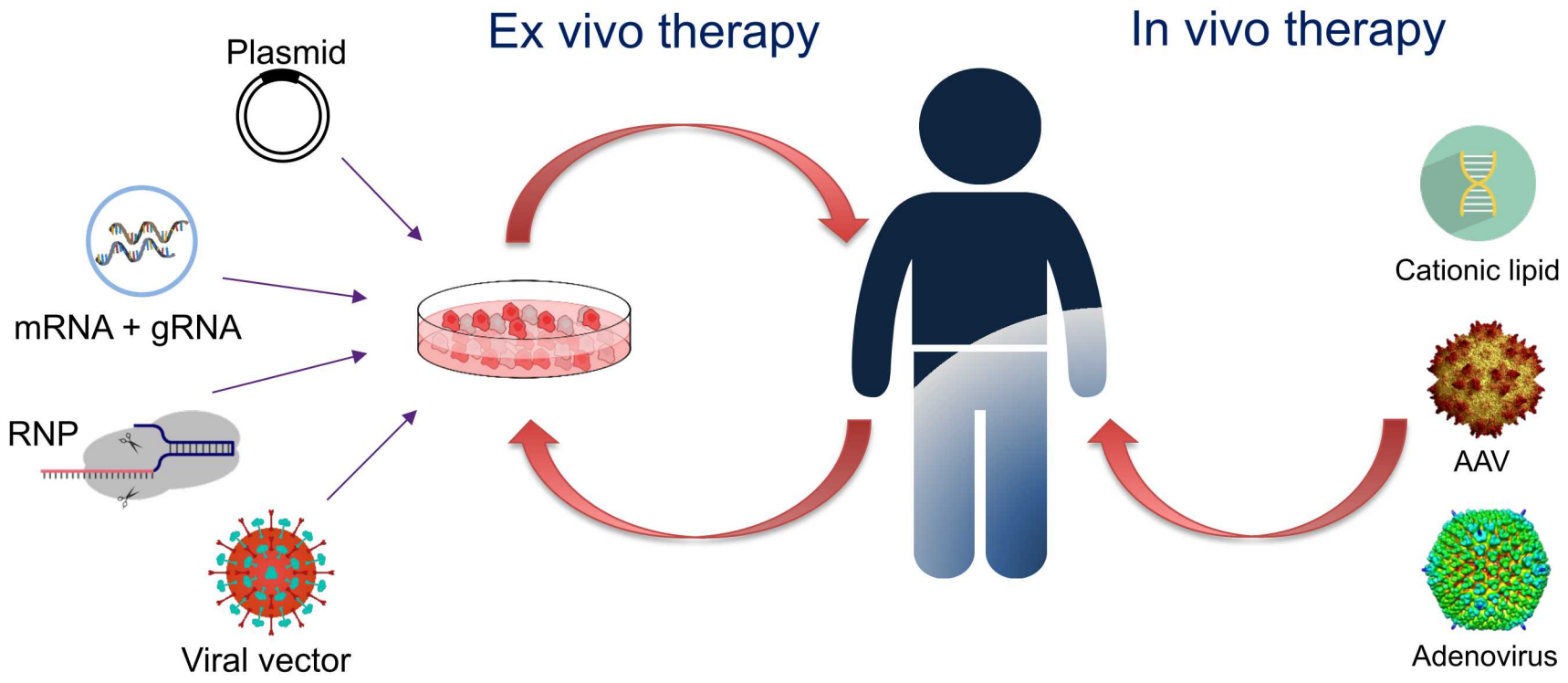
- Identified over 100 hits from the primary screen and approximately half were divided into genome and anti-genome targeting sequences
- Cloned top 40 hits from the anti-VEEV RNA targeting screen into pSaguide vector and performed validation studies

SaCas9-BFP with anti-NPC1 sgRNA (neg control)

SaCas9-BFP with anti-VEEV sgRNA



Current CRISPR delivery methods

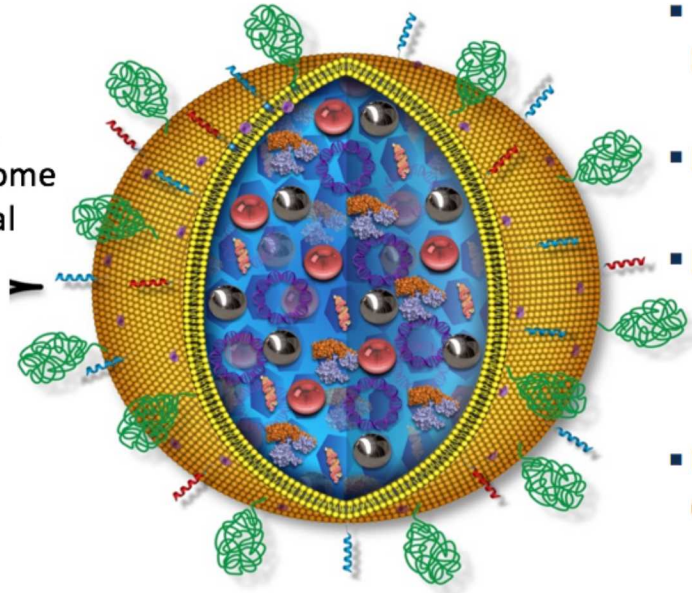


Lipid-coated mesoporous silica nanoparticle (LC-MSN) therapeutic cargo delivery platform

Sandia National Laboratories

Therapeutic Cargos

- Small molecule drugs
- Plasmids for gene therapy or vaccine applications
- CRISPR Cas9/gRNA complexes for genome editing and antiviral countermeasures



Properties of the Mesoporous Silica Core

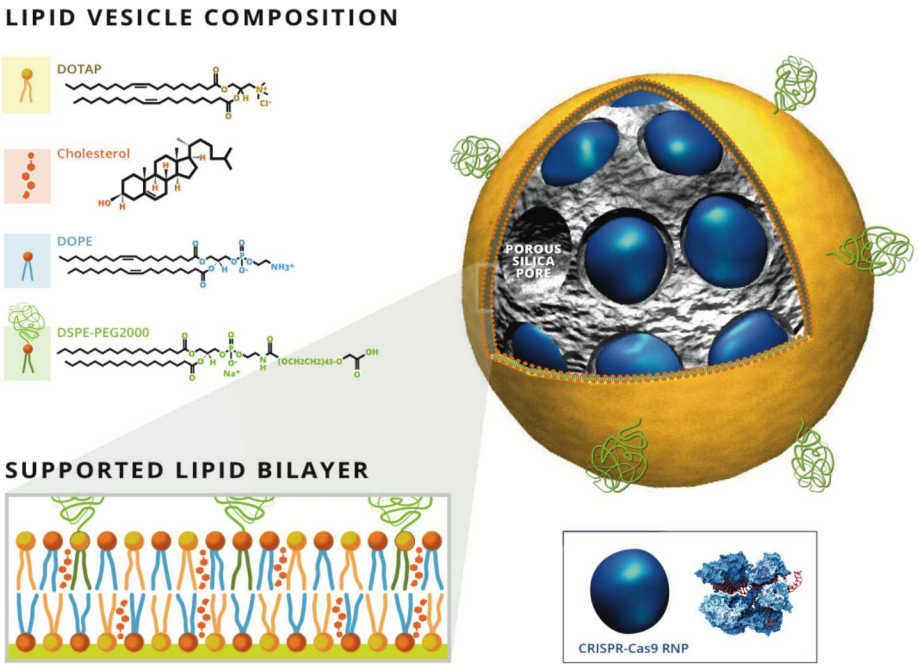
- **Particle Size & Distribution**
  - Biodistribution
- **Particle Charge**
  - Bilayer Stability
- **Pore Size/Chemistry**
  - Loading Capacity
  - Type of Cargo(s)
  - Release Rates
- **Degree of Silica Condensation**
  - Release Rates
  - Biodegradability

Properties of the Supported Lipid Bilayer

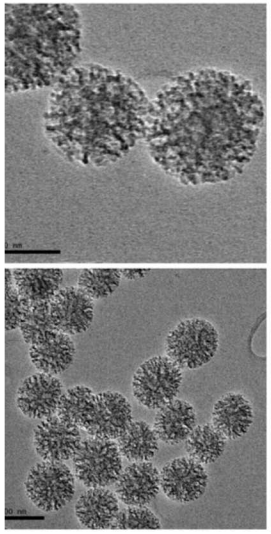
- **Type of Lipid**
  - Bilayer Charge → Unwanted Uptake
  - Bilayer Fluidity → Bilayer Stability
- **Degree of PEGylation**
  - Bilayer Stability → Cargo Retention
  - Colloidal Stability→ Biocompatibility

- Lipid-coated mesoporous silica nanoparticle (LC-MSN) technology is a flexible, modular platform for delivery of small molecule and other therapeutic cargos
- LC-MSNs possess advantages of both MSNs and liposomes, including high loading capacity, controlled release, targeting specificity, colloidal stability, and biocompatibility

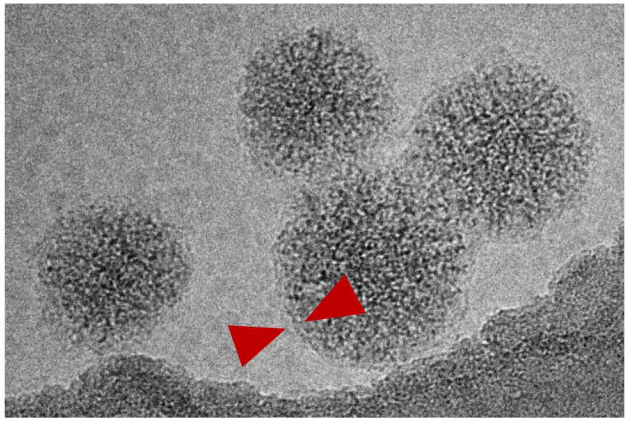
Engineering lipid-coated mesoporous silica nanoparticles for delivery of CRISPR ribonucleoprotein (RNP) complexes



TEM:MSN only

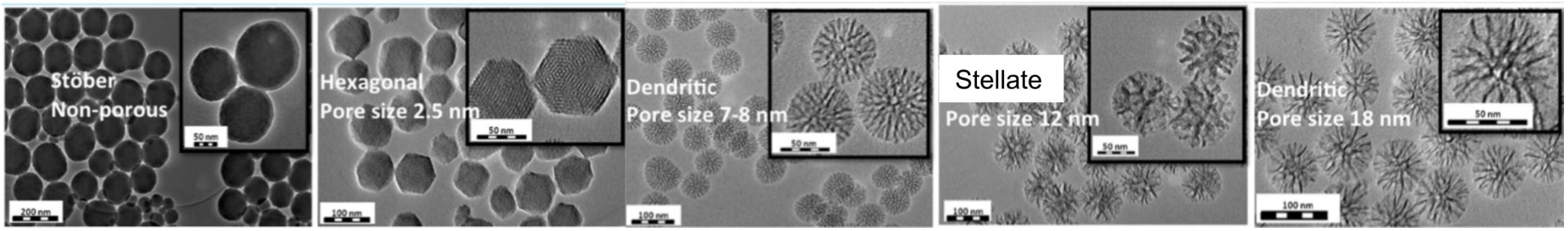


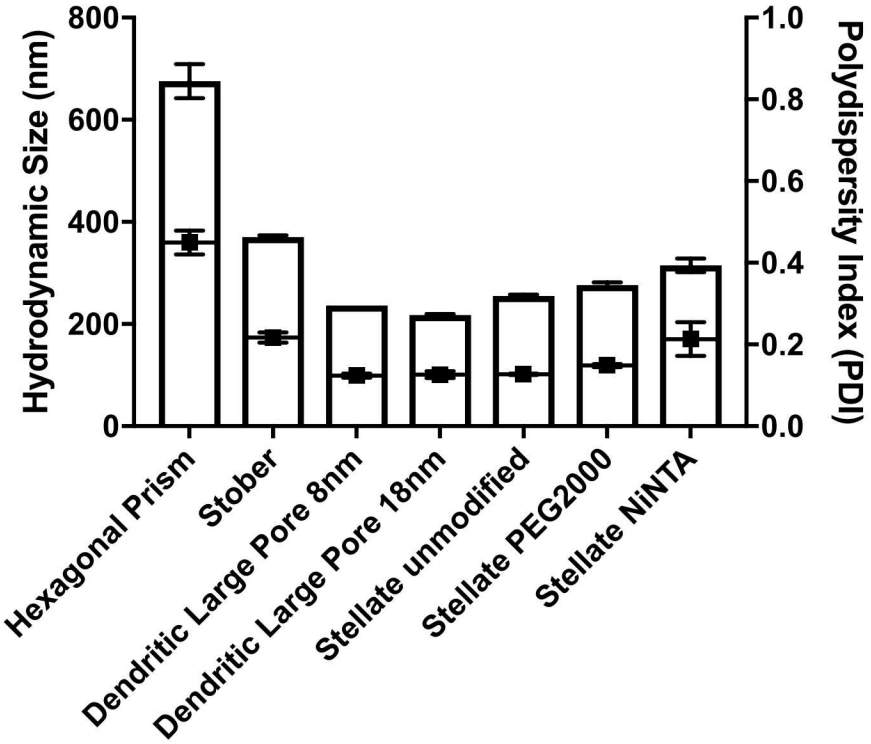
Cryo-EM of RNP loaded LC-MSN

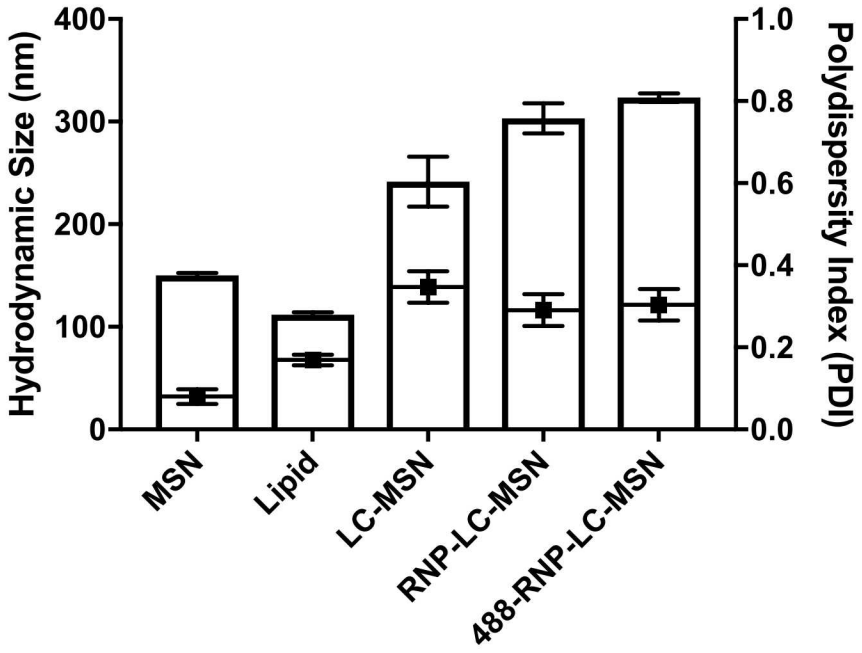


Bilayer coating is present on RNP loaded nanoparticles

LCMSN-RNP particle size and polydispersity



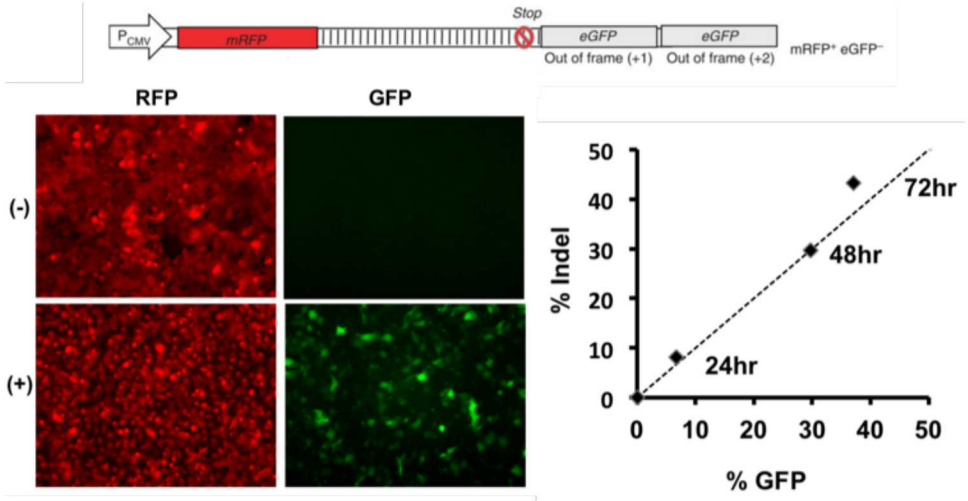




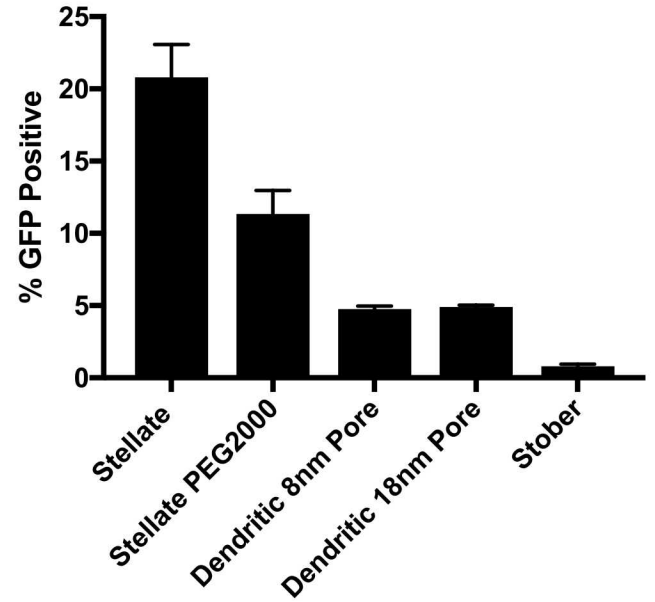
In vitro editing of a reporter cell line using LCMSN-RNP delivery technology

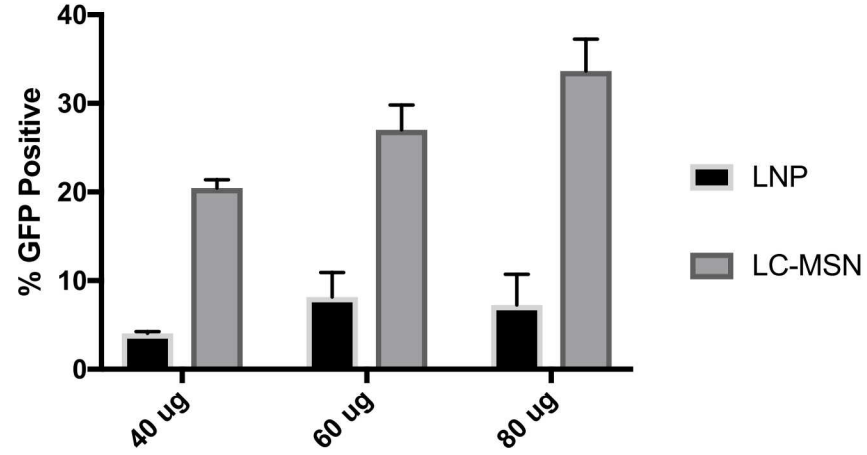
Sandia National Laboratories

CRISPR reporter – A549 cell line



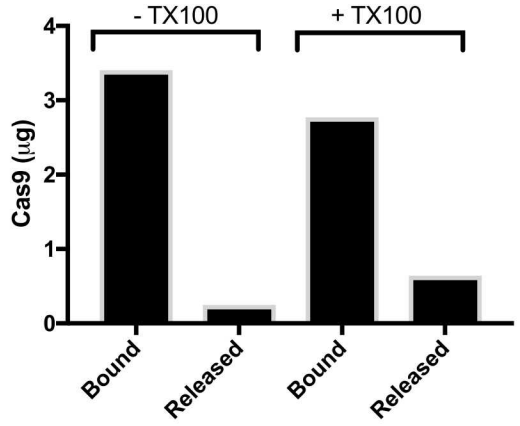
- This cell reporter expresses GFP upon successful indel formation and allows for rapid screening of LC-MSN formulations

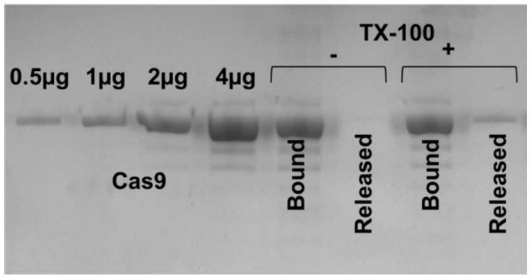




LCMSN-RNP loading, release, activity and protection

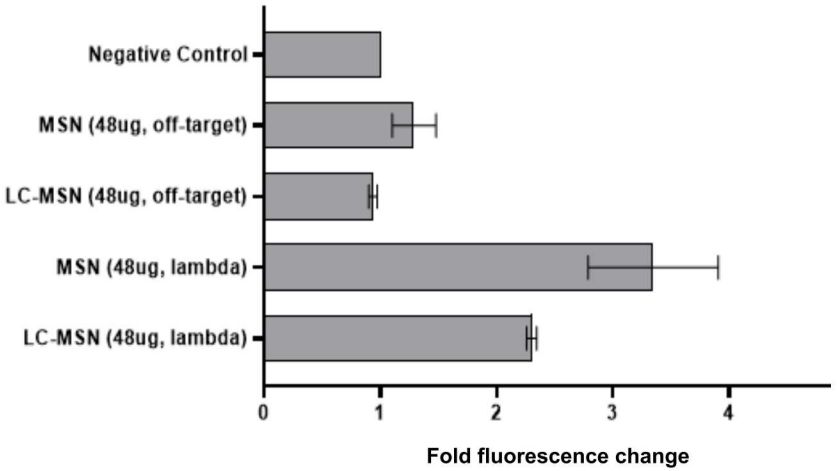
A





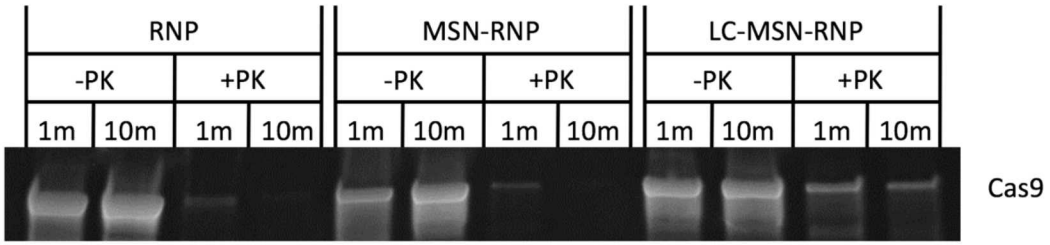
On average LC-MSNs bound 3ug of Cas9-RNP per 25ug of MSNs (or 120ug Cas9/1mg MSN) and released a fraction of RNP (~16%) under these simulated conditions

B



A FRET-based CRISPR activity assay demonstrated active RNP complexes on particles with and without lipid coatings

C

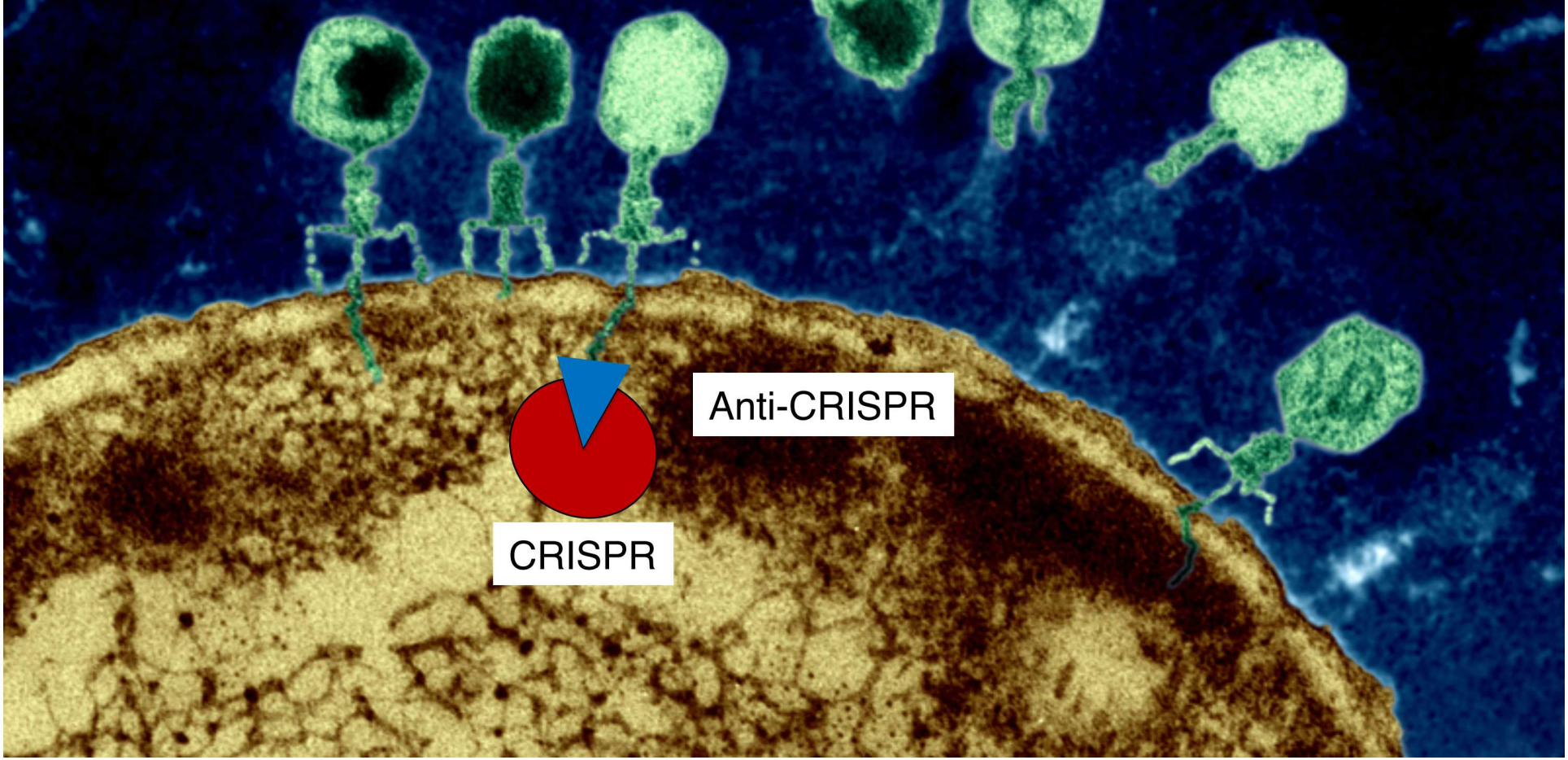


A protease degradation assay was used to measure how well RNPs were protected when loaded on to LC-MSNs.

- HOLD FOR **In Vitro and In Vivo** delivery of NPC1 guides using LC-MSN-RNP platform (we will have data this week for incorporation into the presentation)

Safe Genes TA2: Anti-CRISPR (Acr) proteins

- Small proteins in bacteriophage genomes
- CRISPR/Cas inhibitors that teach us about mechanism
- Provide approaches for regulating CRISPR-based tools

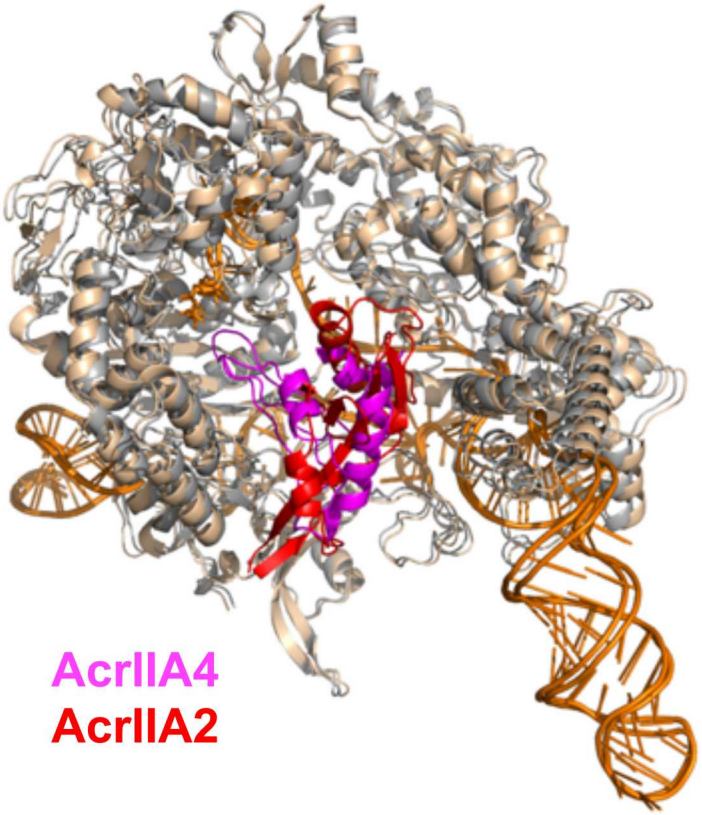


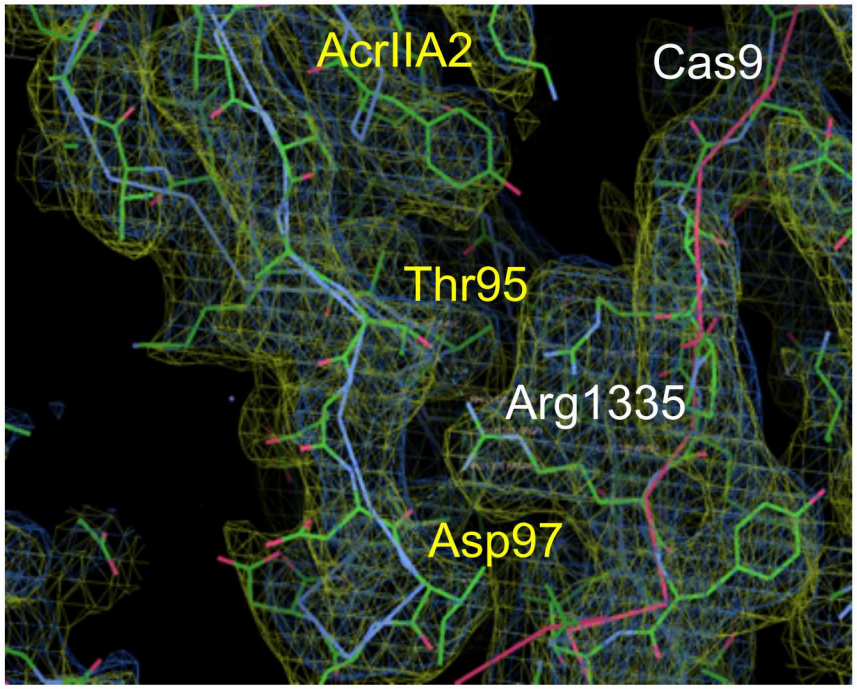
Acr Discovery and Utilization

- Acrs have now been discovered by the Bondy-Denomy and Doudna labs that inhibit several of the most important Cas tools
  - AcrIIA4: SpyCas9
  - AcrIIC1: CjeCas9, GeoCas9, NmeCas9
  - AcrIIA7: SauCas9
- Typically smalll proteins, 100-200 amino acids
  - We have overexpressed and purified 10-100mg of the above
- If Acrs can be delivered *in vivo,* they can be used to block effects of Cas9 gene editing or sequence targeting
- If they can be delivered to cells *in vitro,* they can be used in experiments to control the kinetics of Cas9-derived tools, **including those based on dSpyCas9**

3.4-Å Cryo-EM reconstruction of the AcrIIA2-bound SpyCas9

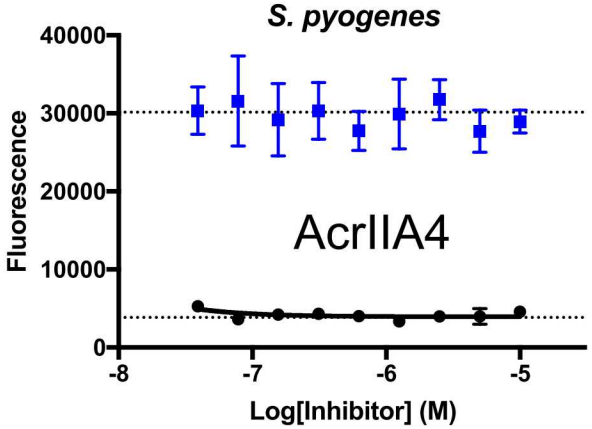
AcrIIA2 is larger than AcrIIA4 (123 aa vs 87aa) and mainly beta-sheet
Biochemistry reveals Cas9 binding prevents DNA binding/cleavage

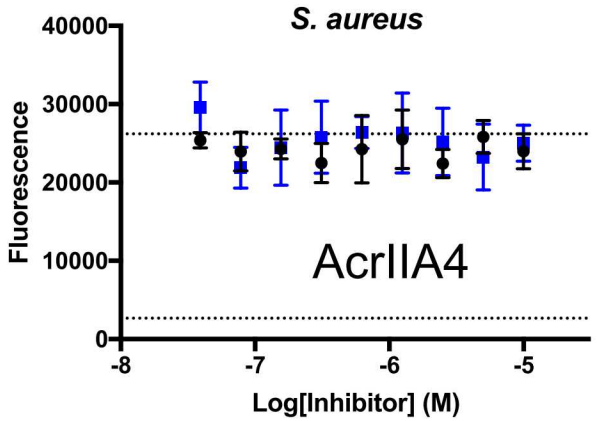




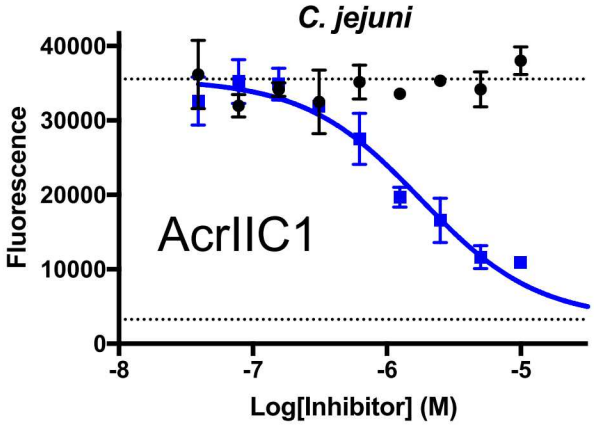
Fuguo Jiang
(Doudna Lab)

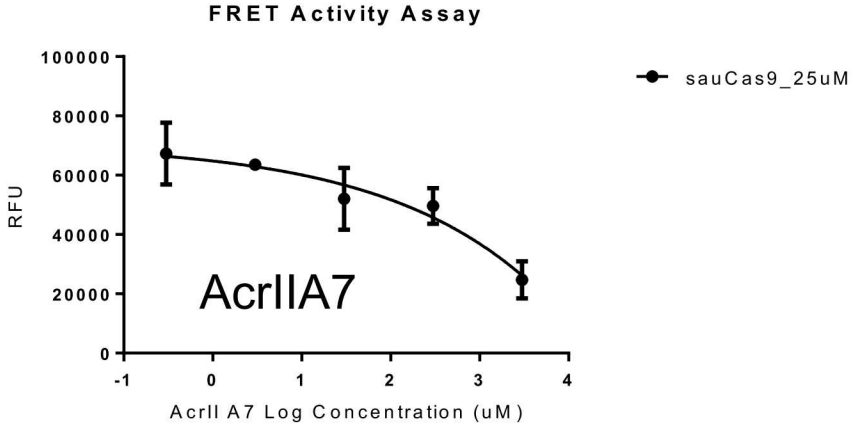
Flexible High-Throughput Cas9 Biochemical Cleavage Assays Allow Quantitative Profiling of Acr Specificity and Screening of Acr Variants





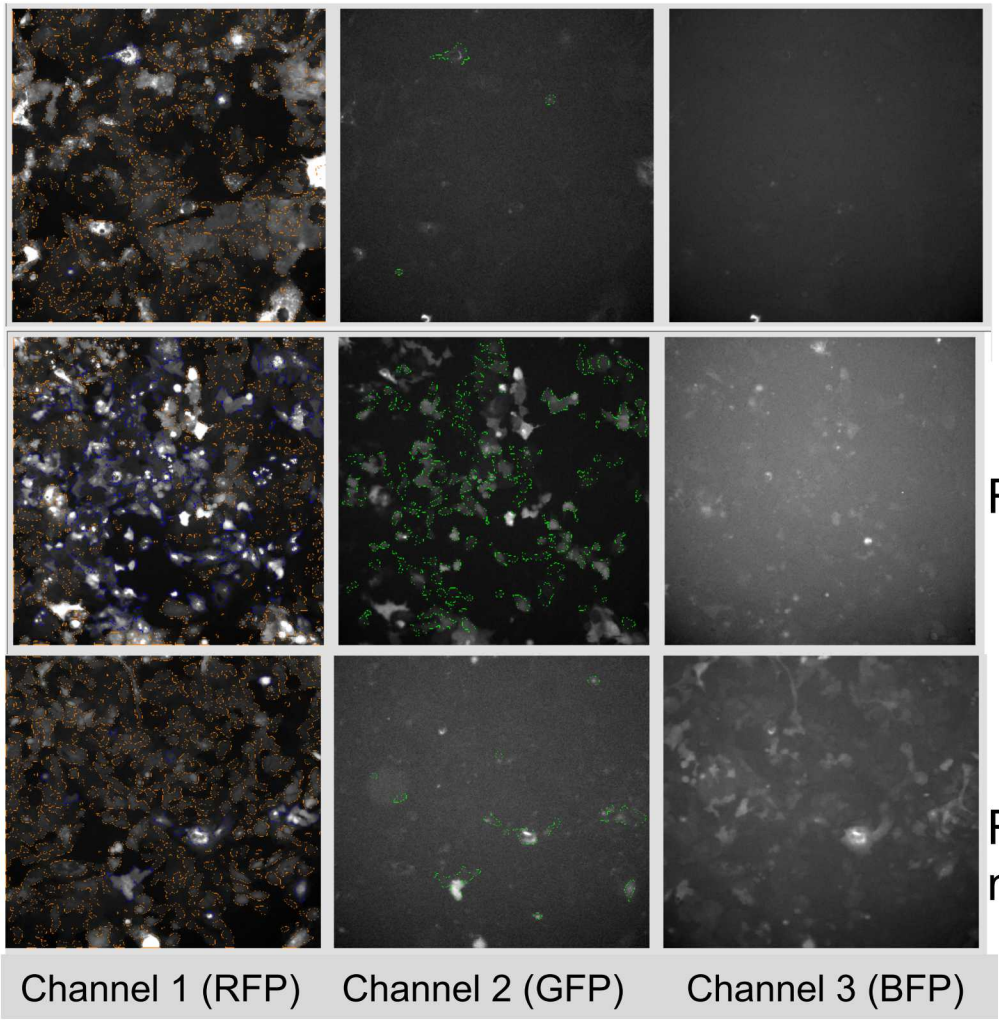
- AcrIIA4 inhibits SpyCas9 with nM IC_{50} (<30 nM)
- AcrIIC1 inhibits CjeCas9 but is weaker (µM) IC_{50}
- AcrIIA7 inhibits SauCas9 but is even weaker (mM) IC_{50}





Anal.Chem., **2018**, *90* (11), pp 6913–6921 **DOI:** 10.1021/acs.analchem.8b01155

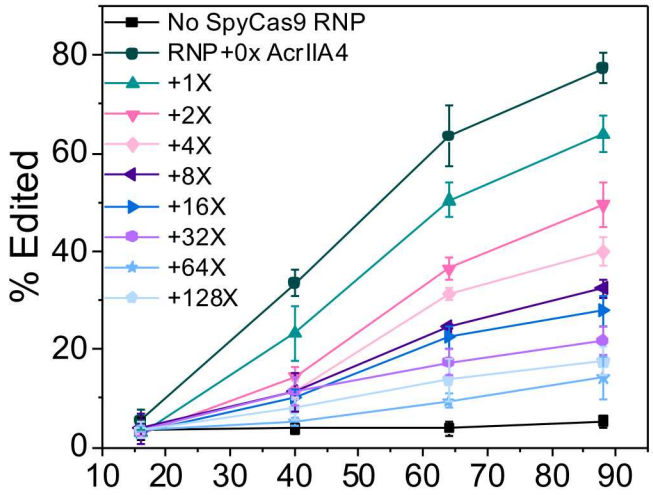
Cell Assays for Gene Editing Inhibition With High Content Imager: Representative CX7 images at 136 h



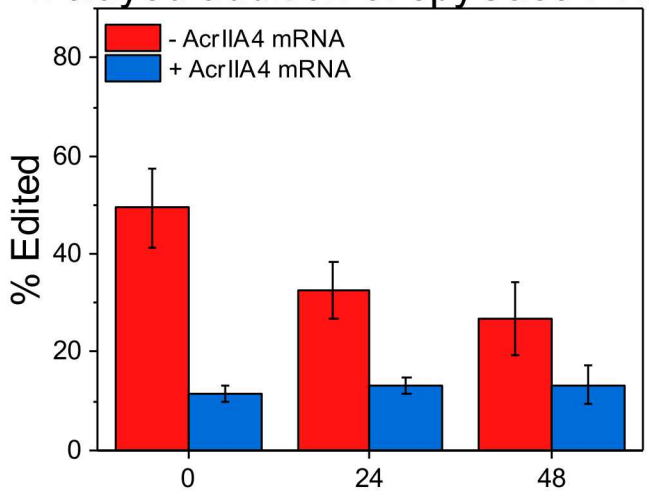
AcrIIA4 mRNA blocks editing by SauCas9 specifically in A549 Cells

- AcrIIA4-BFP mRNA shows strong, dose dependent inhibition of SpyCas9 RNP
- Crossover control shows no inhibition of SaCas9 RNP editing confirming expected AcrIIA4 mRNA function
- There is persistent, functional AcrIIA4 to at least 48 h after mRNA addition
- Next steps are kinetic studies of AcrIIA4 delivery after RNP and mRNA delivery with LC-MSN

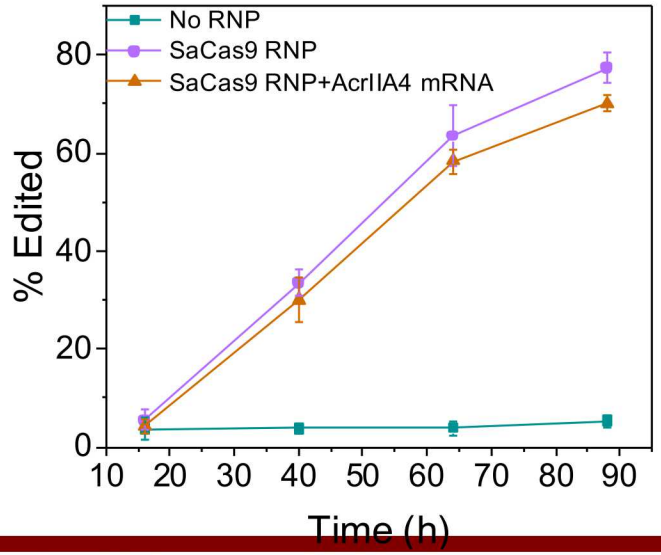
spyCas9 RNP AcrIIA4 mRNA editing inhibition



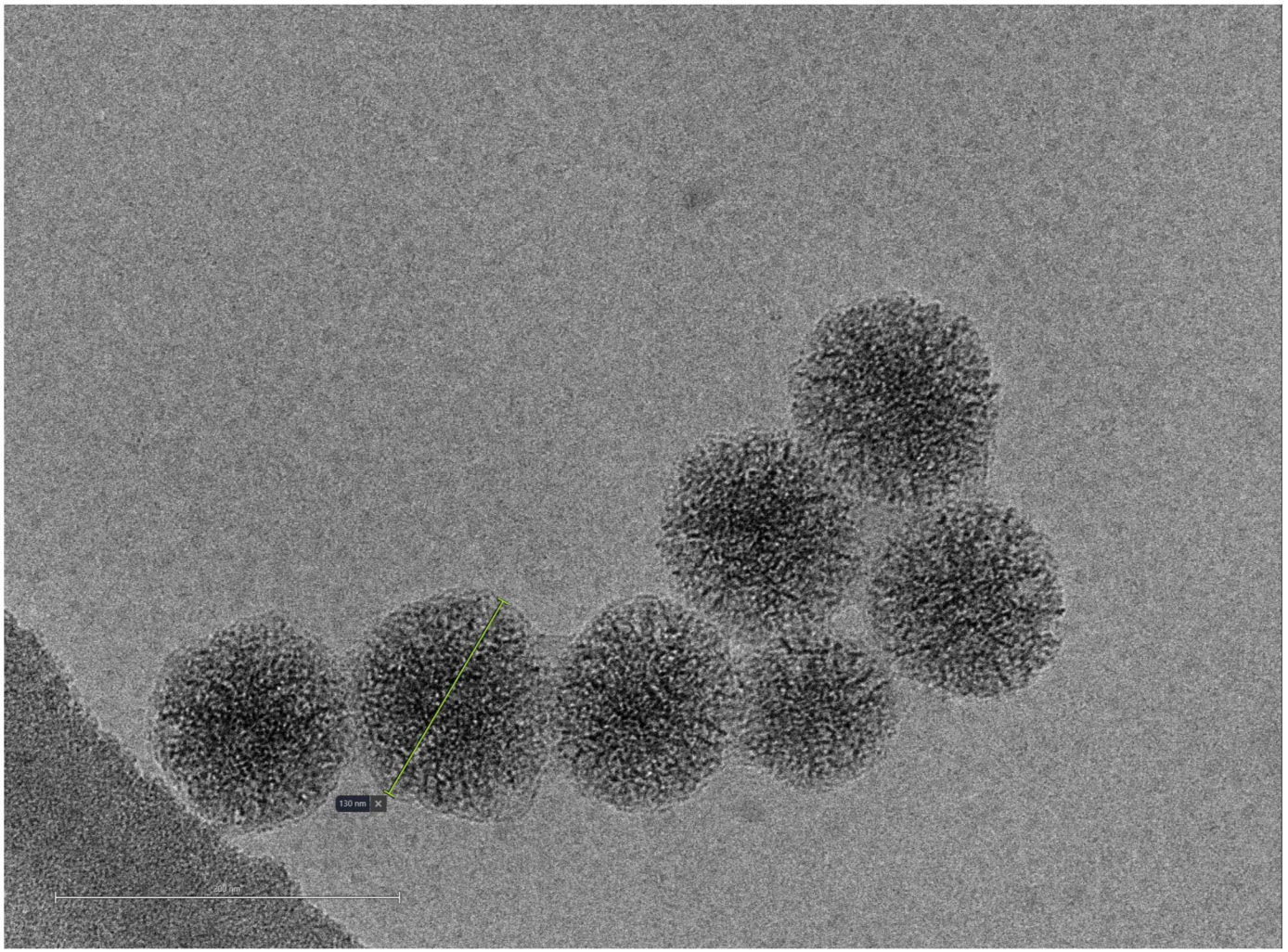
Delayed addition of spyCas9 RNP



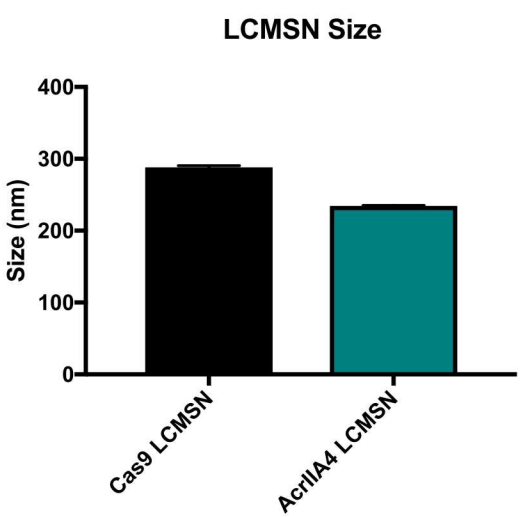
Crossover control confirmation

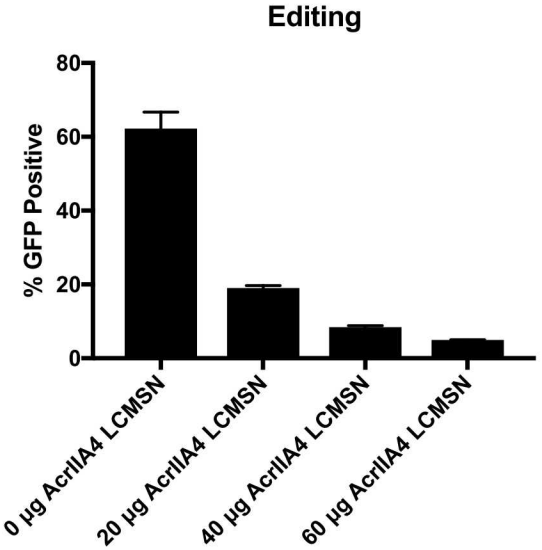


AcrIIA4 Incorporated into LCMSNs: CryoEM shows particles with lipid layer

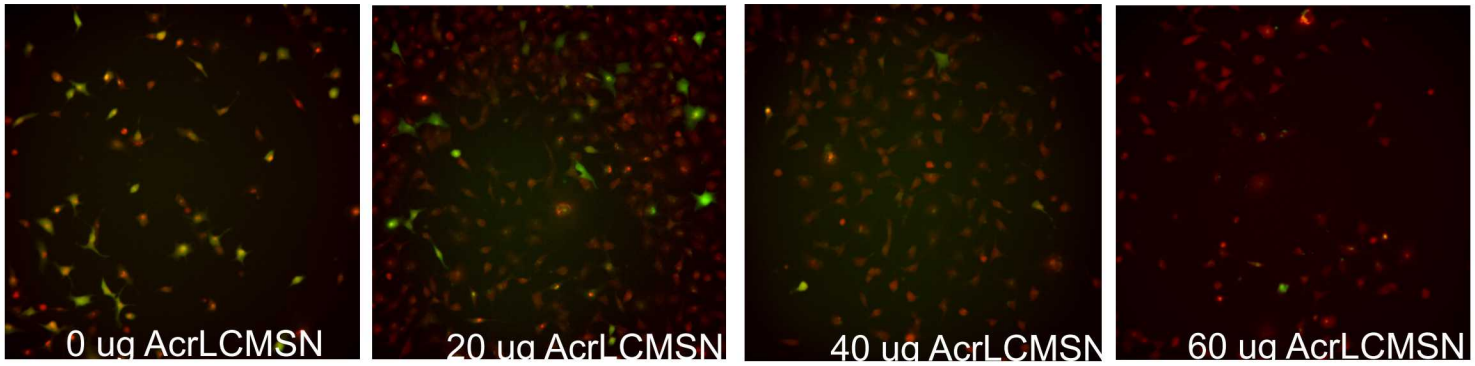


AcrIIA4 was Incorporated into LCMSNs: Size, and Inhibition of *Spy*Cas9



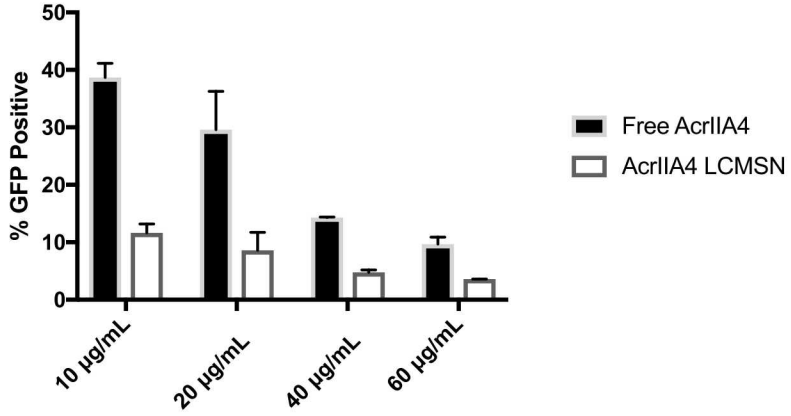


- DLS was measured of resulting LCMSN (compared to typical Cas9 RNP LCMSN)
- Percent GFP positive cells assayed with Accuri flow cytometer (graph) and CX7 (images)

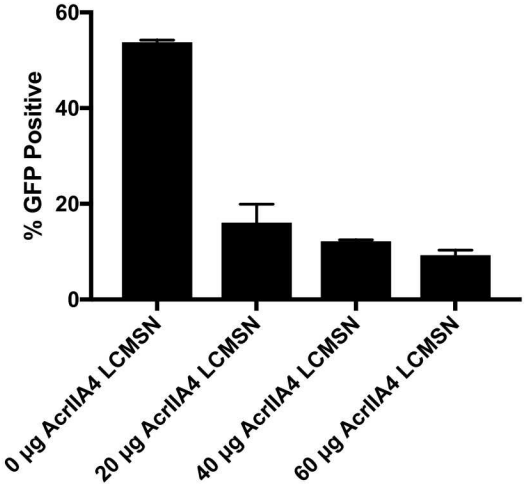


AcrIIA4 is much more potent formulated in nanoparticles than if applied directly in solution

Free AcrIIA4 vs LCMSN Delivery



AcrIIA4 LCMSN Pretreatment (24hrs)



- Free AcrIIA4 equivalent to 10 µg/µL LCMSN = 2 µg/µL etc. following ratio of protein to MSN used 1:5.

Current Status of Acr Work

- Discovery of Acrs continues against new Cas targets
- Delivery formulations of AcrIIA4 protein and mRNA are being developed
  - Measure dose-response and kinetics (pre-exposure versus post-exposure to Cas9) tested on cell lines
- AcrIIA7 protein has been expressed and tests are beginning with cross-over AcrIIA4/7 & Spy/SauCas9 experiments
- mRNA production of AcrIIA4 and AcrIIA7 constructs is underway
- Experiments for measuring AcrIIA4 activity on Cas9 mice are being planned

Acknowledgements

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Luke Gilbert

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Georgia Tech

Phil Santangelo

SNL

Joe Schoeniger

Oscar Negrete

Kim Butler

Kyle Seamon

Edwin Saada

Robert Johnston

Andrew Gomez

Annette LaBauve

Colleen Courtney

DARPA

Safe Genes Award HR0011-17-2-0043

Sandia National Laboratories

GC LDRD Award (190245- NanoCRISPR)