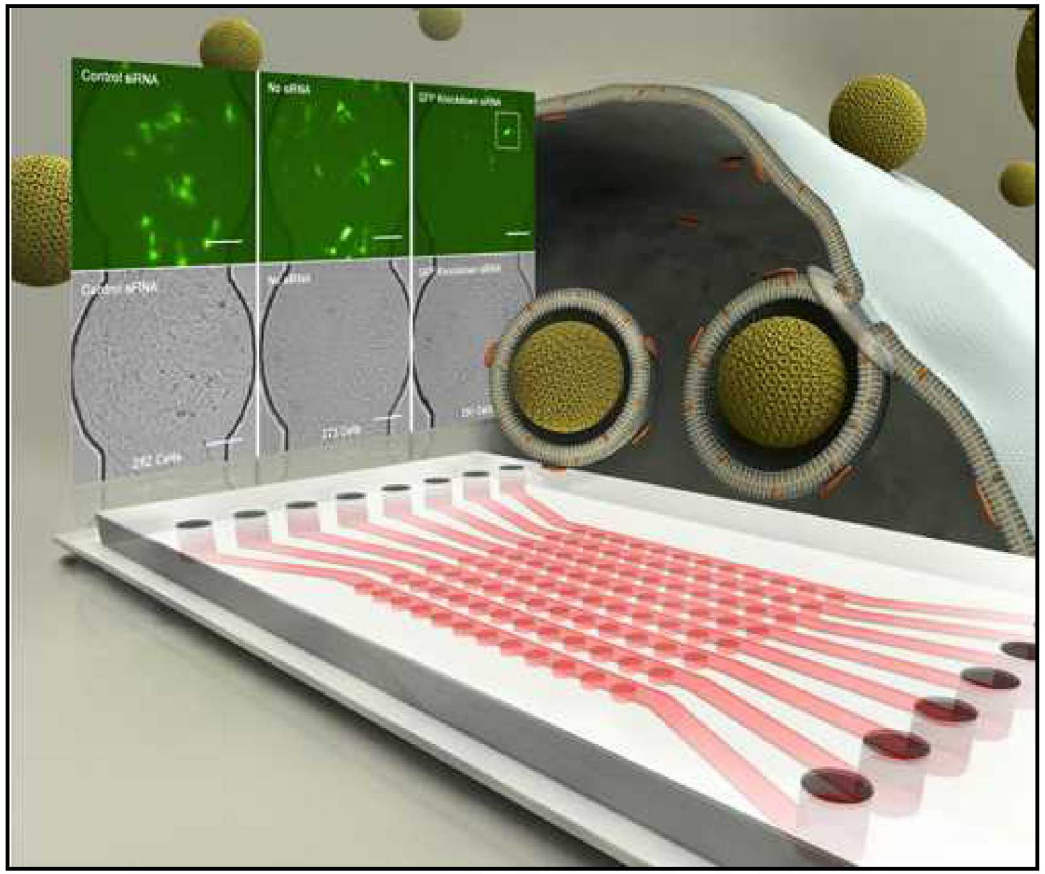
SANDIA BIOSCIENCE

SAND2016-4870C

Functional Genomics Screening Using RNAi and CRISPR/Cas9 Technology to Identify Drug Targets and Pathways of Rift Valley Fever Virus Infection



Spring 2016 NCASM Meeting

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Sandia National Laboratories

March 4, 2016

Sandia National Laboratories

- Department of Energy (DOE) National Laboratory contracted to Sandia Corporation, a Lockheed Martin company
- Two main campus located in Albuquerque, New Mexico and Livermore, California
- National Nuclear Security Administration (NNSA)
- Bioscience Research Foundation focuses on Biofuels and Biodefense and Emerging Infectious Disease (BEID) Research

Viruses of biodefense concern

NIAID Biodefense Category A, B, C Priority Pathogens

Category A

Smallpox related-viruses

Viral Hemorrhagic fevers
- -Arenaviruses
- -Bunyaviruses
  - -Rift Valley Fever Virus
- -Filoviruses
  - -Ebola virus

Category B

Mosquito-borne encephalitis viruses
- -Venezuelan, Eastern and Western Equine Encephalitis viruses

Category C

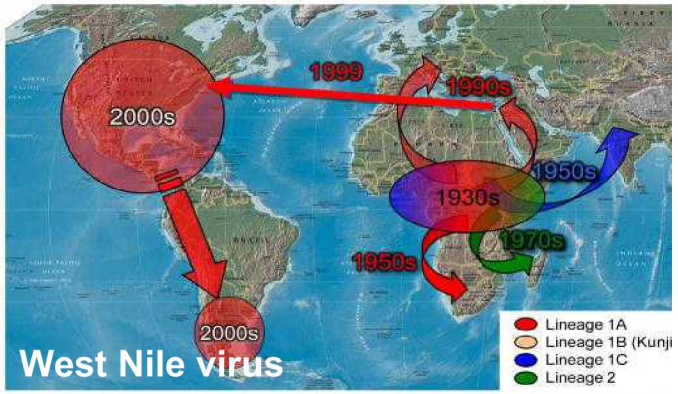
Nipah and Hendra viruses

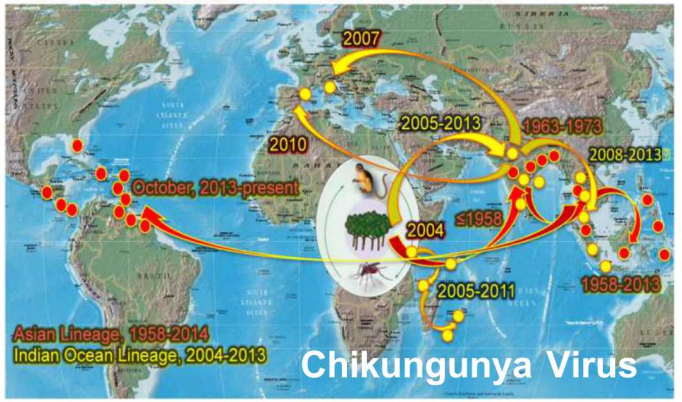
Chikungunya virus

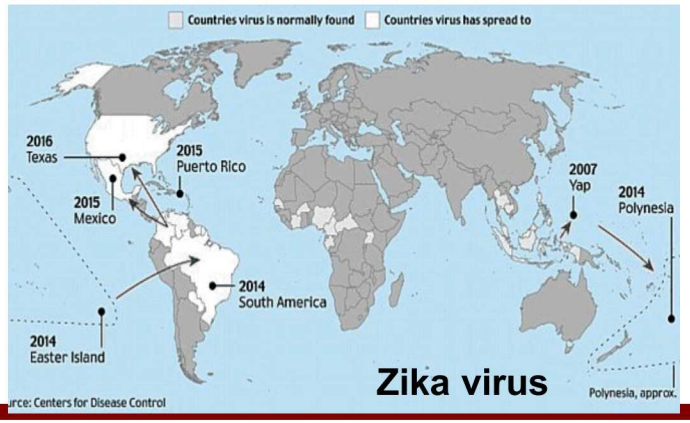
SARS, MERS-CoV

Arthropod-Borne Viruses (Arboviruses)

- **Includes flaviviruses, alphaviruses and bunyaviruses**
- Fever, headache, and malaise are most common but encephalitis and hemorrhagic fever may also occur.
- Circulate among wild animals and many cause disease in humans and domestic animals
- Some viruses including Dengue, Chikungunya (CHIKV), and Zika do not require enzootic amplification
- Dramatic Geographic Expansion: West Nile virus CHIKV, and Zika virus



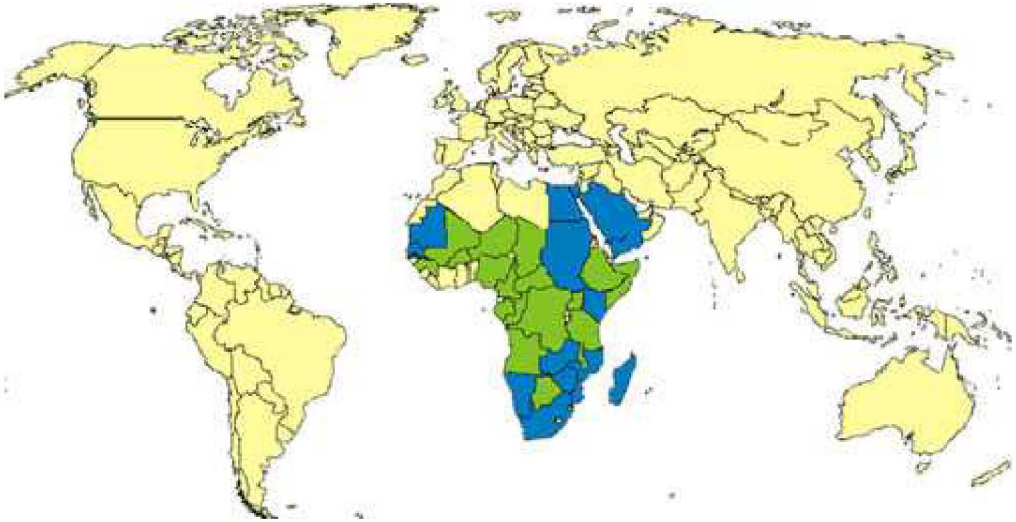


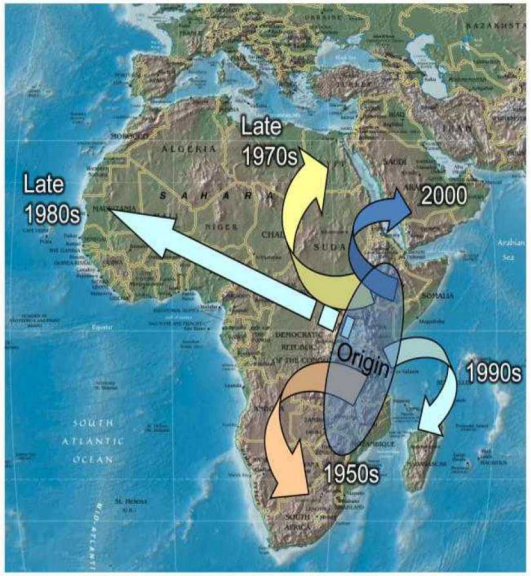


Bunyaviridae: > 350 members; 5 genera

	Genus	Vector	
Plant-infecting viruses	Tospovirus	→ **Thrips**	
Animal-infecting viruses	Orthobunyavirus	Arthropods → **Mosquitoes** Members of Arboviruses	
	Phlebovirus RVFV	→ **Phlebotomine flies** Mosquitoes Ticks	
	Nairovirus	→ **Ticks** Mosquitoes Culicoid flies	
	Hantavirus	Rodents → **Mouse** **Rats**	

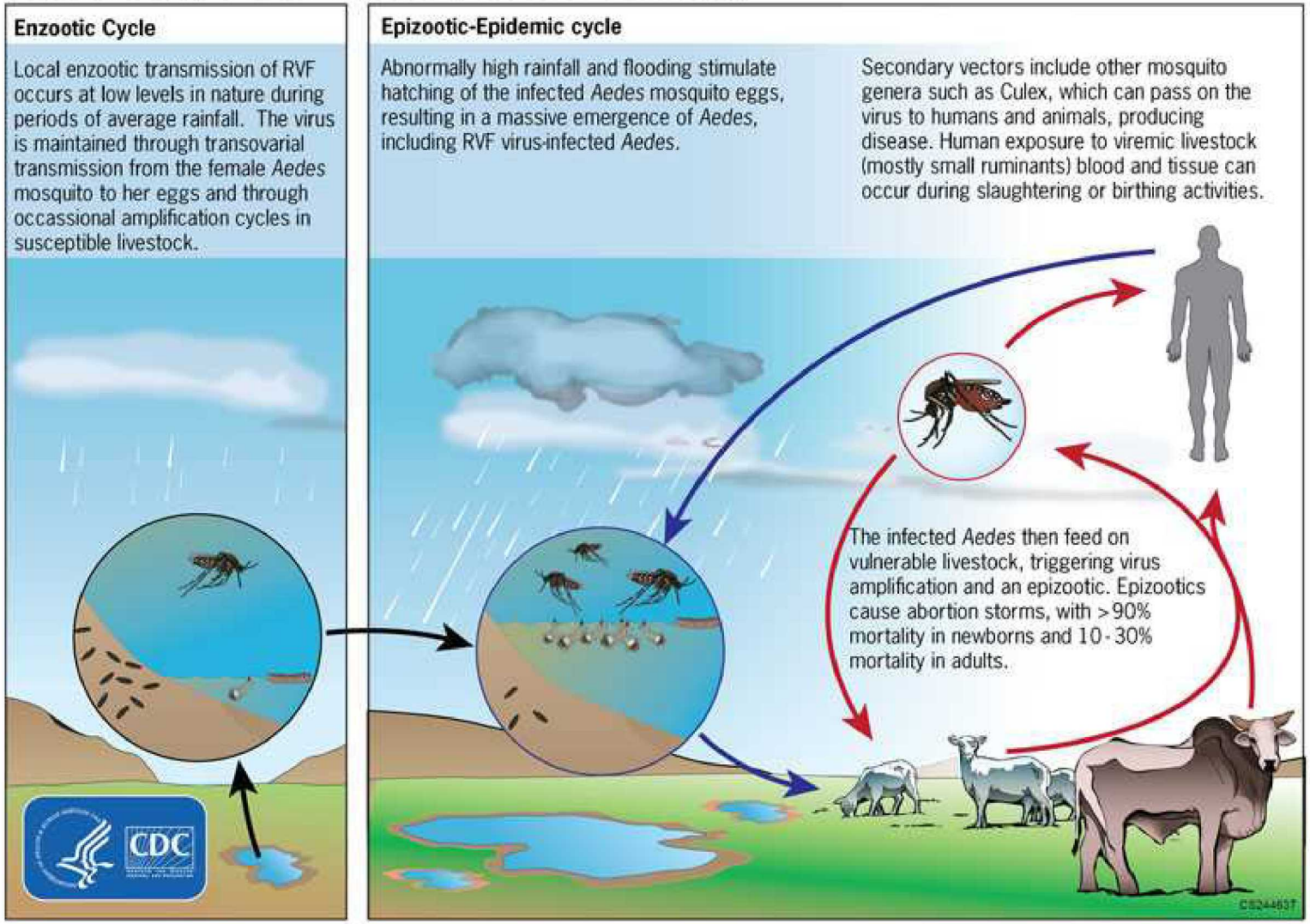
Geographical Distribution of RVFV





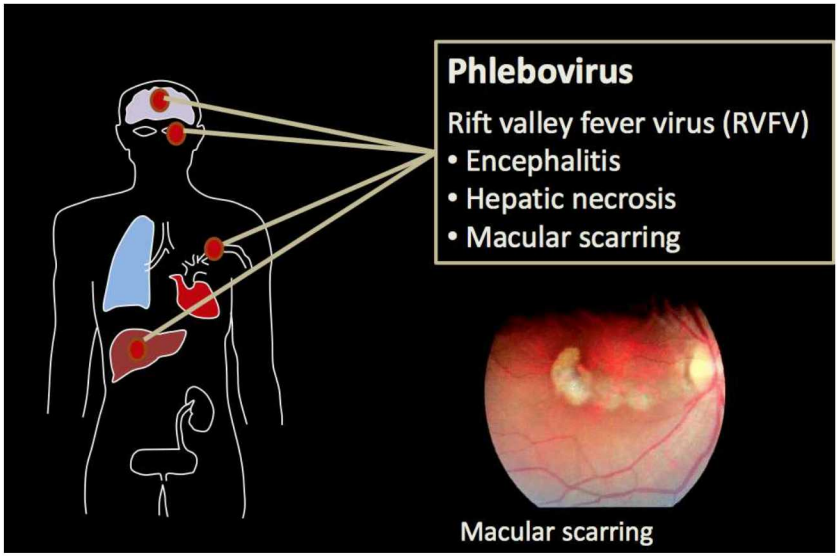
Blue, countries with endemic disease and substantial outbreaks of RVF; green, countries known to have some cases, periodic isolation of virus, or serologic evidence of RVF

Rift Valley Fever (RVF) virus ecology



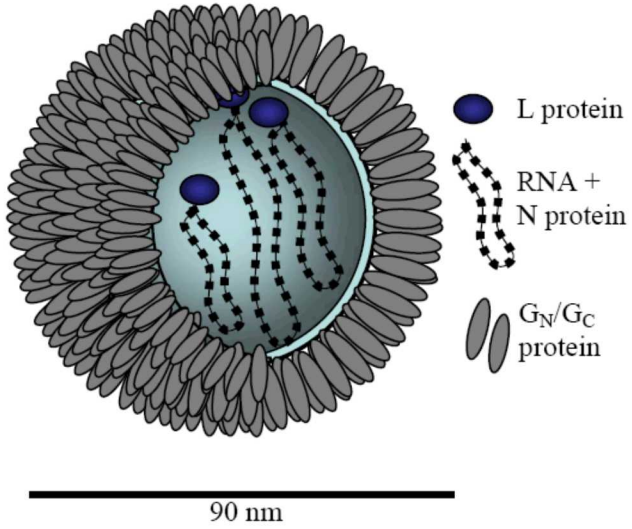
RVFV impact on animal and human health

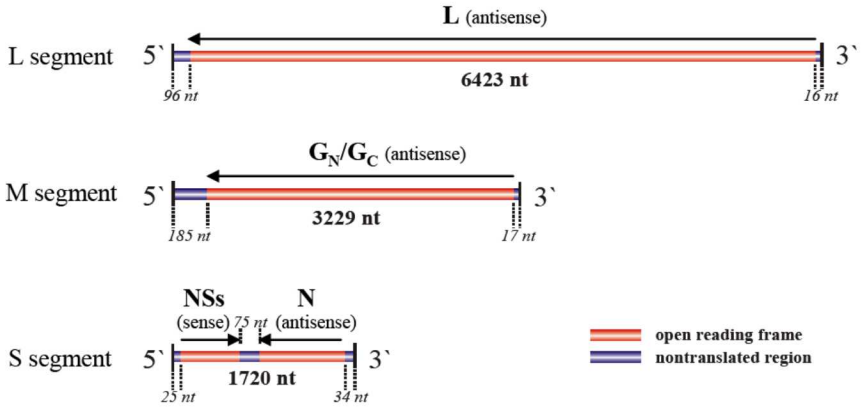
- RVFV mainly causes disease in ruminants (sheep, goats, and cattle) leading to frequent abortions in pregnant animals and a newborn mortality rate as high as 95%.
- Human infection with RVFV is typically associated with an acute febrile illness but can lead to more severe symptoms such as retinal vasculitis, encephalitis, and fetal hepatitis with hemorrhagic fever.
- Transmitted by *Aedes* and *Culex species*
- The U.S. NIAID lists RVFV as a **category A** biodefense priority pathogen
- **No** licensed vaccines or treatment options to combat RVFV infection



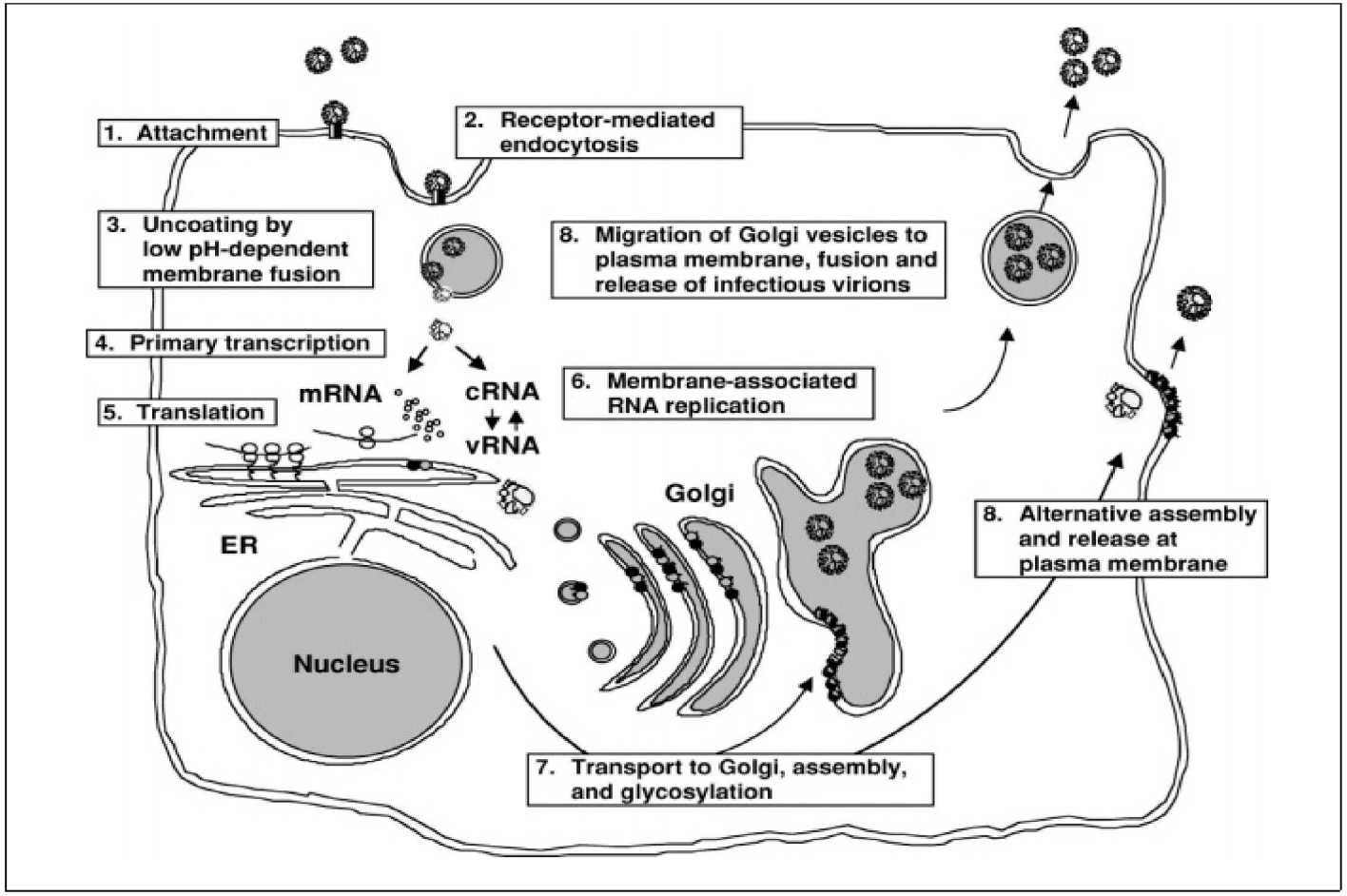
RVFV characteristics

- *Bunyaviridae* family, Phelbovirus genus
- Spherical enveloped virion: 90 to 100 nm in diameter
- Triparite, single-stranded, negative-sense RNA genome, ~11kb total.
- RVFV genome
  - L segment encodes the L polymerase protein
  - M segments encodes the Gn/Gc envelope proteins arranged in a T-12 icosahedral symmetry, the non-structural protein NSm.
  - S segment is ambisense and encodes N nucleoprotein and the non-structural protein NSs important in host transcriptional downregulation and IFNβ inhibition





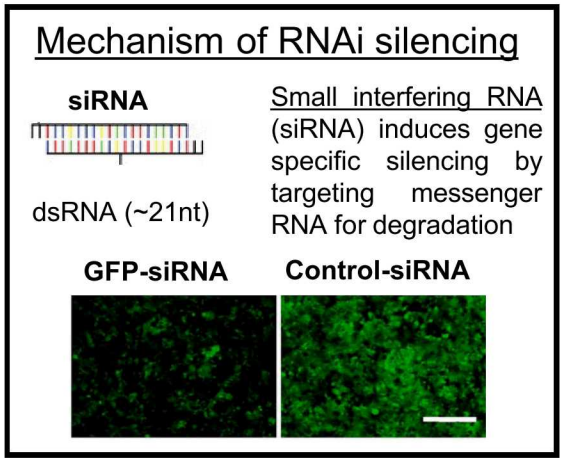
RVFV life cycle



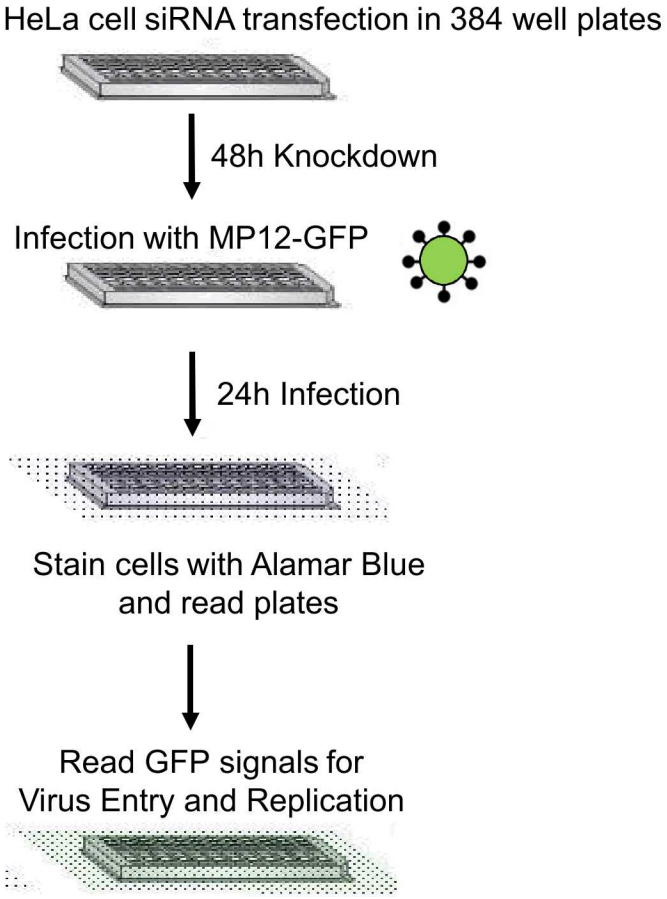
Genome-wide RNA interference screening

Qiagen Genomewide siRNA library

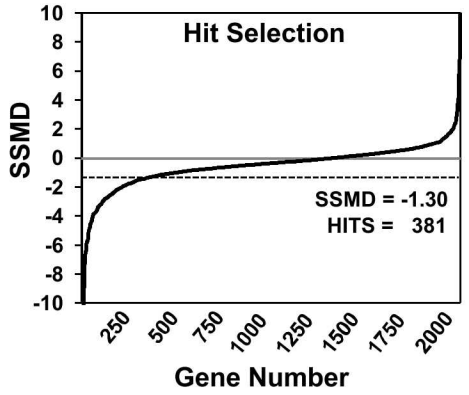
- Pools of 4 siRNA against ~22,000 genes



RNAi screening schematic



Gene Hit selection

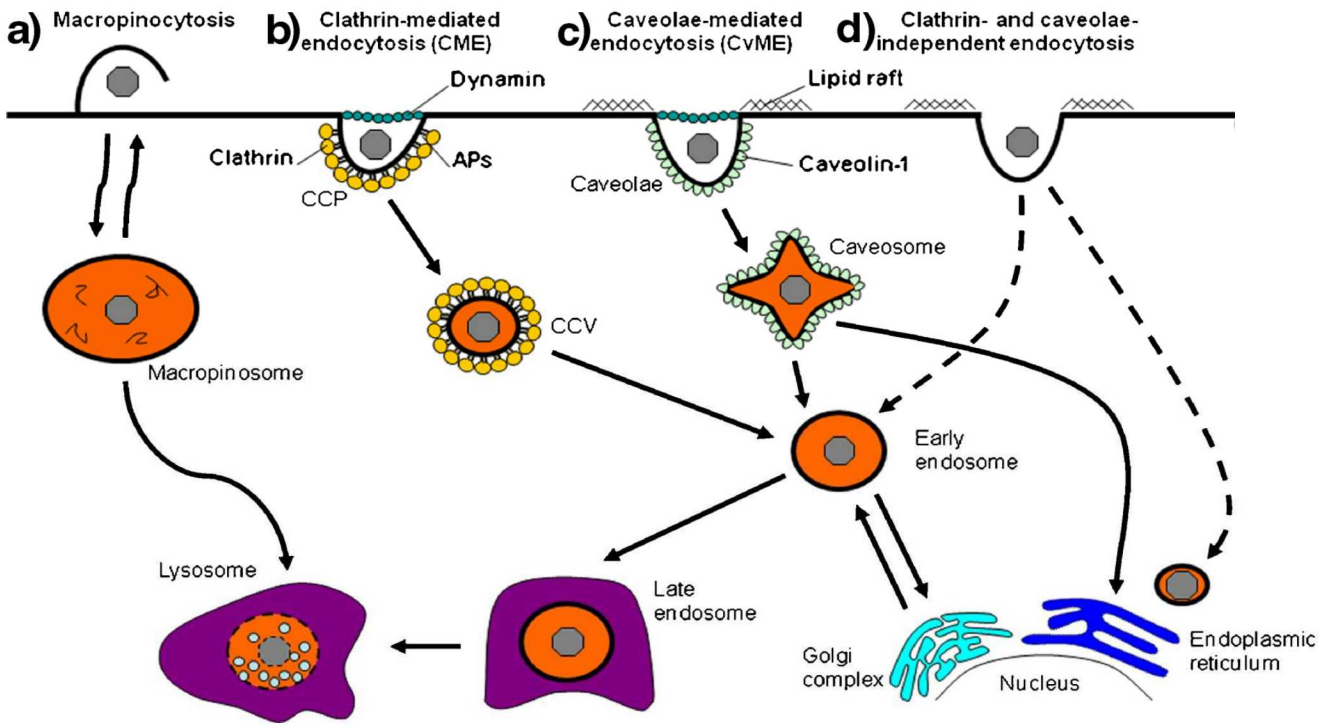


Entry related Hits:

- vATPases
- Caveolin 2 and 3
- Dynamin (DMN)
- UCGC: Ceramide Glucosyltransferase
- Integrins

Characterization of endocytosis pathway involved in RVFV infection

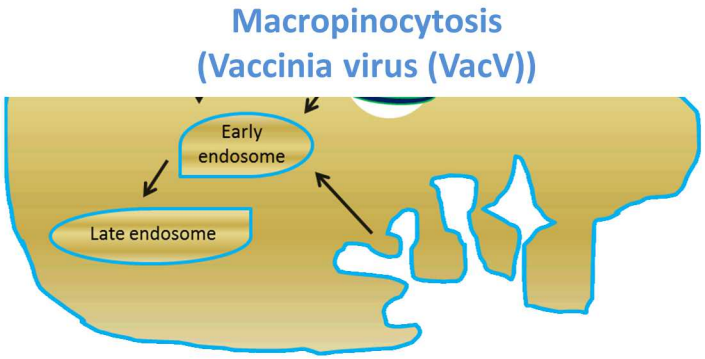
Endocytosis pathways involved in virus uptake



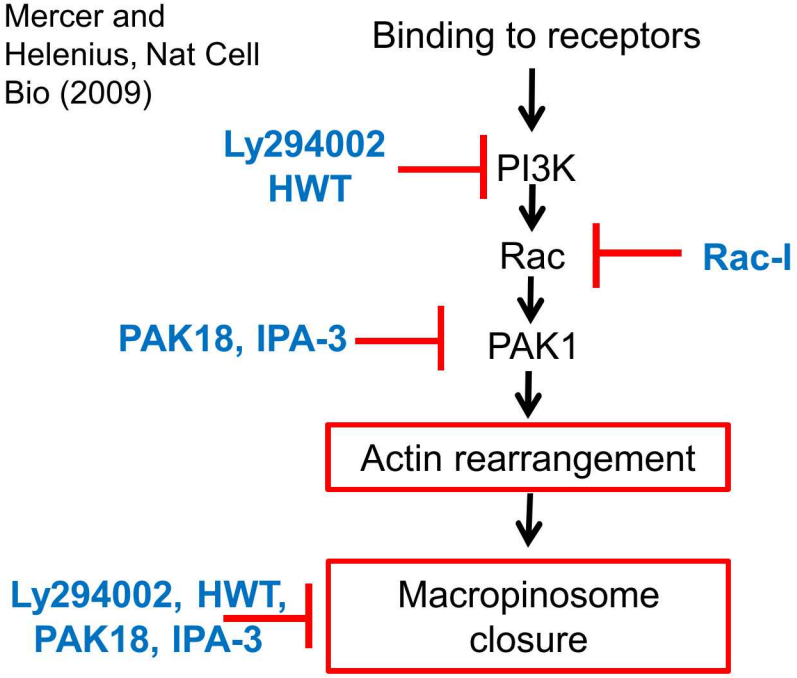
- Electron microscopy of RVFV cell infection indicated a potential role of **clathrin-mediated endocytosis** in entry (Fields Virology 2007)
- Small molecule drug screen against RVFV-MP12 found over-represented classes of inhibitors known to target **macropinocytosis**, including protein kinase C (PKC) inhibitors.

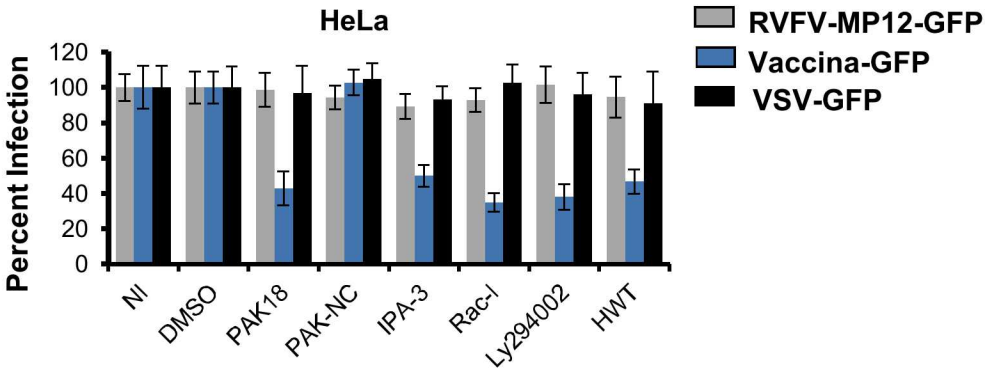
PLoS One (2010) 5(11): e15483

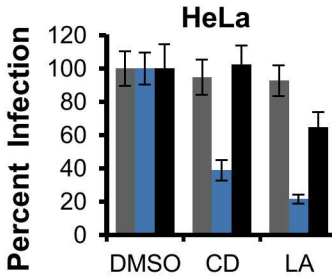
Testing inhibitors of macropinocytosis



Mercer and Helenius, Nat Cell Bio (2009)







Actin dynamics inhibitors:

cytochalasin D (CD)

latrunculin A (LA)

Inhibitors of caveolar but not clathrin-mediated endocytosis block RVFV infection

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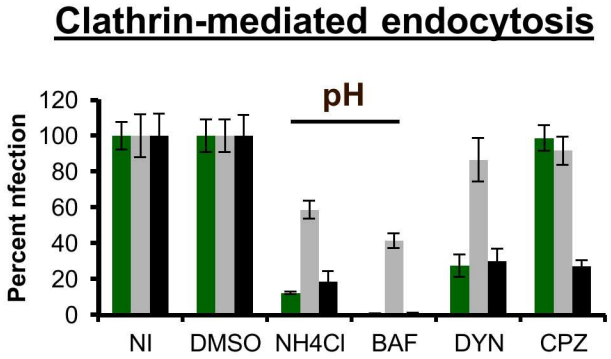
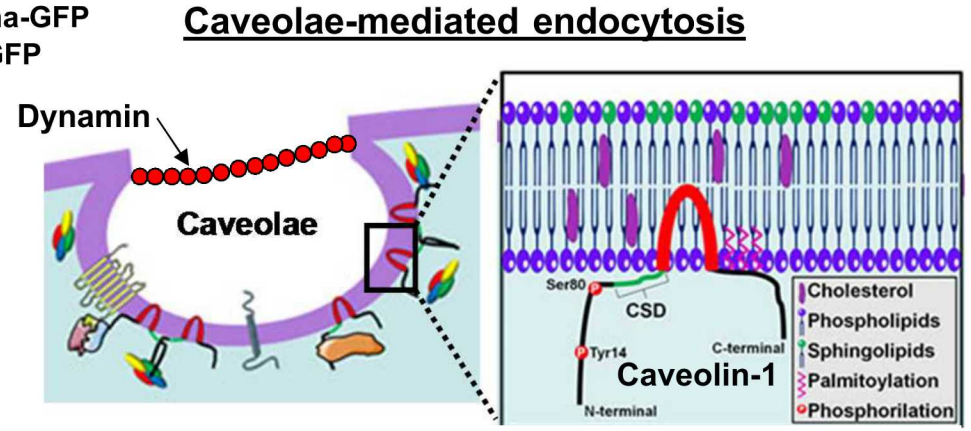
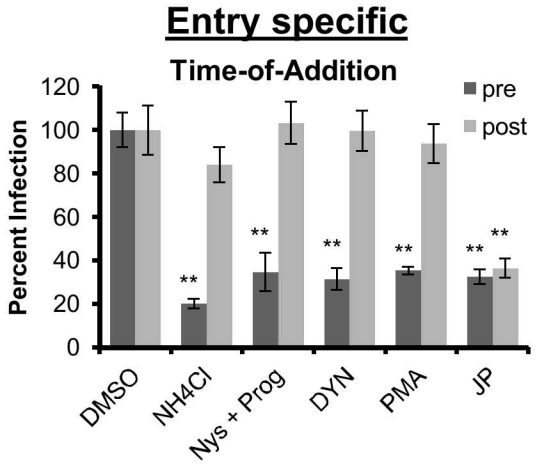
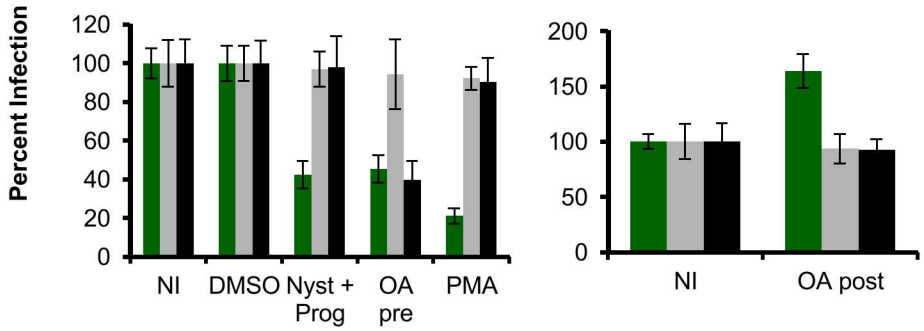


Dynasore (DYN) – Blocks dynamin II

Cholorpromazine (CPZ) – prevents clathrin pit assembly





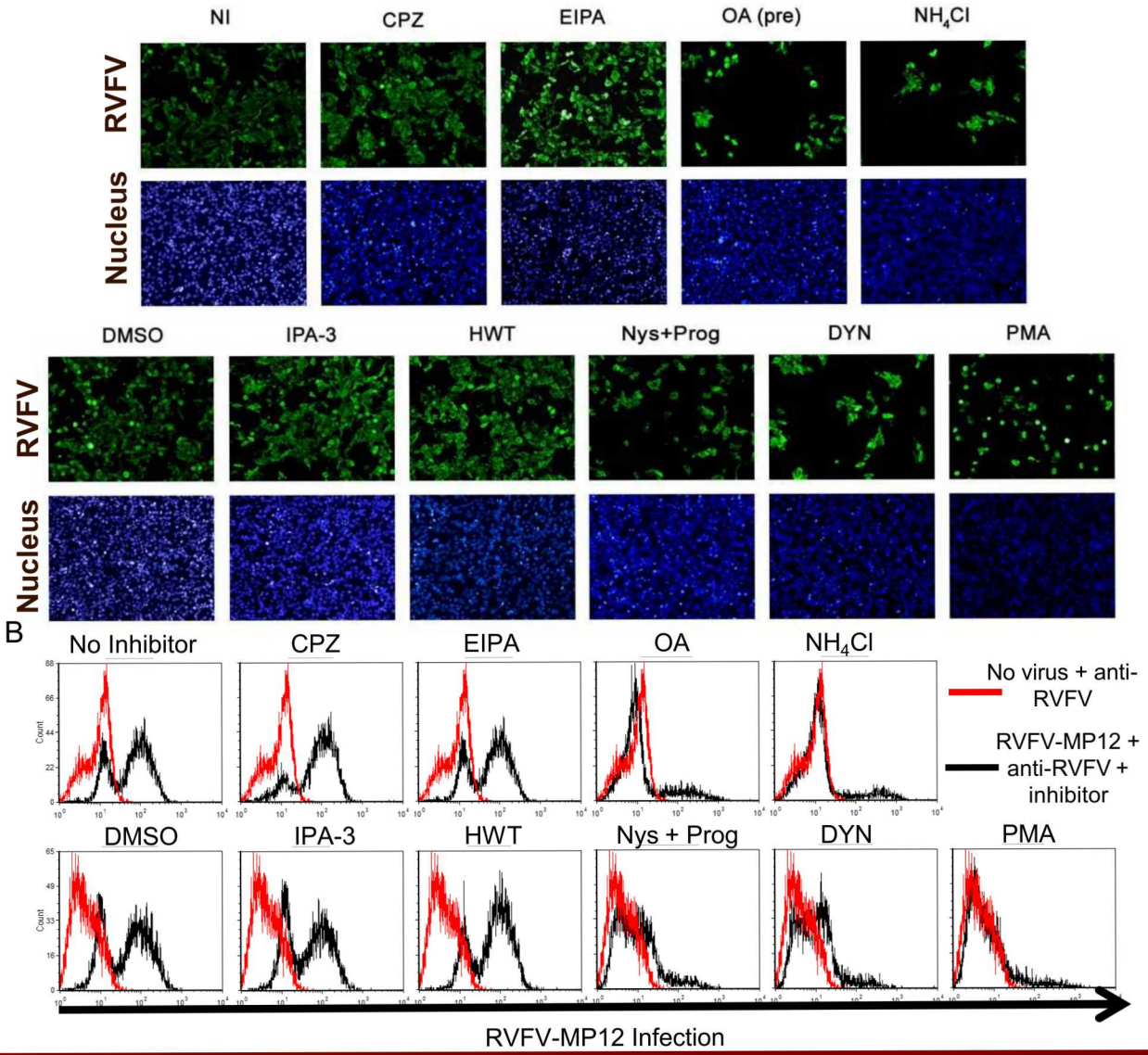


Nystatin/Progestrone (Nyst+Prog) - Sequesters and blocks cholesterol

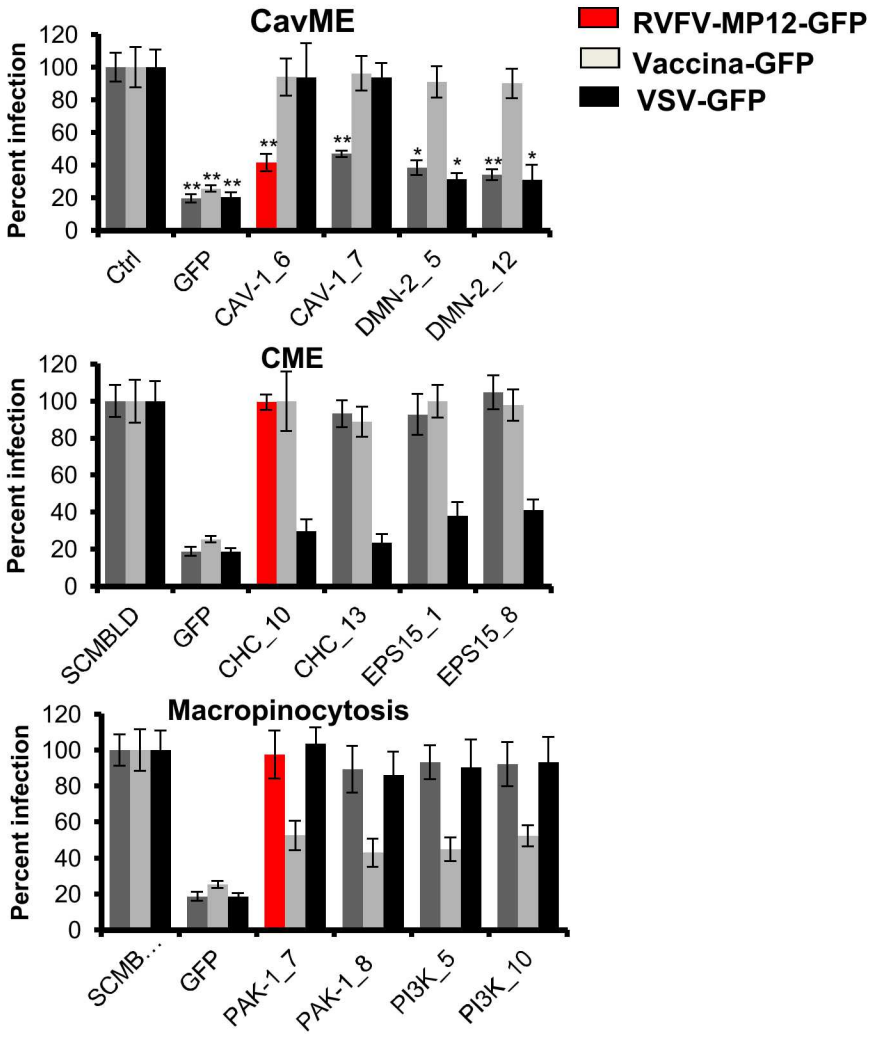
Okadaic acid (OA) – Serine/Threonine phosphatase inhibitor

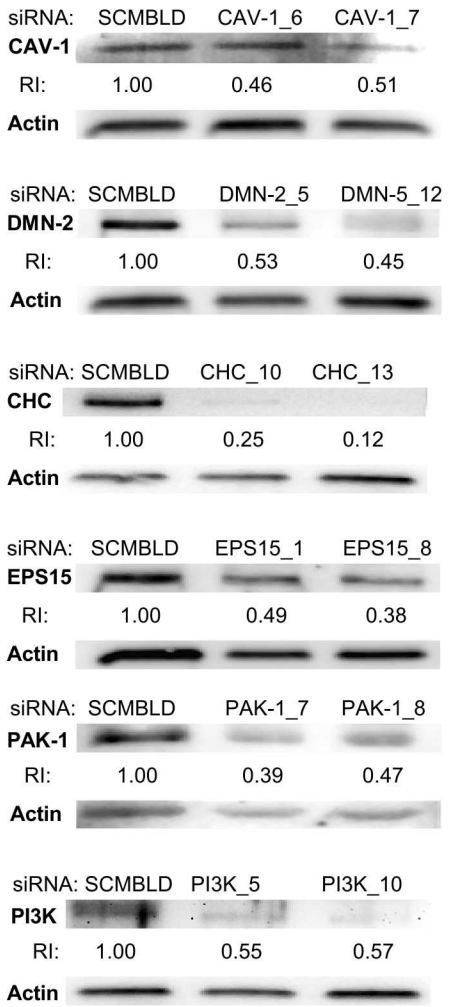
Phorbol 12-myristate 13-acetate (PMA) – Increases degradation of CAV1

Inhibitors with authentic RVFV MP-12

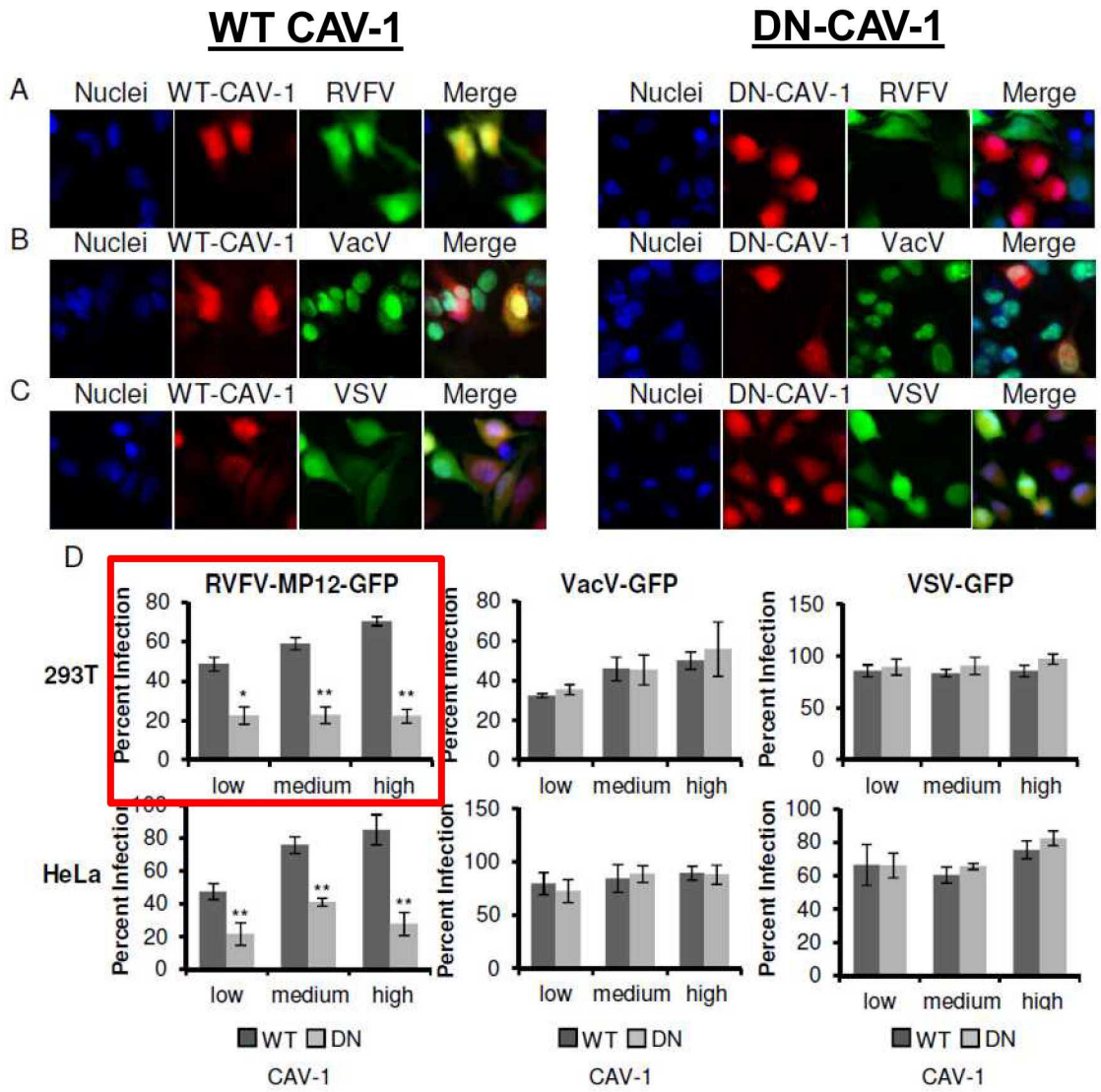


Caveolin-1 downregulation with siRNA reduces RVFV infection



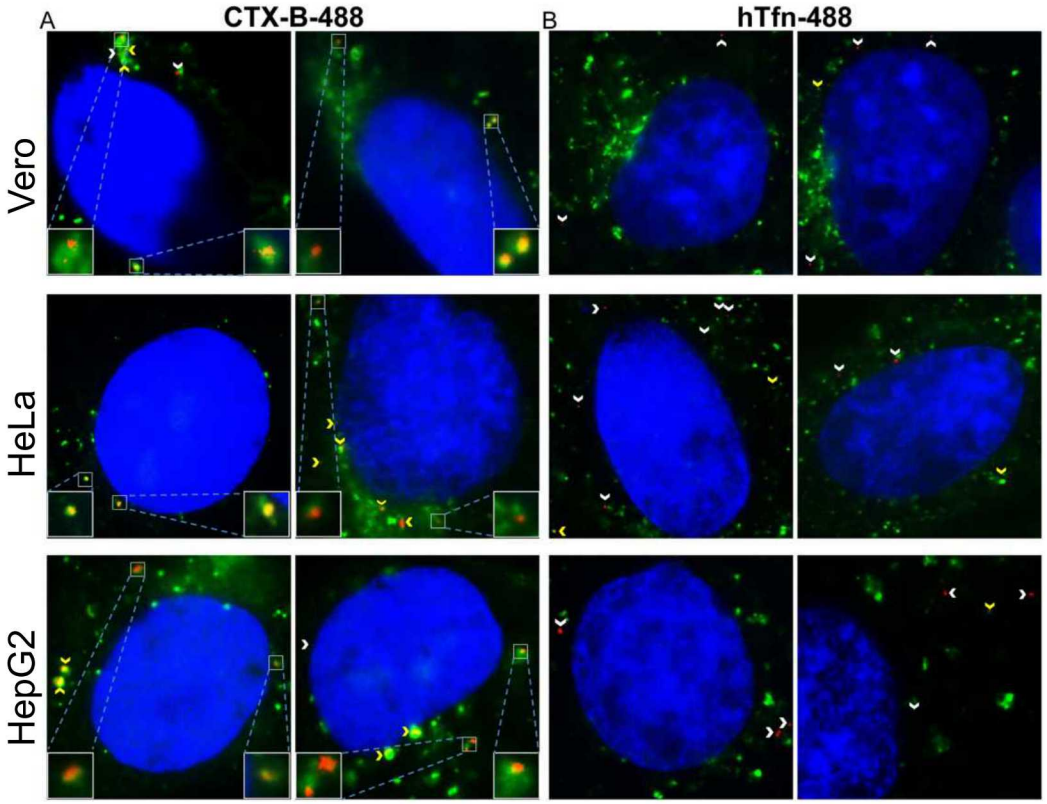


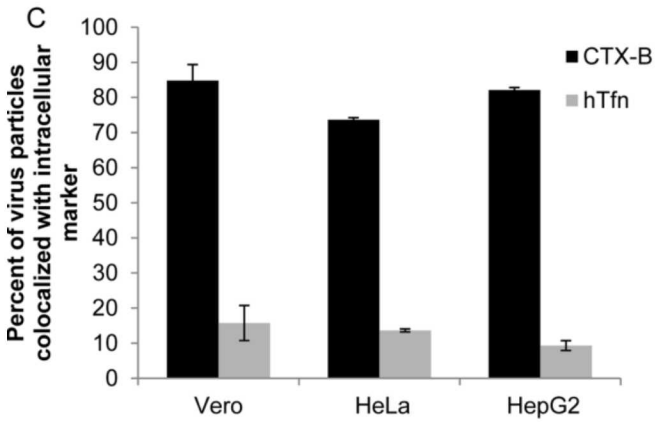
Expression of dominant negative caveolin-1 blocks RVFV infection



- Experiments in more than one cell type (HeLas and 293Ts)
- Used all three viruses to test each dominant negative construct (Cav-1, EPS15, PAK1)

RVFV particles co-localize with CAV marker CTX-B





RVFV – Labeled w/TS-Link BODIPY TR C_5-thiosulfate

A role for Wnt signaling in bunyavirus infection

Bioinformatics analysis of RNAi screening data identifies a role for Wnt signaling in RVFV infection

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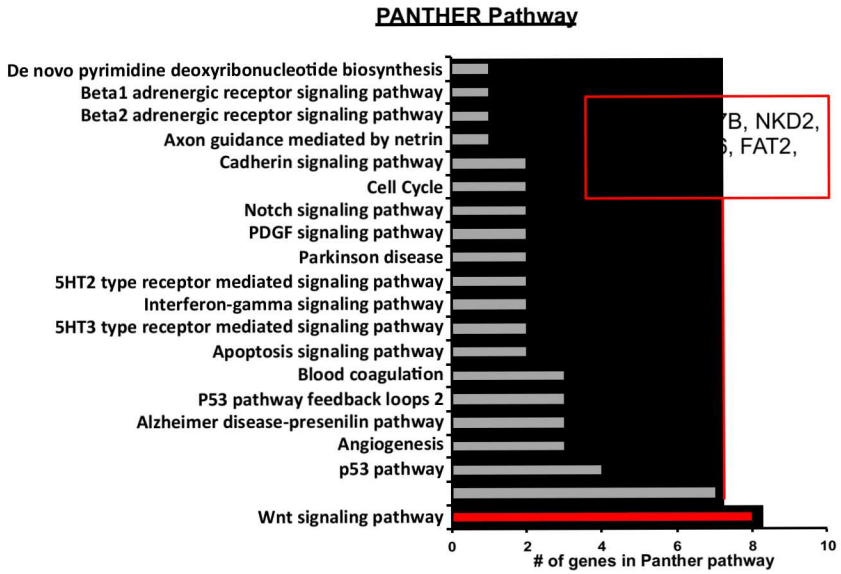
A



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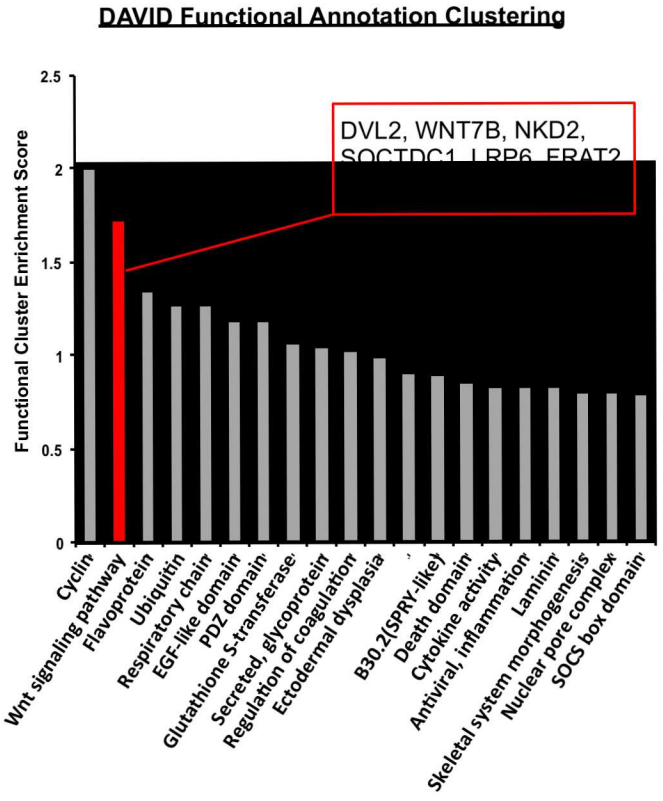

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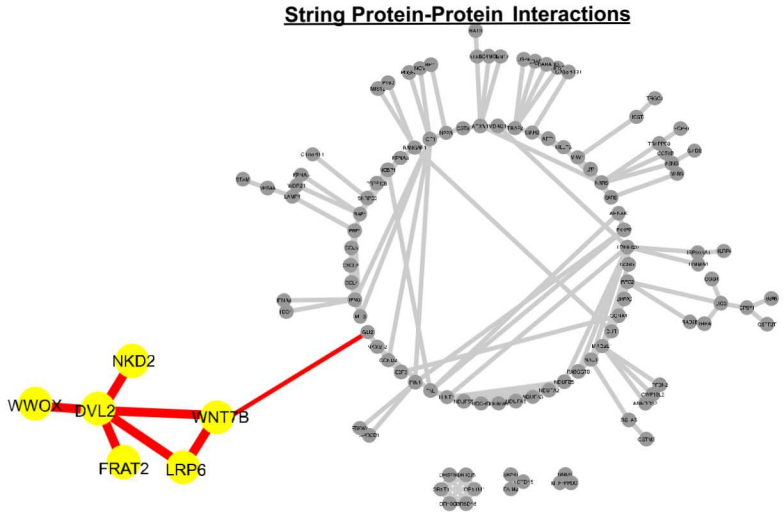
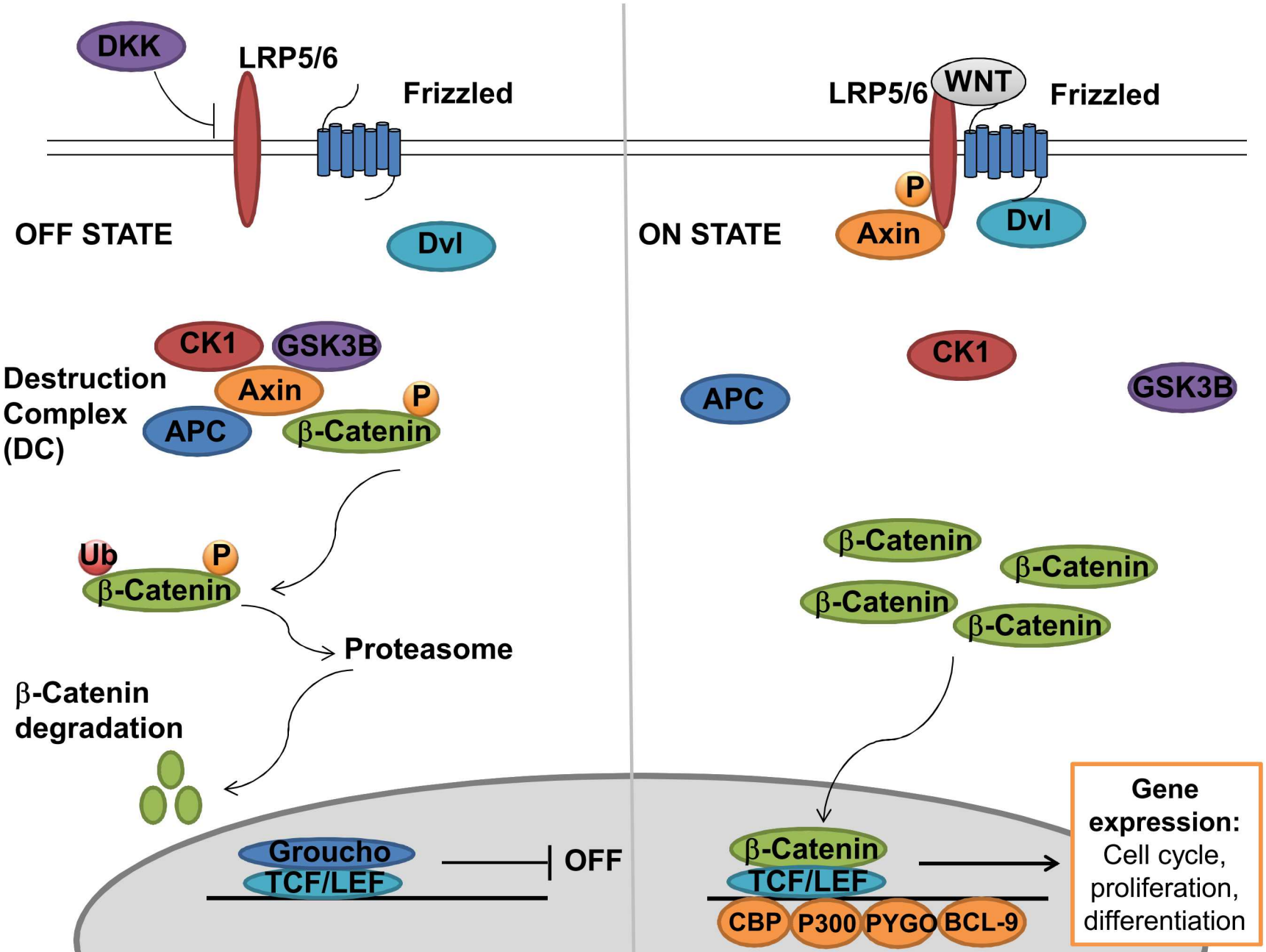


Canonical WNT/β-Catenin

- A highly conserved signal transduction pathway involved in many processes from early embryogenesis to adult multi-organ homeostasis.
- The well-studied canonical WNT pathway signals through ß-catenin and ***regulates the cell cycle, cell growth, and proliferation***.
- Involved in cancer progression, including tumor initiation, tumor growth, cell death, differentiation and metastasis.
- Improved drug-discovery platforms and new technologies have facilitated the discovery of agents that can alter WNT signaling in preclinical models, thus setting the stage for clinical trials in humans.

Canonical Wnt signaling



Activation of Wnt/β-catenin by RVFV infection

Wnt reporter Assay

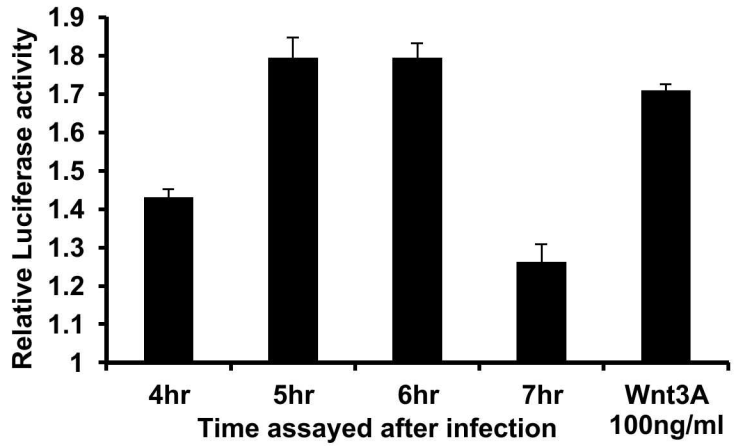
Transfection

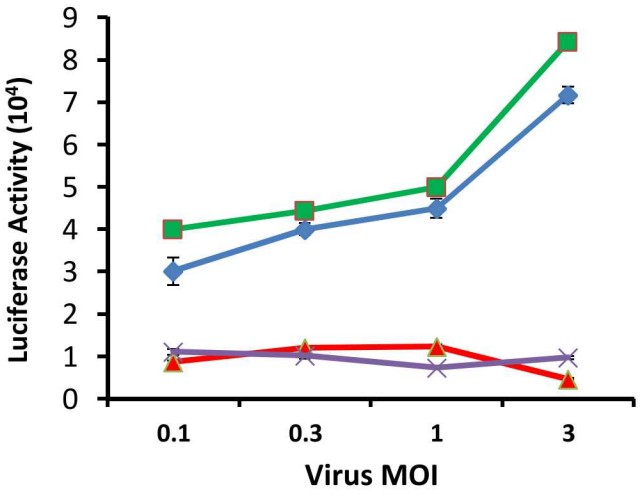
18hr

Virus infection

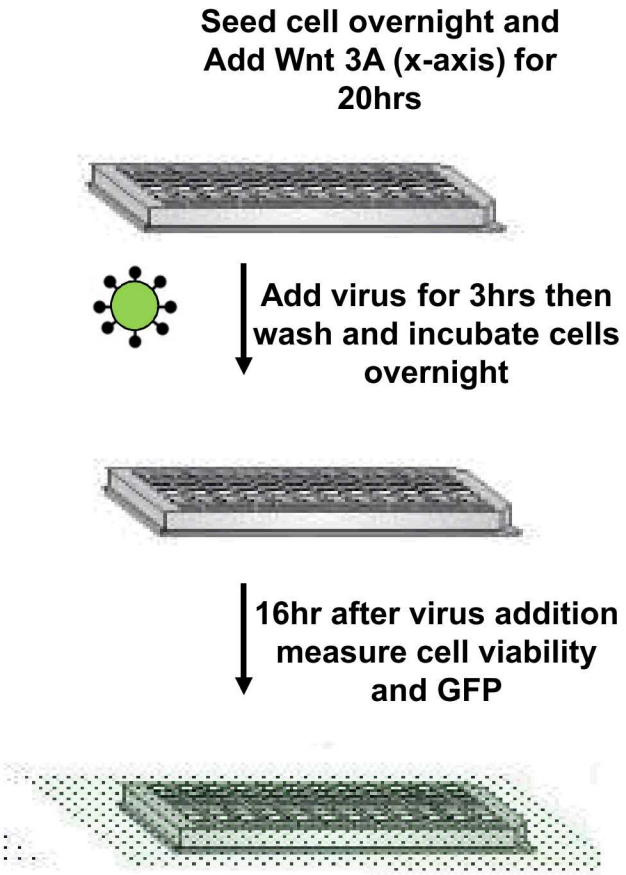
4-7 hours post-infection

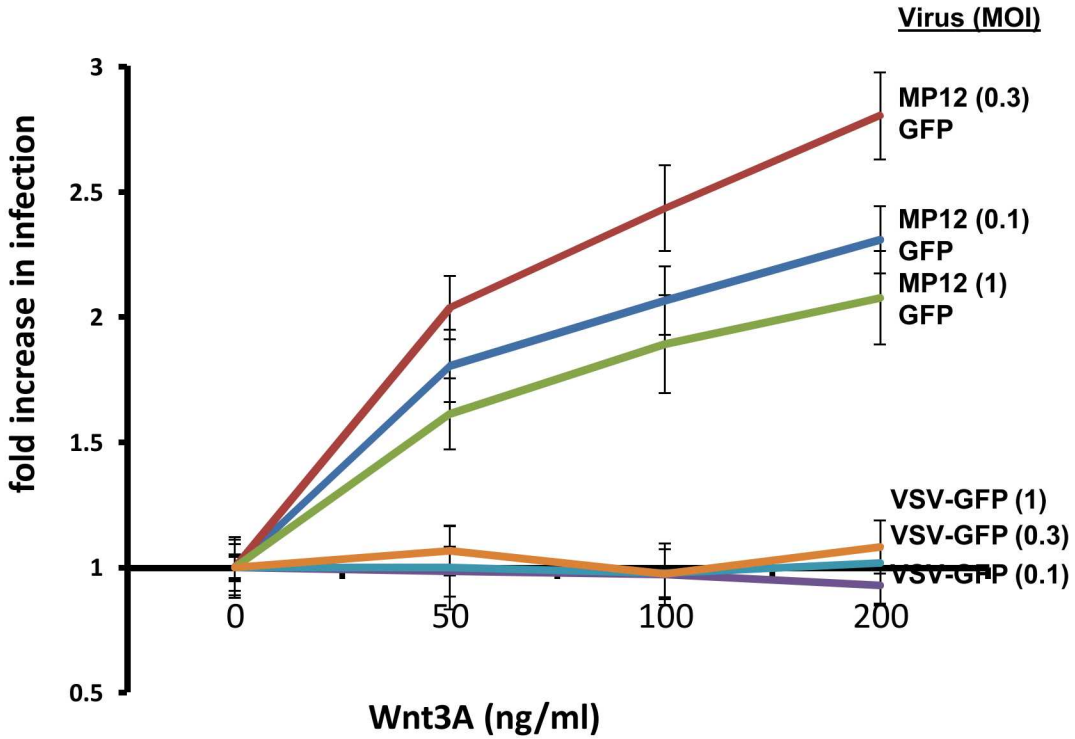
Read Luminescence



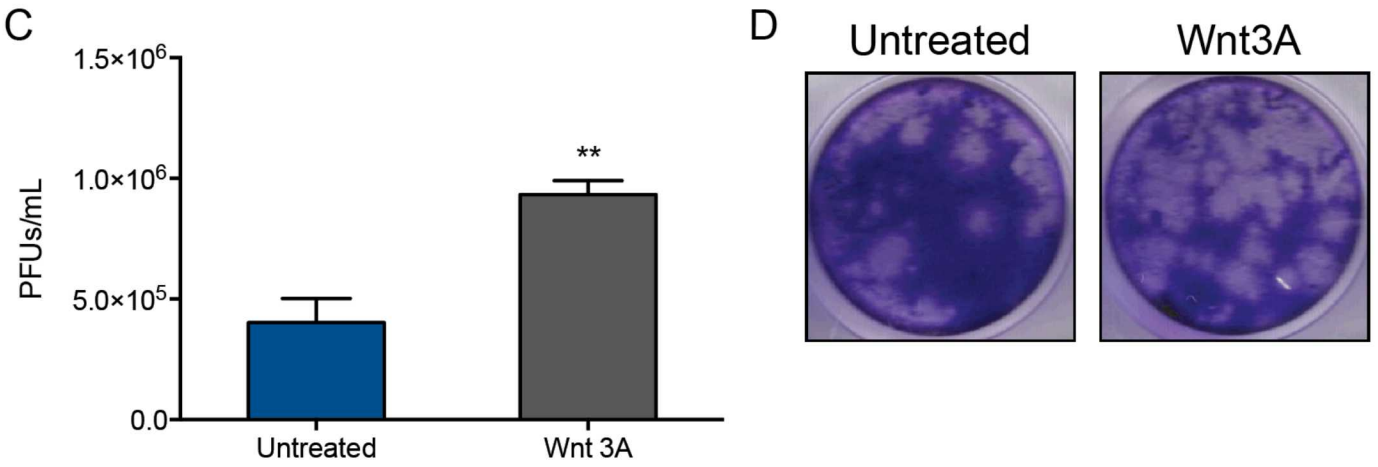


Enhancement of RVFV replication by pre-activation of Wnt/β-catenin

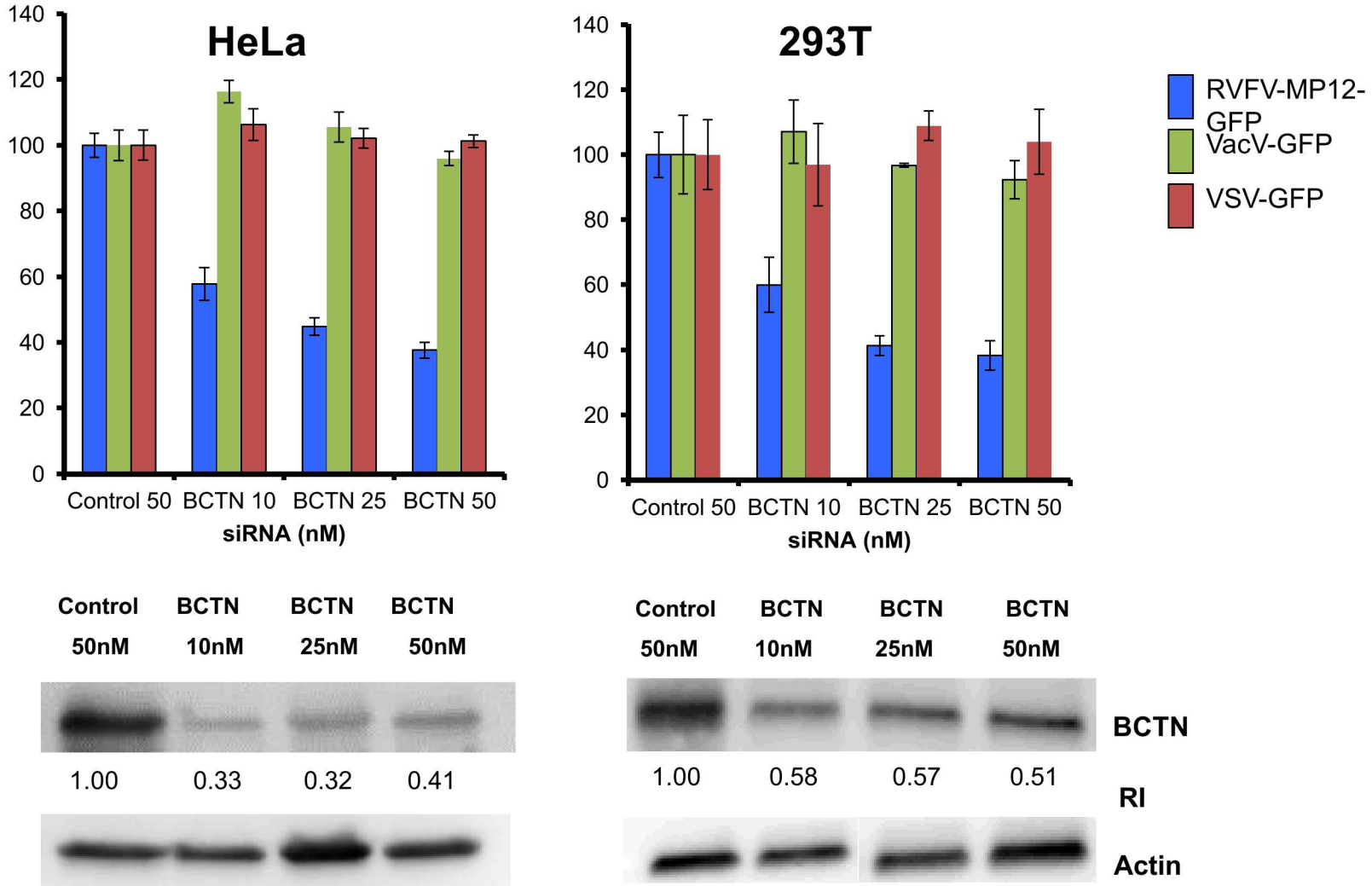




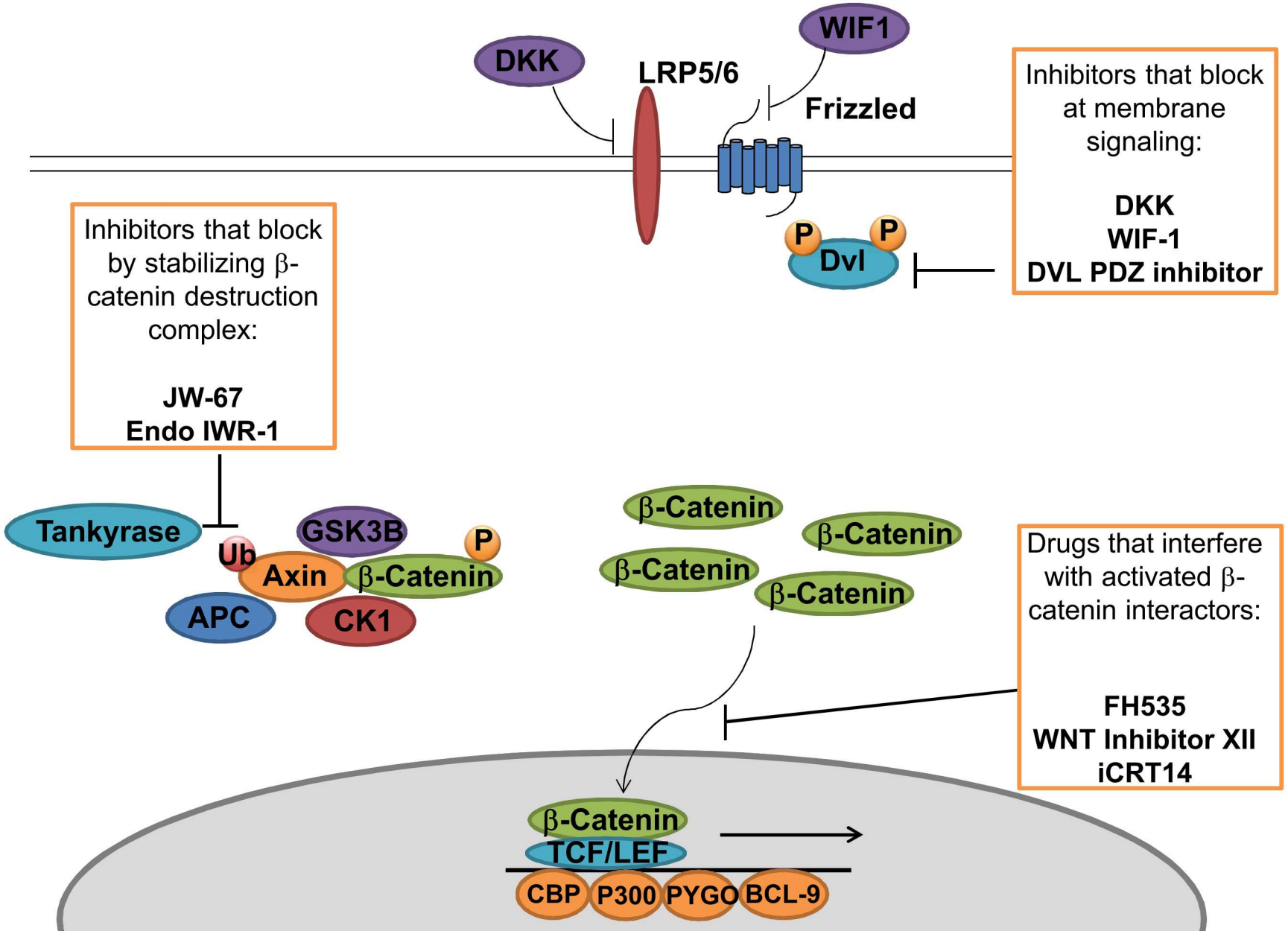
Enhancement of WT RVFV ZH501 infection by pre-activation of Wnt/β-catenin



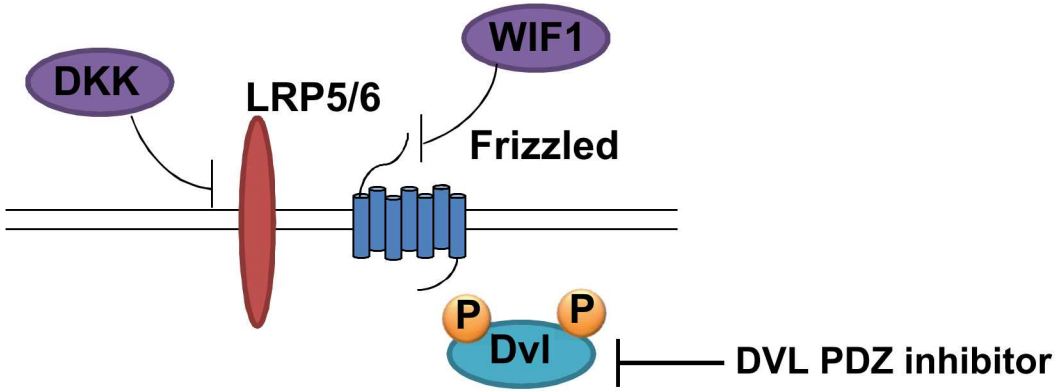
siRNA against β-catenin inhibits RVFV

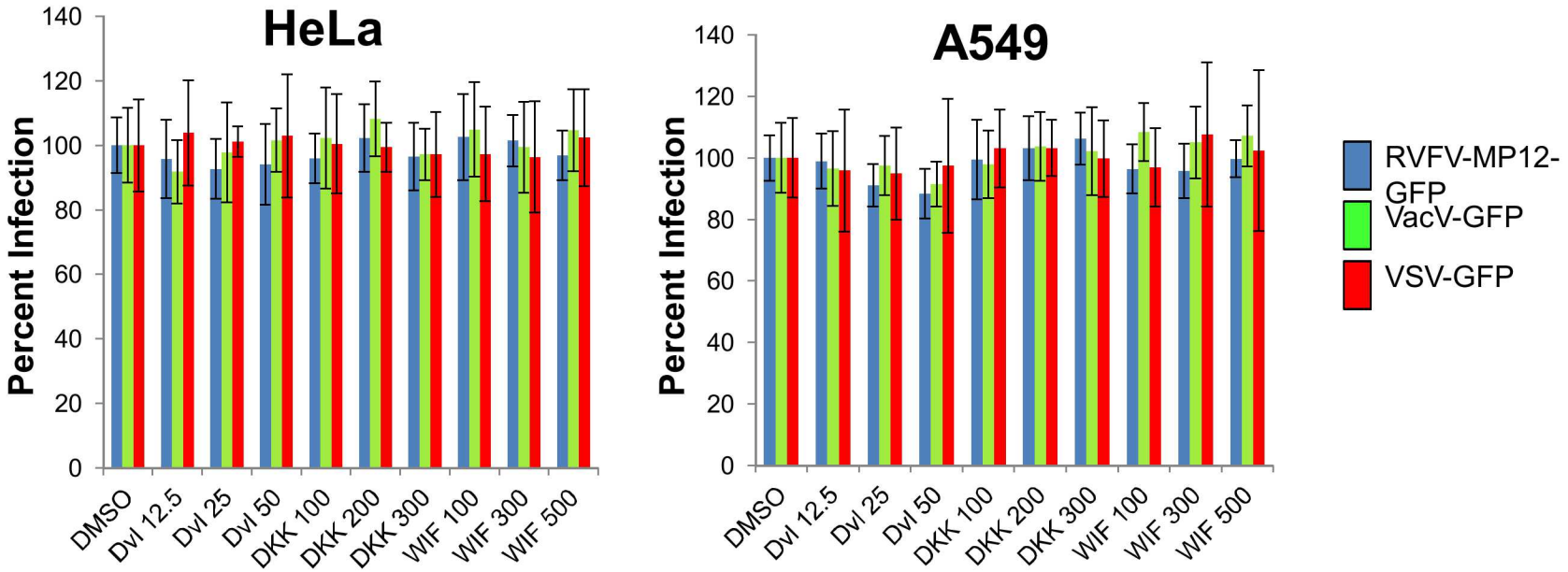


Inhibitors of Wnt signaling

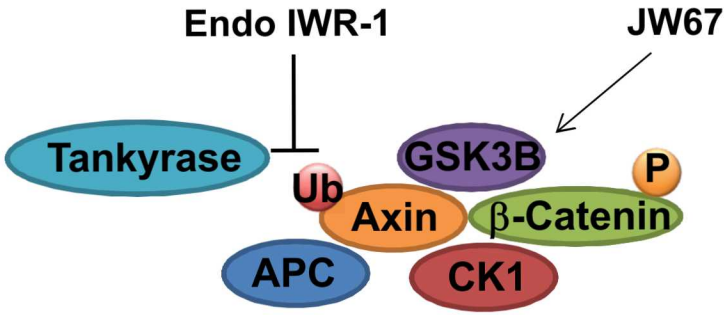


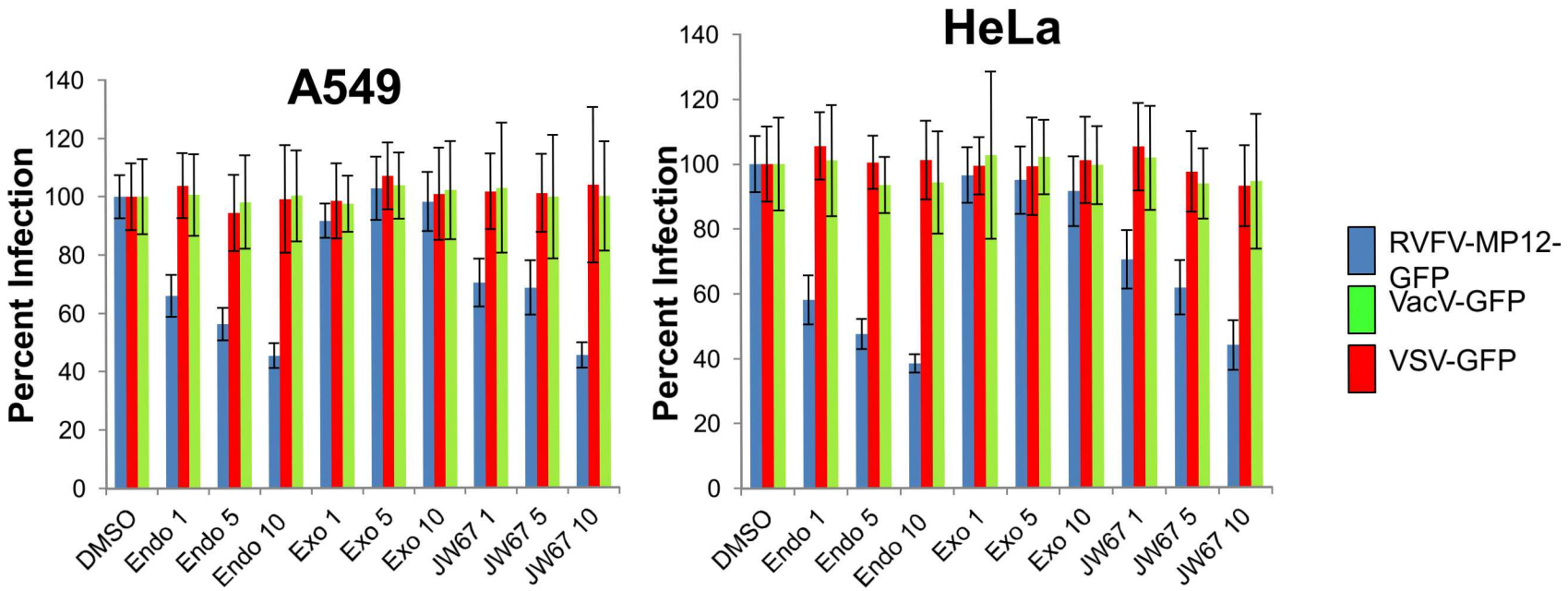
Inhibitors that Block Wnt Signaling Upstream of the DC Have No Effect on RVFV Infection



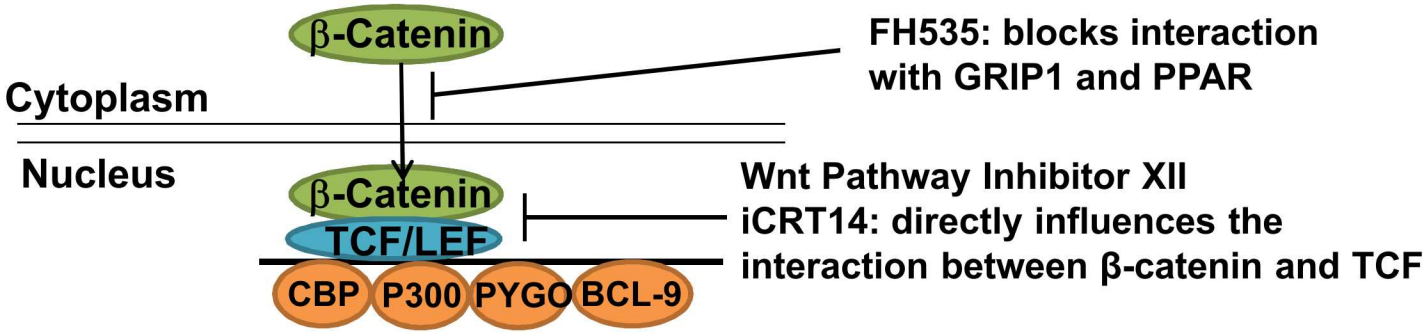


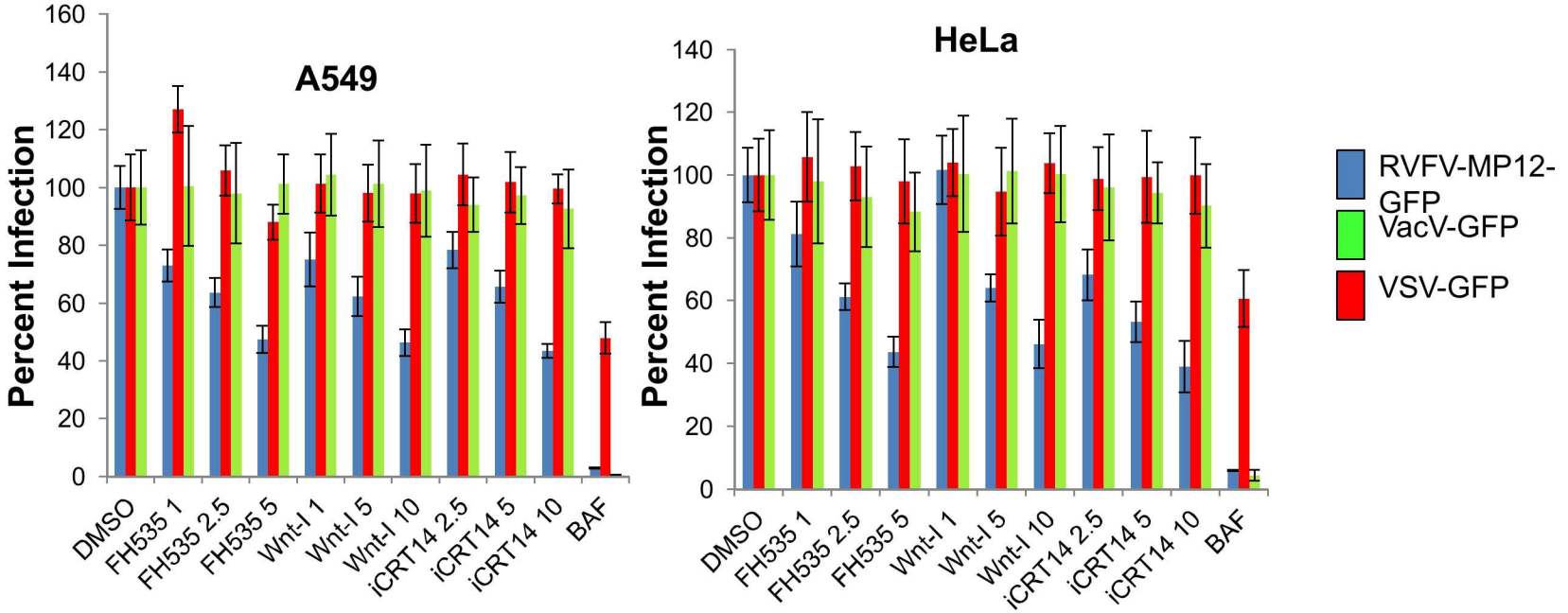
Inhibitors that Stabilize the Destruction Complex Specifically Decrease RVFV infection



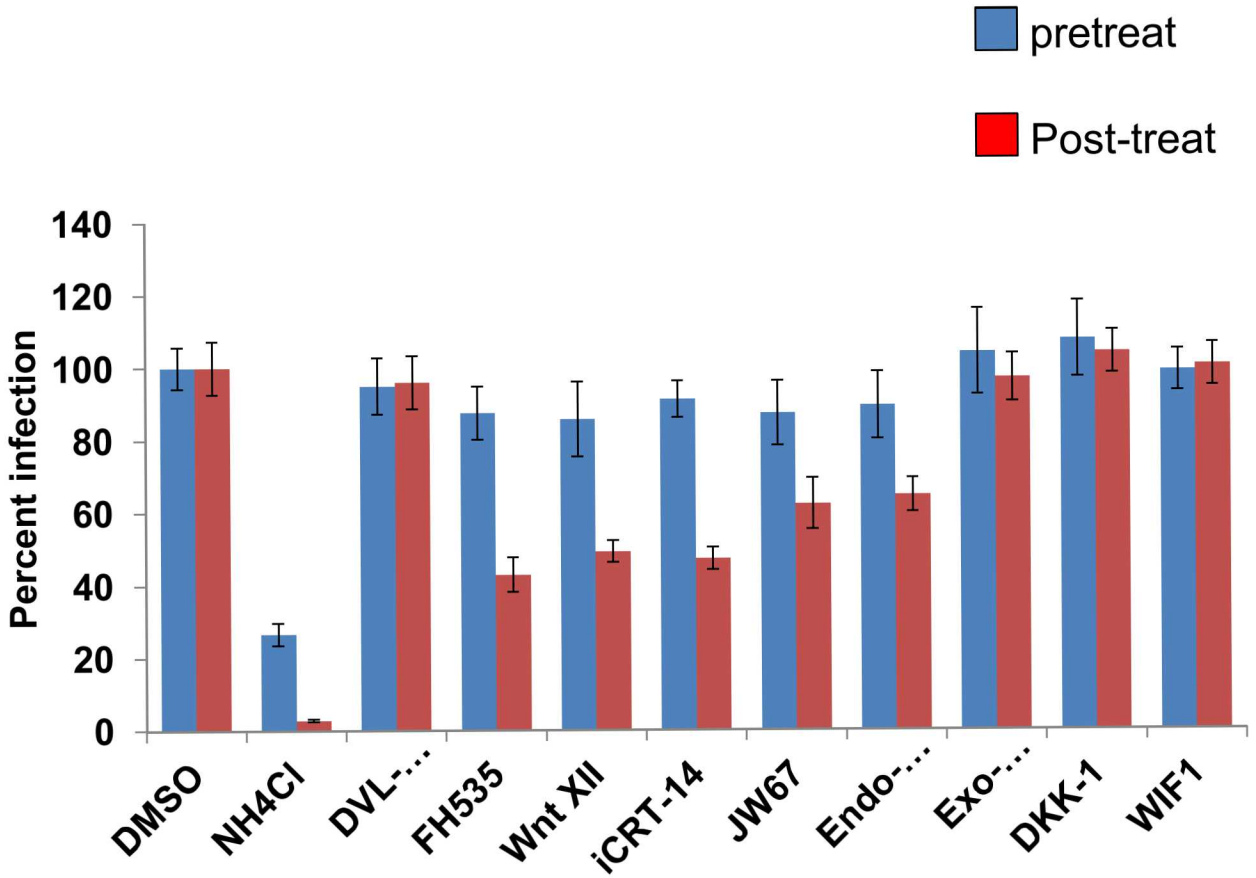


Inhibitors that Block Activated β-catenin Decrease RVFV Infection



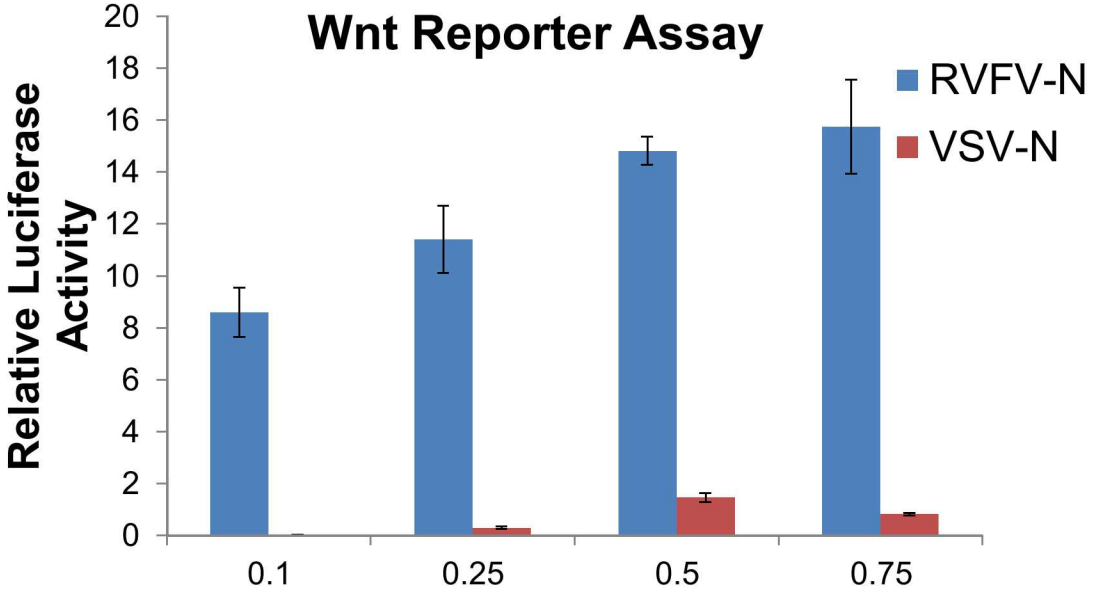


Inhibitors that stabilize the DC specifically decrease RVFV infection at a post-entry step



What is the mechanism of activation?

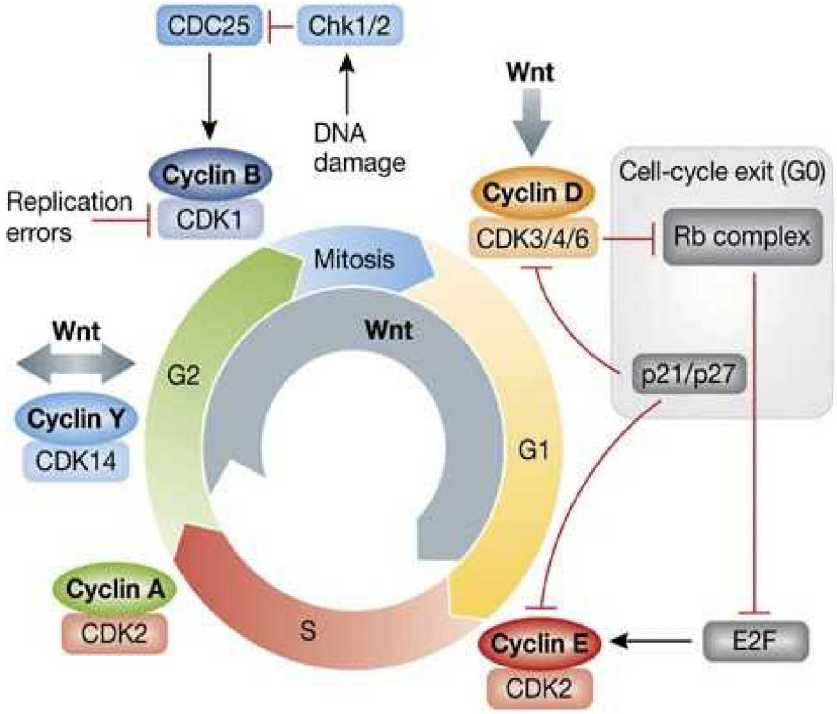
- Is viral protein expression responsible for activation?
- NSs doesn't appear to be involved since both MP12 with and without NSs activate Wnt signaling
- RVFV-N is most abundant viral protein at early time points of infection



RVFV N protein induces β-catenin activation

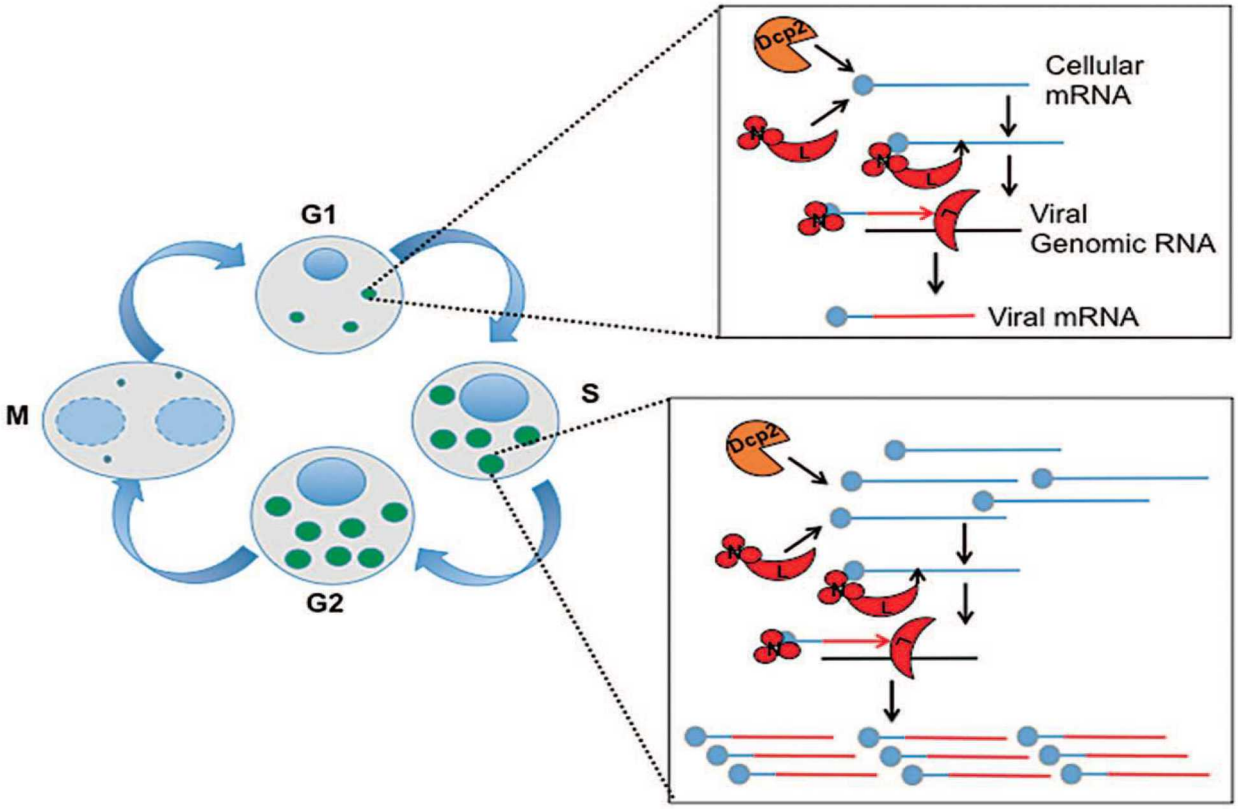
Wnt signaling and the Cell Cycle

- Wnt signaling induces cell cycle shift and cell proliferation



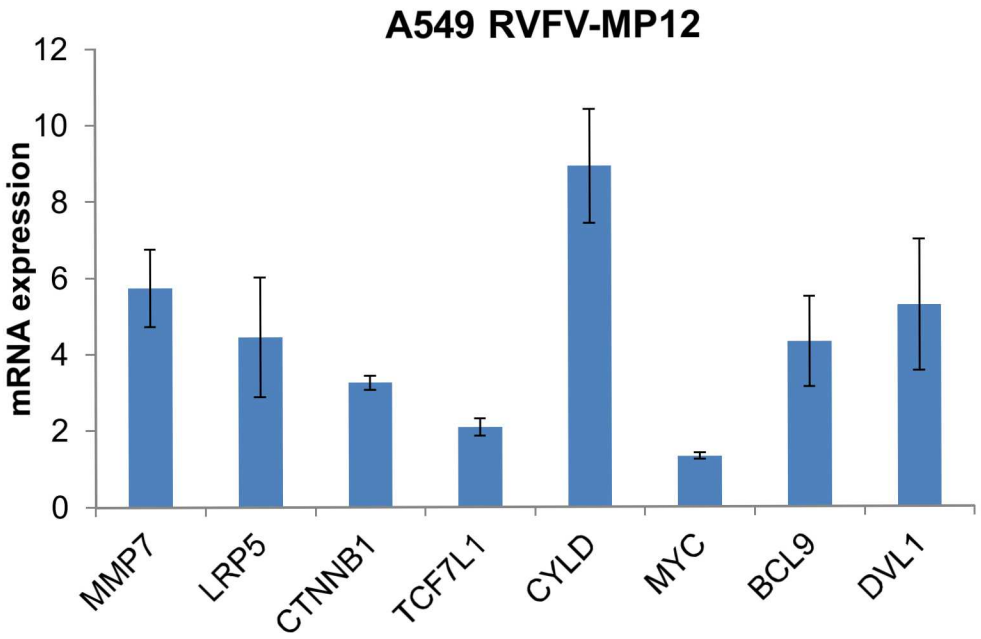
Decapping and Bunyaviral cap-snatching machinery compete for cell cycle regulated mRNAs

- **Bunyaviruses** cap their mRNAs at the 5' end by "cap-snatching"
- **Bunyaviruses** prefer S phase where cellular mRNAs are abundant in P-bodies and available for cap snatching
- Half of the mRNAs snatched by the virus had gene ontology (GO) terms associated with the cell cycle.

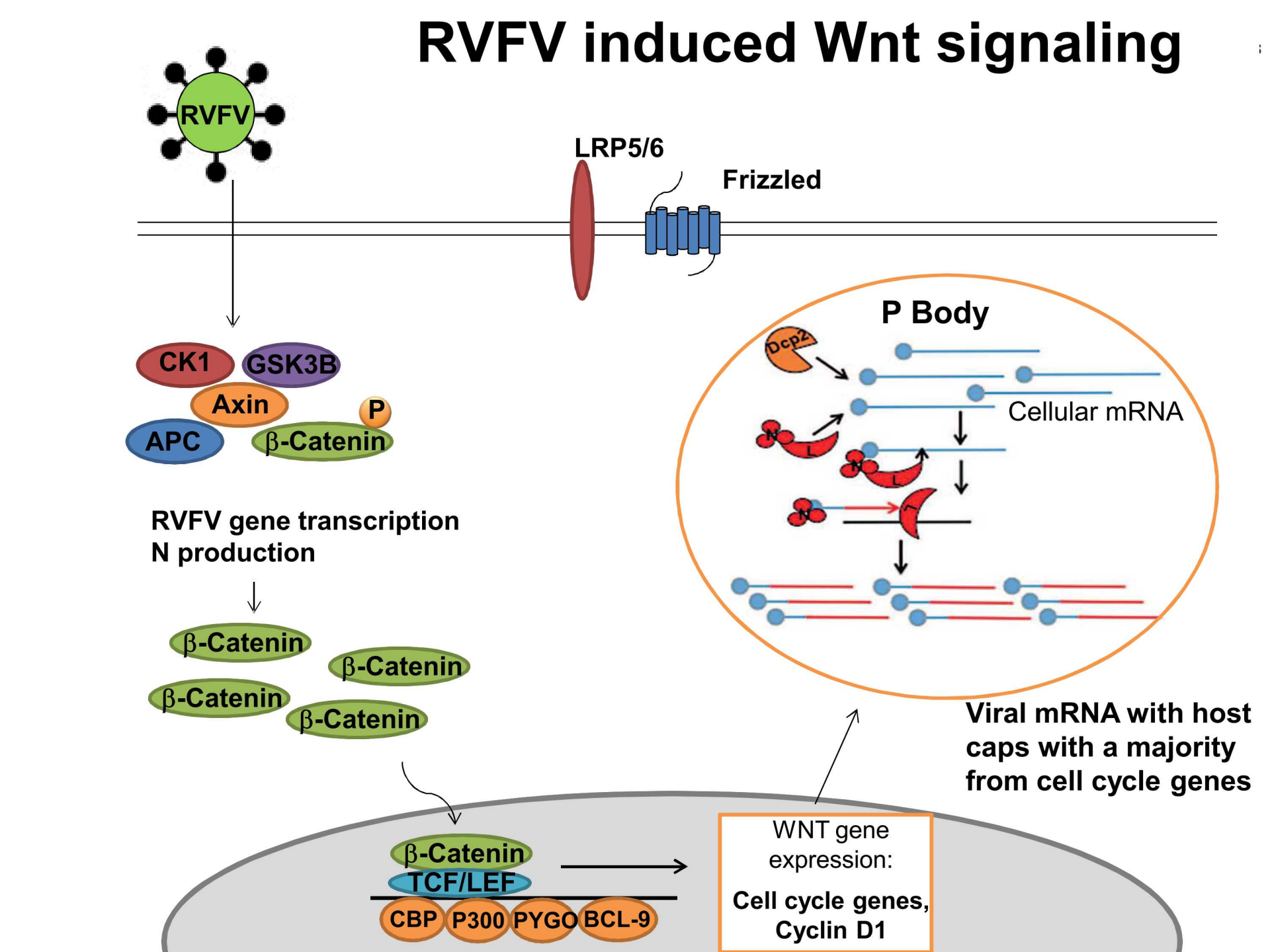


Hopkins et al., Cell Cycle 12:24, 3711–3712; December 15, 2013; Landes Bioscience

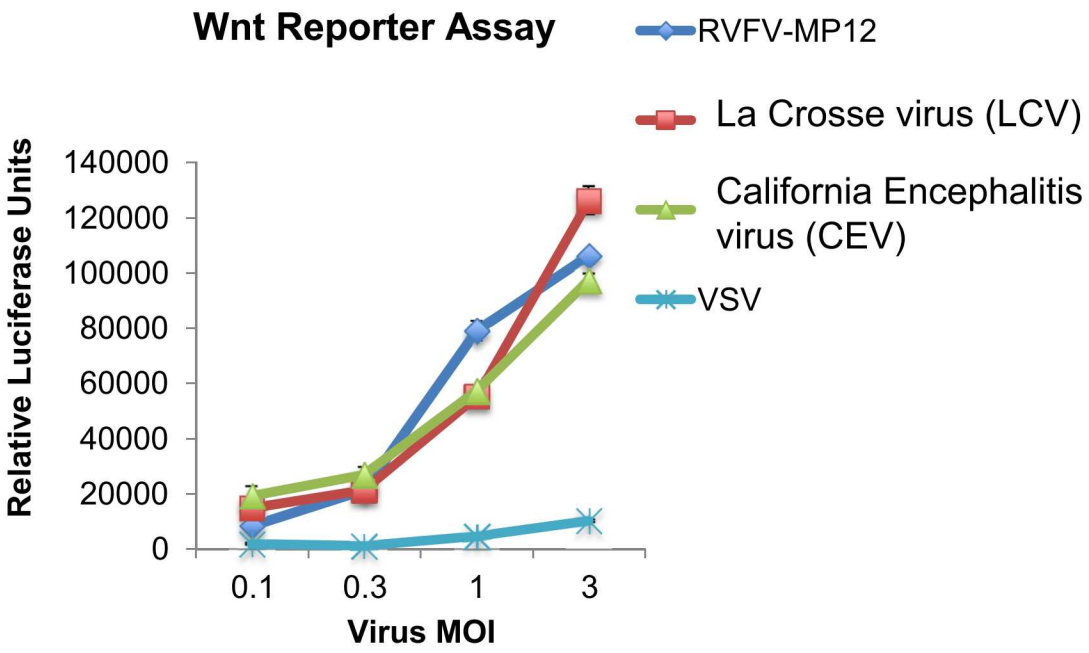
Wnt gene expression upon RVFV infection



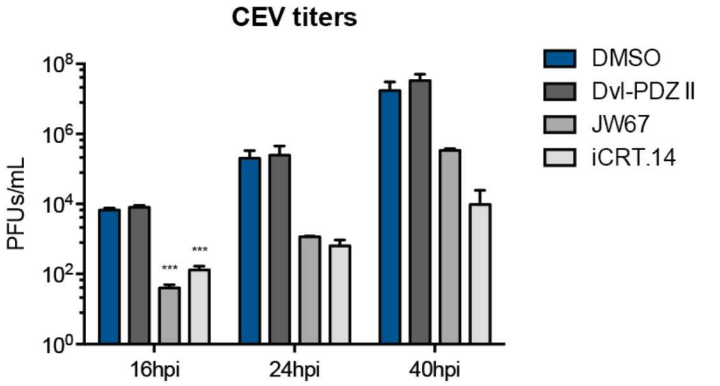
RVFV induced Wnt signaling



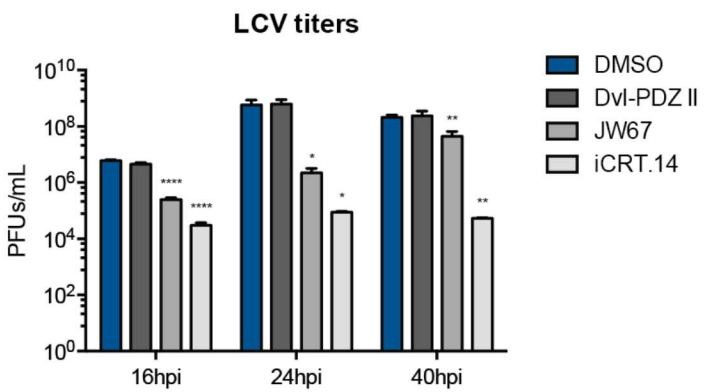
Distantly related bunyaviruses induce Wnt/β-catenin signaling upon infection and are inhibited by Wnt signaling inhibitors.



B



C



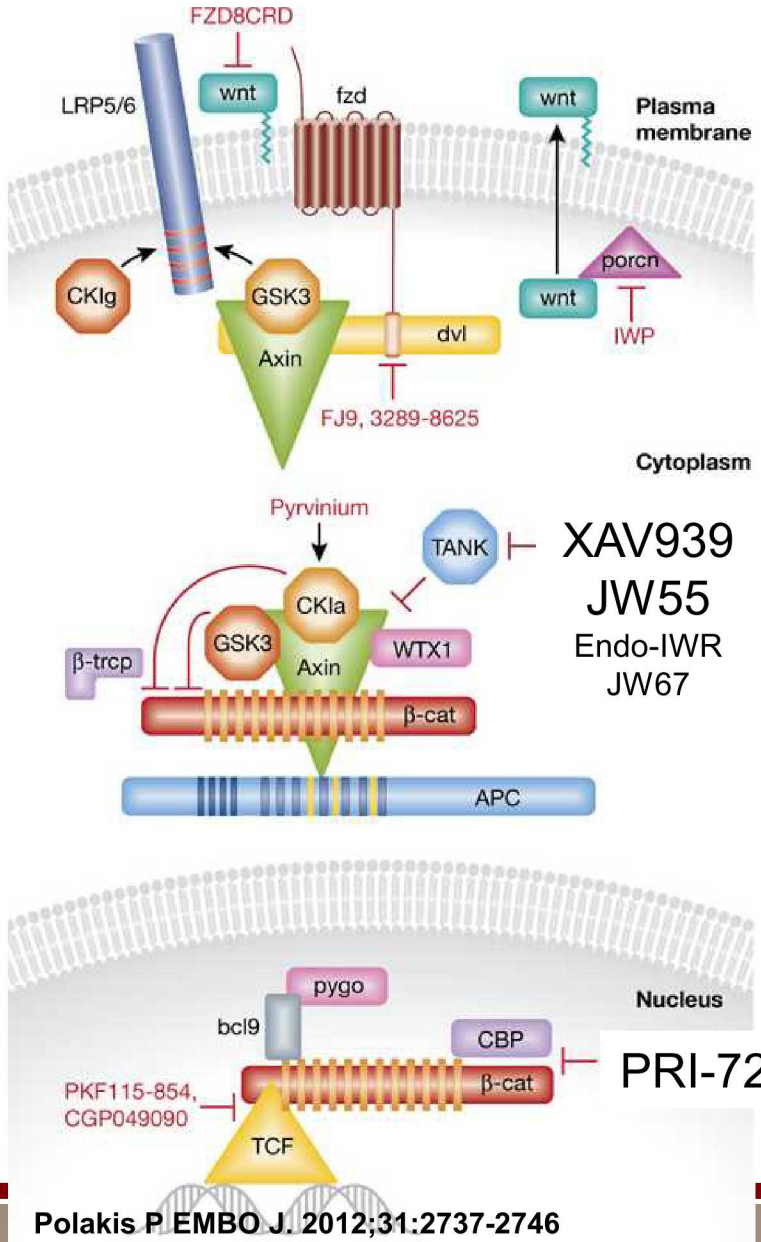
Therapeutic intervention in Wnt signaling

Phase II/I clinical trials:

- **PRI-724** (Prism Pharma Co, Ltd/Eisai)
  - Disrupts the interaction between β-catenin and CBP
  - It is being studied in both solid tumors and myeloid malignancies

Preclinical Studies

- **XAV939** (Novartis Pharmaceuticals)
  - This small-molecule poly(ADP ribose) polymerase (PARP) inhibitor has demonstrated efficacy in cellular models of cancer survival.
  - Axin protein is stabilized and can promote the degradation of β-catenin
- **JW55** (Tocris Bioscience)
  - This selective tankyrase1/2 inhibitor has been shown to inhibit the growth of colon cancer cells in both cell and animal models.
  - Axin protein is stabilized and can promote the degradation of β-catenin



Polakis P EMBO J. 2012;31:2737-2746

CRISPR library screening for RVFV-host interactions

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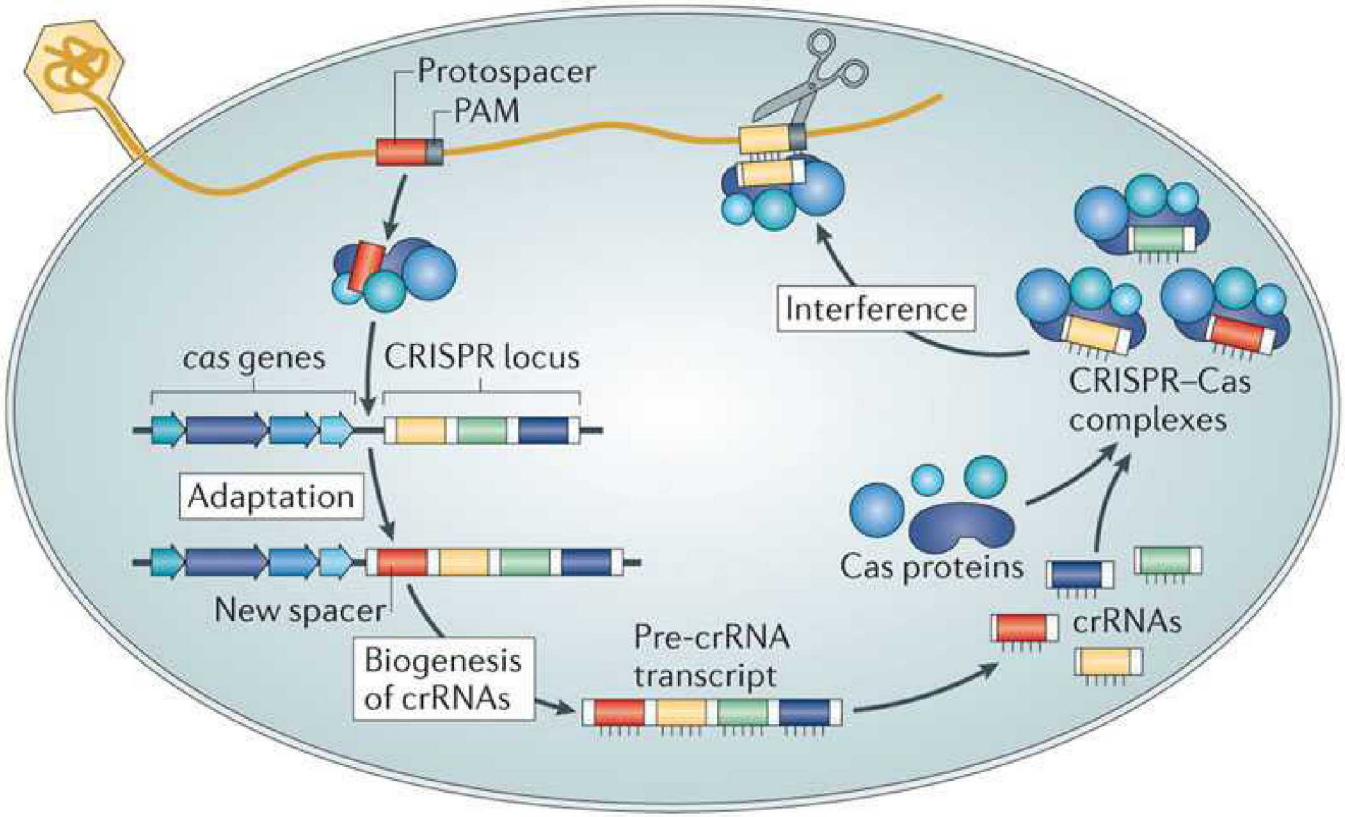
III. CRISPR/Cas9 technology

- 2015 – Science's Breakthrough of the Year
- Disruptive technology
- Potential Nobel Prize discovery
- Dramatically impacts the speed, rate, efficiency, and flexibility at which you can modify genomes

Image from GEN article "CRISPR-Fast, Easy and Increasingly Accurate" May 1,2014.

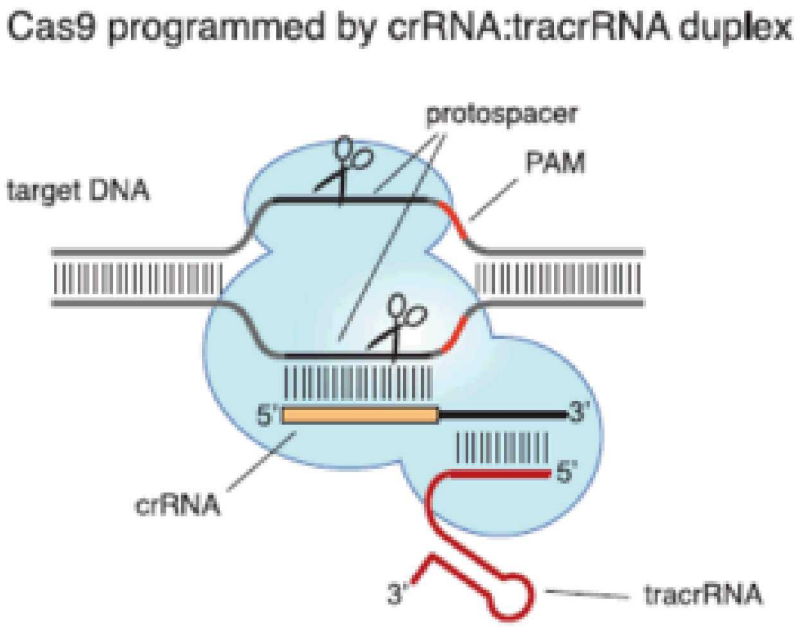
The CRISPR/Cas adaptive immune system

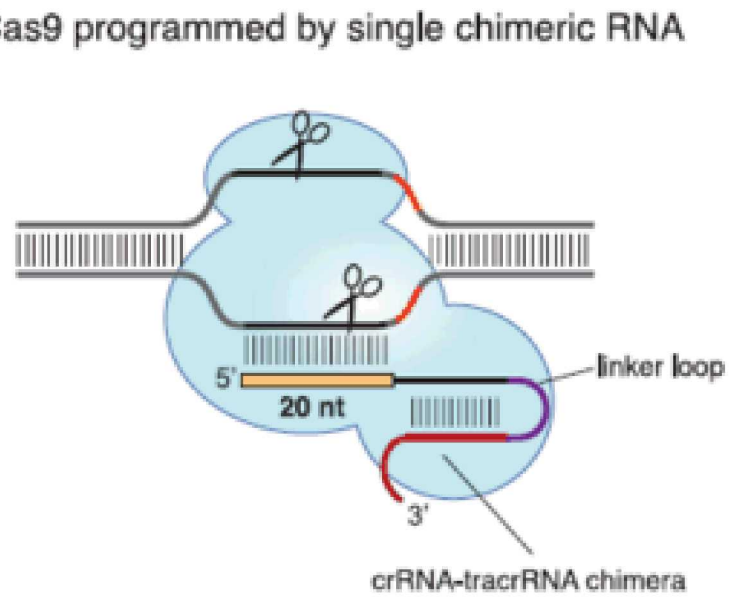


CRISPR: Clustered regularly interspaced short palindromic repeats
Cas: CRISPR-associated systems
Protospacer: DNA sequence targeted by CRISPR/Cas systems
PAM: Protospacer adjacent motif

Image from *Nature Rev. Microbio*. 11:675(2013)

CRISPR technology: A two component system



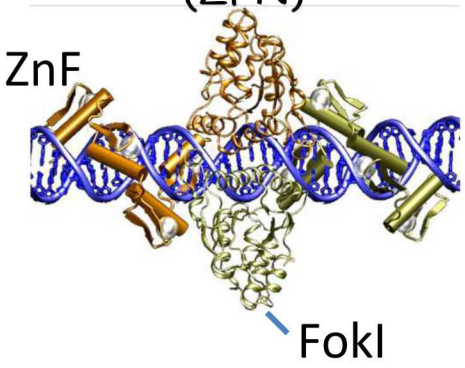


Cas9: RNA-guided endonuclease
gRNA: Synthetic fusion of crRNA and tracrRNA

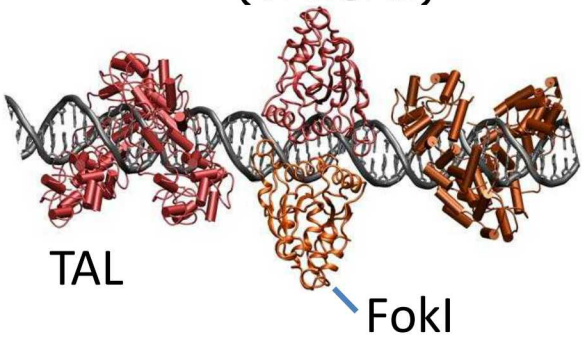
Image from Jinek *et al. Science* (2012)

Genome-editing technologies

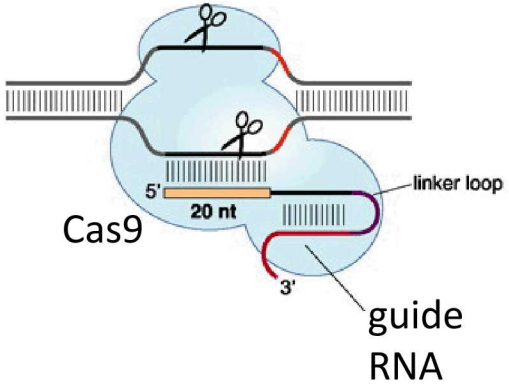
Zn Finger nucleases (ZFN)



TALE nucleases (TALENs)



RNA directed cleavage: Cas9/guide RNA



Code for DNA recognition:

~3 amino acids to 3 bp

~1 amino acid to 1 bp

Watson-Crick bp

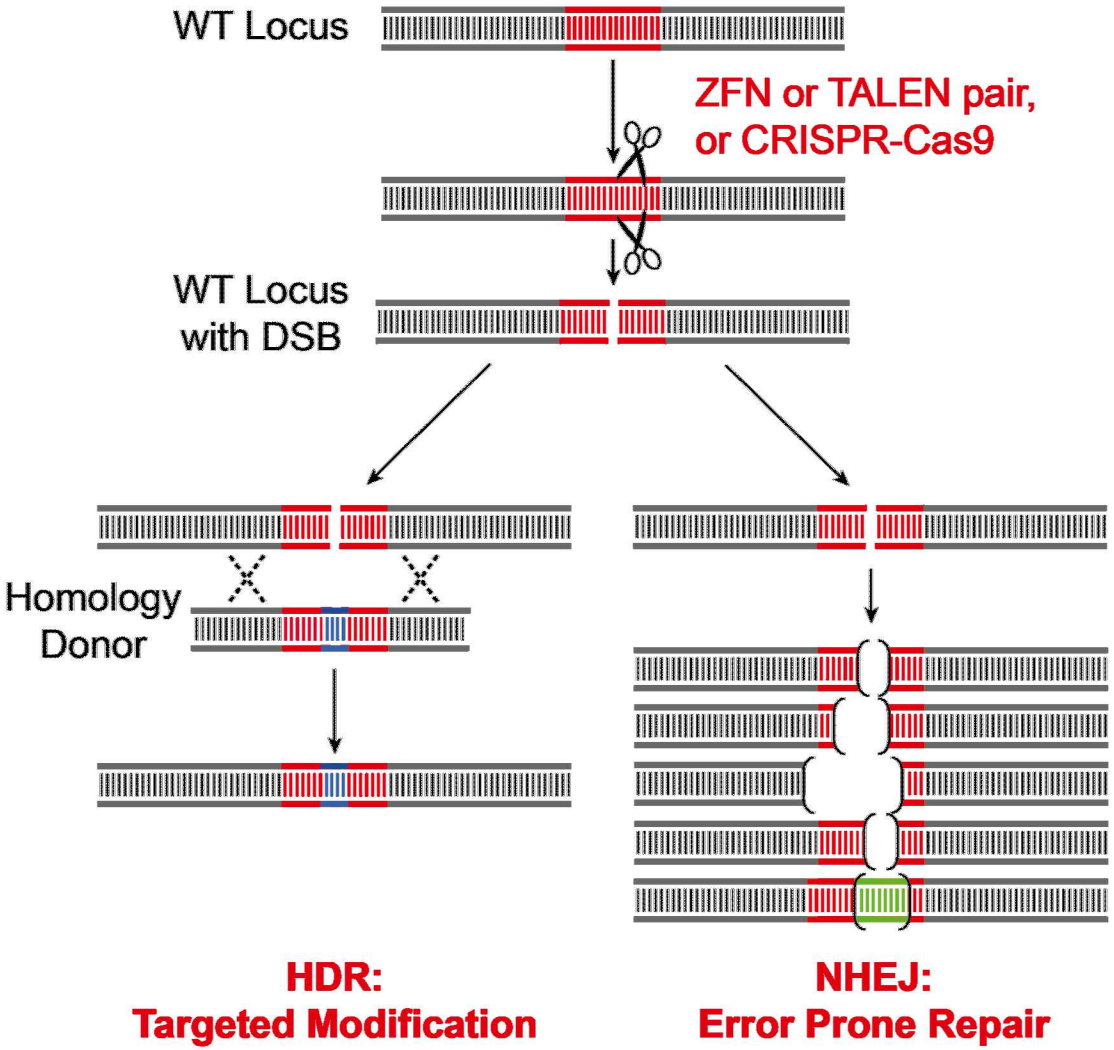
Gene modification:

Modify some genes (2005)

Modify any gene (2009)

Multiple genes (2012)

Genome-editing with sequence-specific nucleases



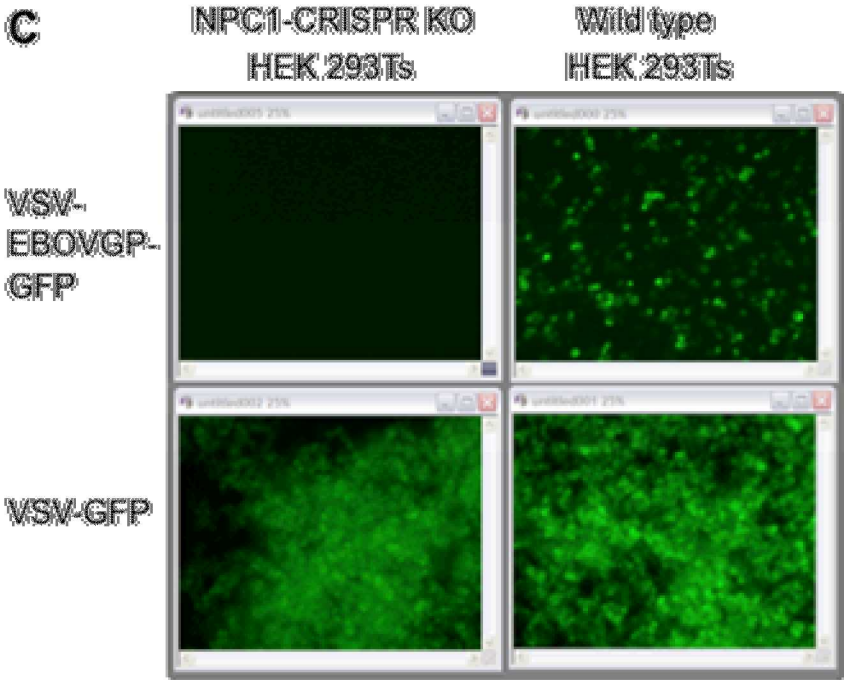
Example: NPC1 knockout cells created using CRISPR are resistant to VSV-EbolaGP infection

Sandia National Laboratories

CRISPR KO experiment

1) Designed 3 gRNA targeting human NPC1 using E-CRISP
2) Cloned gRNAs into pCas9 vector
3) Transiently transfected HEK293Ts cells with constructs
4) Passaged cells for 1 week and then infected with VSV-EBOVGP
5) Passaged resistance cells for 2 weeks post-infection
6) Sequenced resistance cells for NPC1 gene mutation

```
WT      GGGTCACAACCAATCCAGTTGACCTCTGGTCAGCCCCCAGCAGCCAGGCTCG
NPC1-KO GGGTCACAACCAATCCAGTTGAC----------CCCCAGCAGCCAGGCTCG
```

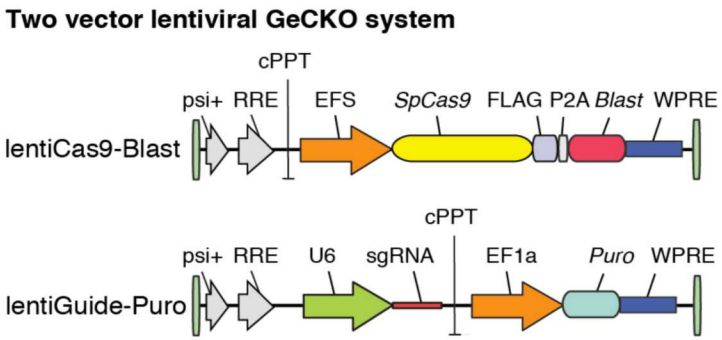


Elucidating virus-host interactions using CRISPR knock-out screens

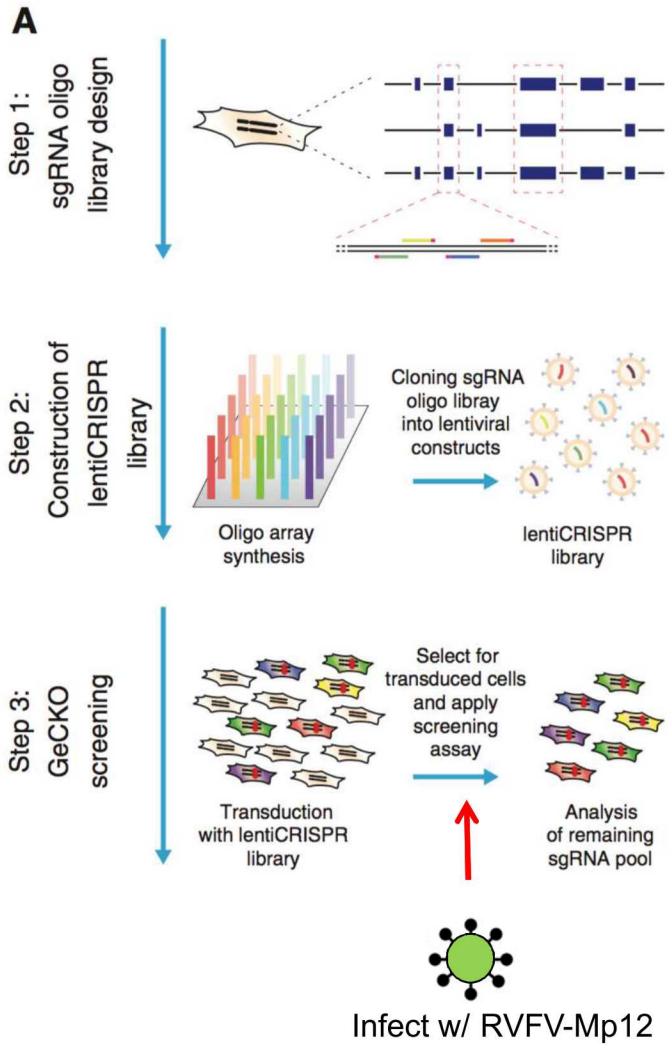
- Similar to functional genomics screening by RNAi, CRISPR knockout (KO) screening can identify host factors involved in virus infection and replication. However, CRISPR screening is a more robust approach for identifying viral-host interactions due to the fact that RNAi gene silencing is typically incomplete and CRISPR can completely suppress gene expression.

- Genome-scale CRISPR KO libraries are inexpensive

    - CRISPR-Cas9 knockout (GeCKO) v2 library consists of 122,417 unique guide sequences targeting 19,052 human genes and including 1000 control (non-targeting) sgRNAs available from Addgene for $500

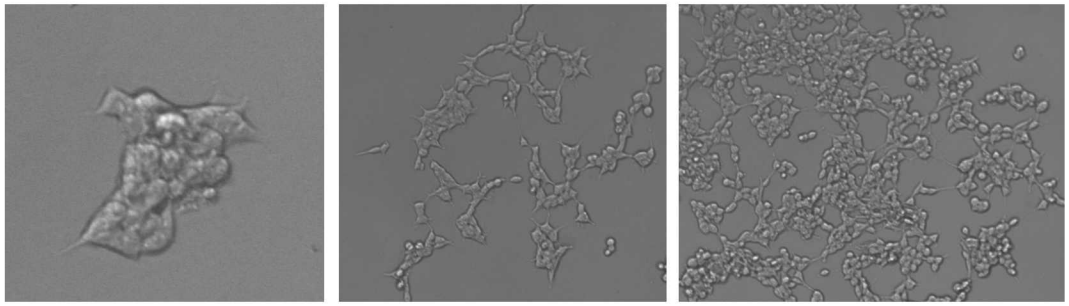


CRISPR KO screening for RVFV-host interactions



Surviving cell colonies resistant to RVFV

~10 ~100 ~1000



Glycosaminoglycans (GAGs) related Hits:

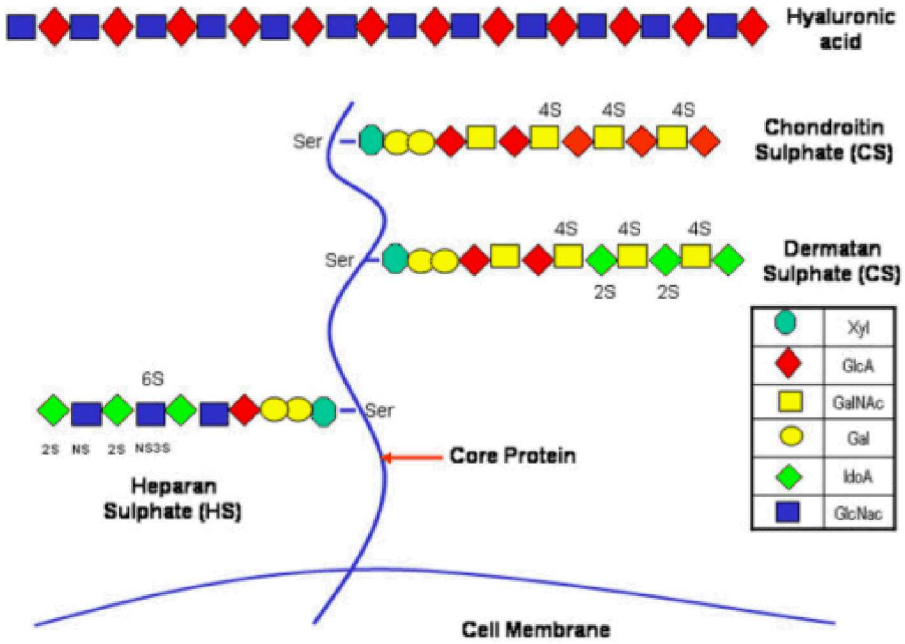
- SLC35B2
- B3GAT3
- EXT2
- B3GALT6

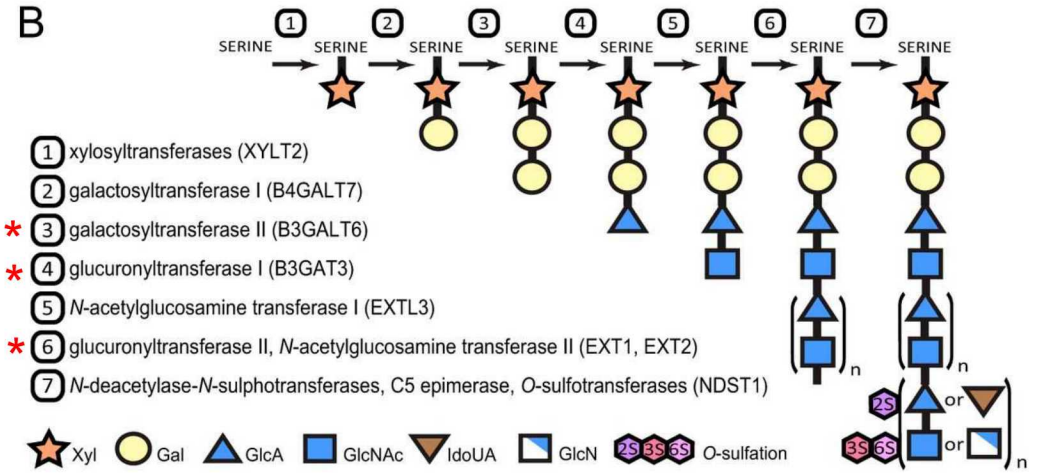
Glycosaminoglycans (GAGs)

GAGs are long unbranched polysaccharides consisting of a repeating disaccharide unit. One or both sugars contain Sulfate Groups (except Hyaluronic acid).

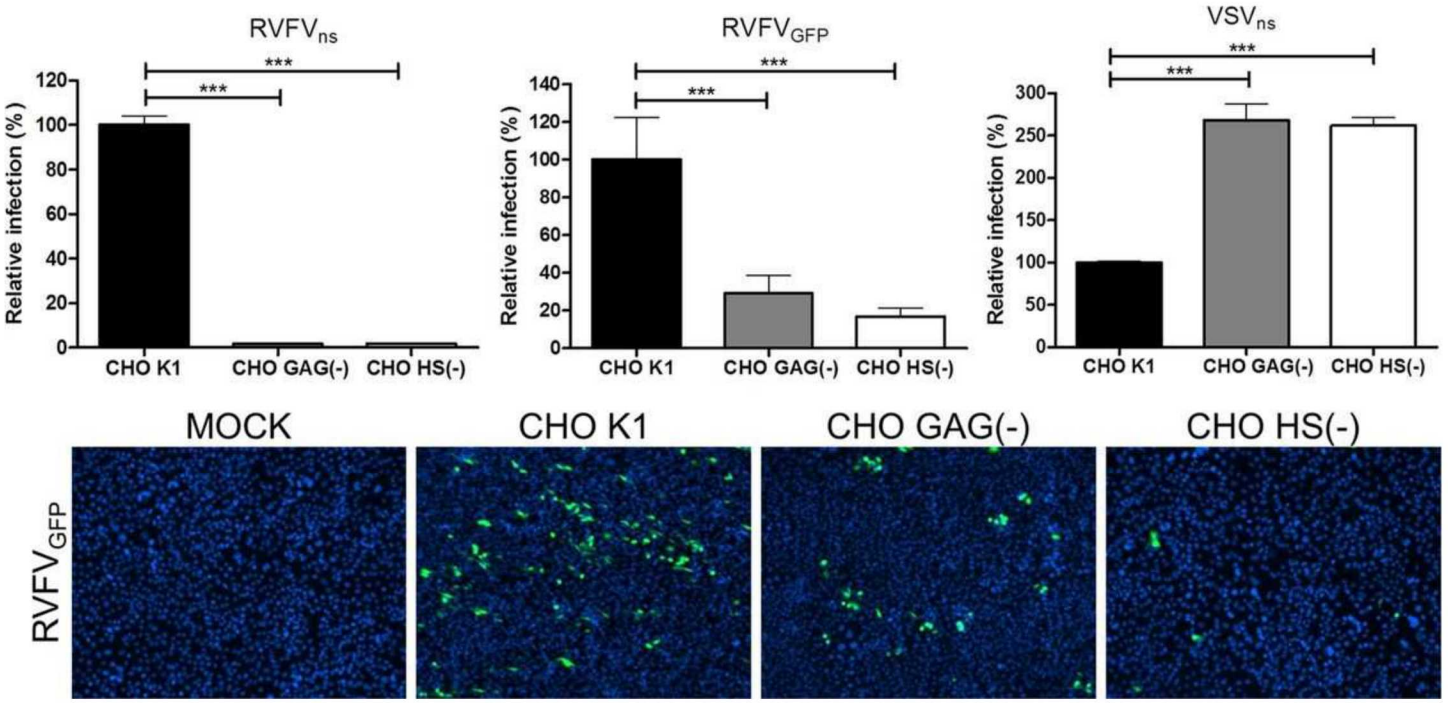
GAGs forming the proteoglycans are the most abundant heteropolysaccharides in the body.

Serve as attachment factors for several viruses including HIV, Dengue virus and Adenovirus.



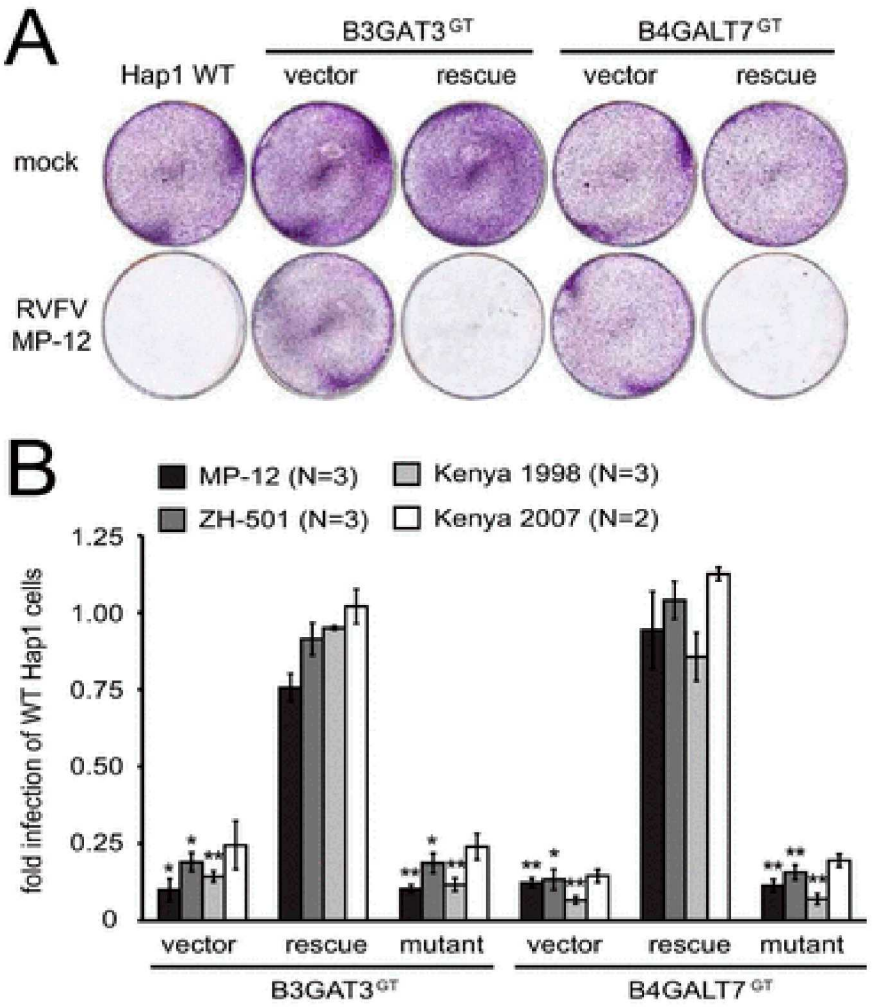


Entry of RVFV into GAG-deficient CHO cells is inefficient due to the lack of heparan sulfate



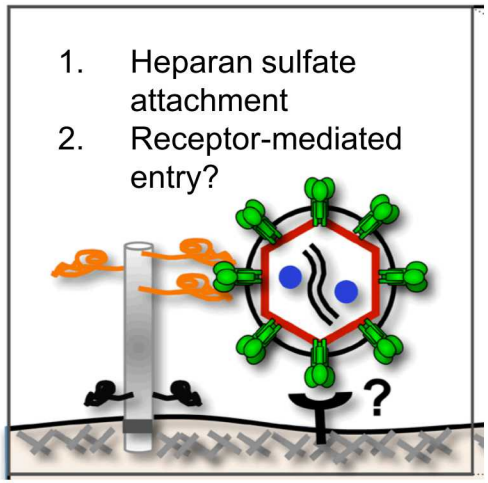
S. M. de Boer et al. J. Virol. 2012;86:13767-13771

GAGs are important for RVFV infection



Amber M. Riblett et al. J. Virol. 2016;90:1414-1423

Other entry factors?



- Some viruses acquire HS binding due to repetitive passaging in cell culture, however, sequence analysis of low passage strains conclude HS requirement is not likely the result of cell culture adaptation.
- There is residual infection in cells deficient in GAG and HS
- Unidentified attachment factor or receptor involved in RVFV entry is still a possibility

Acknowledgements

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Brooke Harmon (RNAi screening, RVFV Entry, Wnt)

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