FOURTEENTH INTERNATIONAL

ROTAVIRUS SYMPOSIUM

MARCH 14–16 **2023** BALI INDONESIA

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Induction of systemic and mucosal neutralizing antibodies to norovirus and rotavirus after oral administration of a live recombinant rotavirus to suckling mice

14th International Rotavirus Symposia, March 14-16, 2023
Bali, Indonesia

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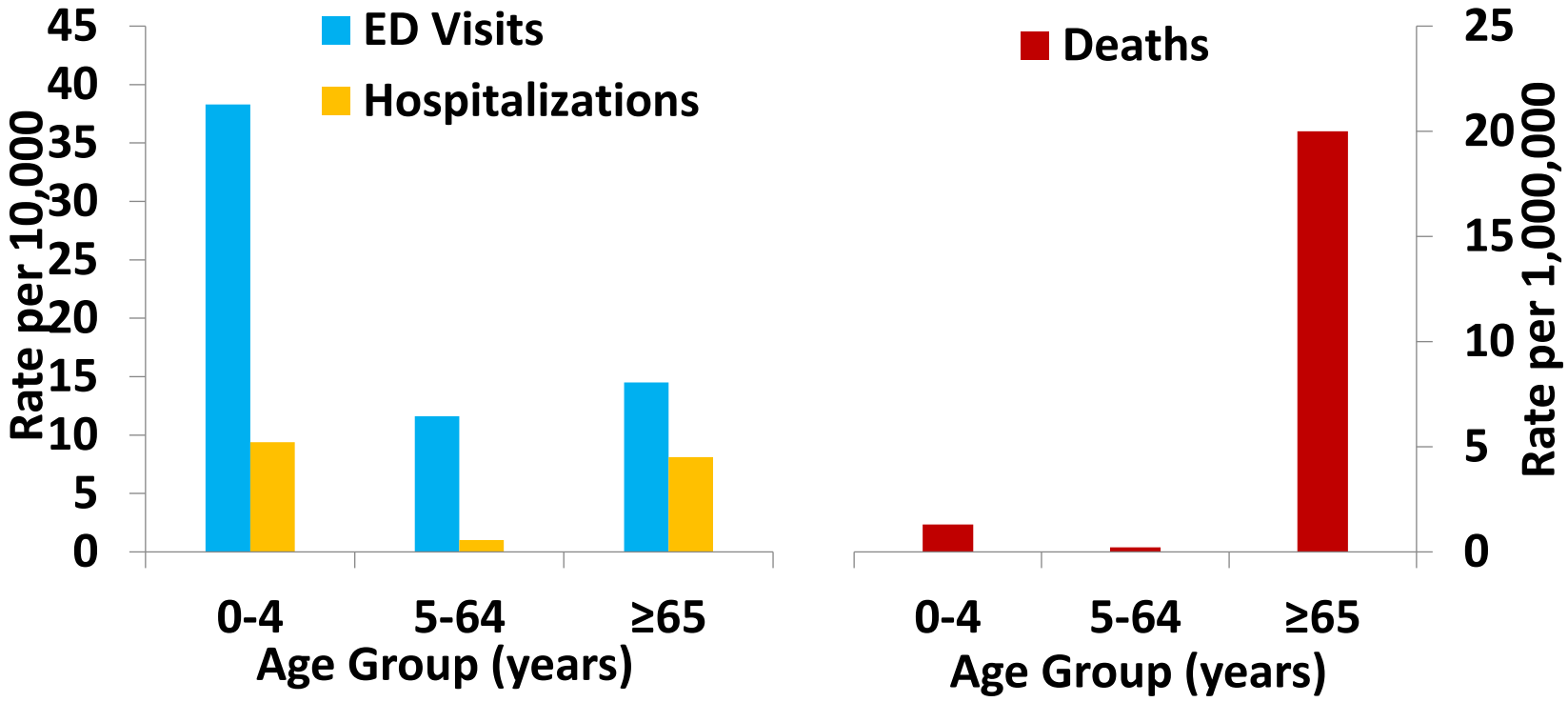
Two major gastroenteritis viruses (Rotavirus and Norovirus)

Rotavirus: two widely licensed vaccines (Rotarix and RotaTeq) now available. Several other approved RV vaccines available on a national/limited number of country basis.

Norovirus: Neither effective vaccines nor antivirals are currently available



Hall, AJ, et al, EID, 2013

Norovirus causes severe disease in *both* young children and the elderly

What was the purpose and rationale for this set of experiments?

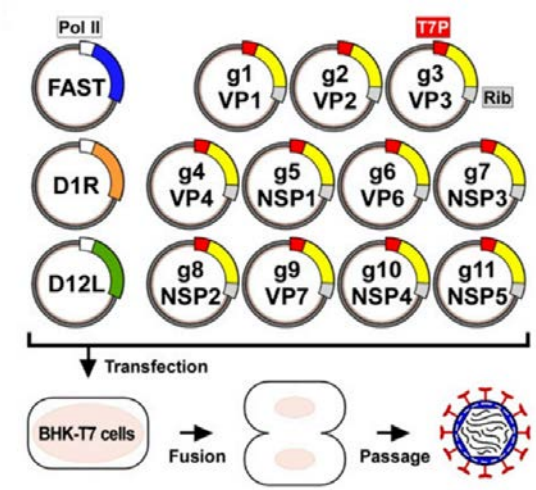
To undertake "Proof of Principle" studies to explore the feasibility of expressing selected Norovirus (NV) structural protein(s) in the small intestine of a test animal using a replication competent RV as a combination NV/RV enteric vaccine and then to assess the ability of such a construct to induce antibody responses to RV, NV, or both

1) RV vaccines have well-established **efficacy** and **safety records** and are in use worldwide.
2) Current RV vaccines, which are delivered orally, are generally thought to work by stimulating neutralizing antibodies, preferentially in the small intestine.
3) There are several animal models available to study the generation of immunity to a vectored NV antigen by orally delivery RV vaccines (infant mouse and suckling pigs being frequently studied).
4) Why use the Rhesus Rotavirus (RRV)? Because RRV has been studied extensively in various animal models; it was previously approved as a licensed human RV vaccine, RRV replicates well in cell culture, and is not the "property" of a commercial entity.

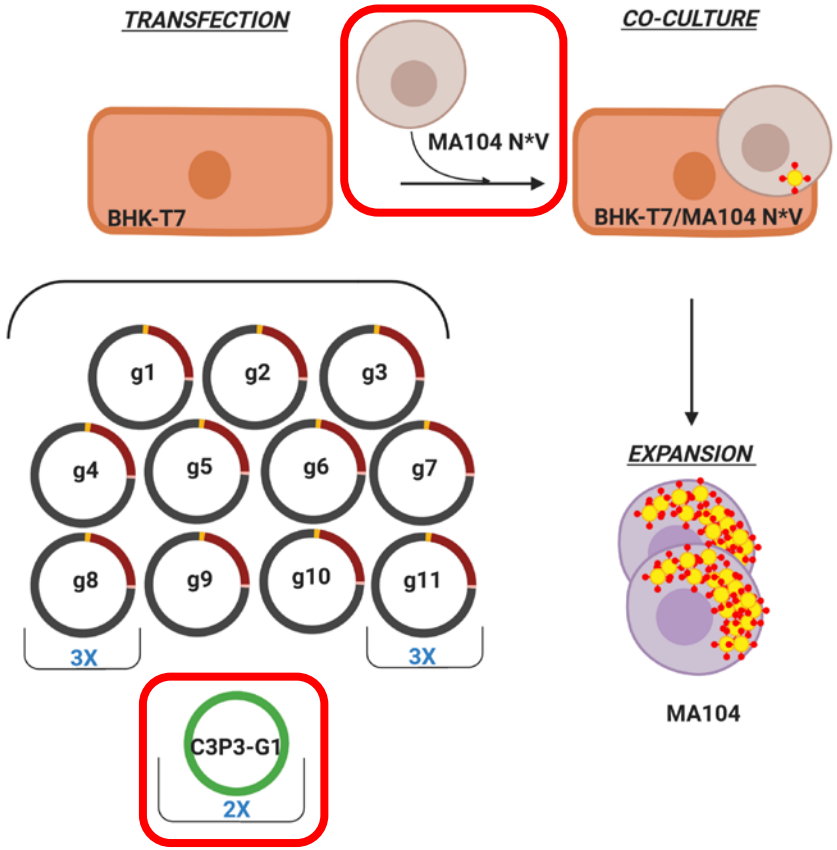
We used an enhanced reverse genetics (RG) system to determine if RRV could express Norovirus VP1 and P proteins and induce systemic and local antibodies *in vitro/ in vivo*

Original RG method

Kanai Y; Kobayashi T *et al* (2017) PNAS



Enhanced RG method



***KEY ENHANCEMENTS*:**

i) **Helper chimeric plasmid: C3P3-G1**
Capping enzyme NP868R of African swine fever virus (ASFV) + T7 RNA polymerase

ii) **IRF3 and STAT1 defective MA104: MA104 N*V cells**
Constitutively expressing: 1) Parainfluenza virus 5 (PIV5) V protein-> STAT1
2) Bovine viral diarrhea virus (BVDV) N protein-> IRF3

Sanchez-Tacuba L *et al* (2020) J Virol

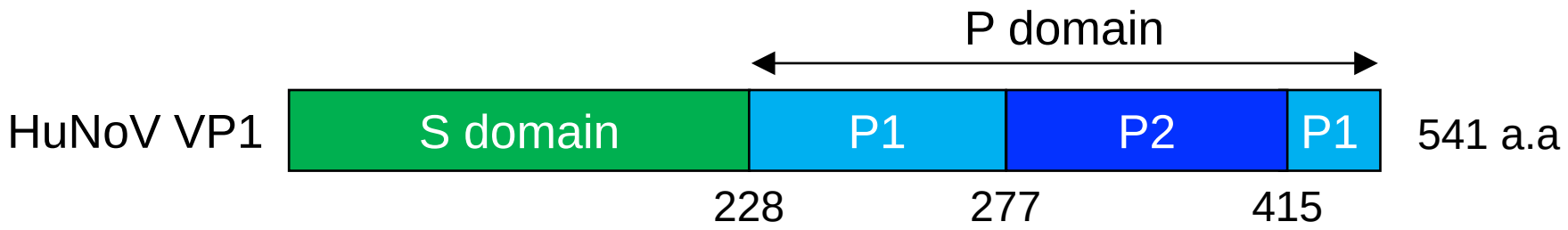
Two human Norovirus (HuNoV) proteins were selected to examine their suitability to induce systemic/ mucosal Abs to HuNoV using the RRV as the enteric expression vector

1. HuNoV VP1 (~58 kDa) can form **virus-like particle** (VLPs)

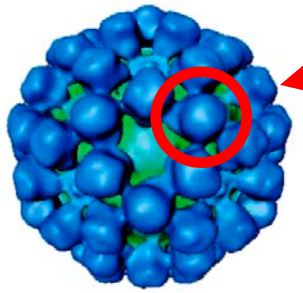
   VLPs have multiple P2 surface subdomains and these are likely targets of protective antibodies

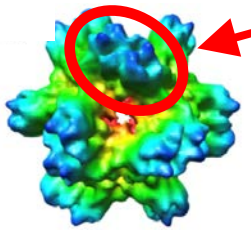
2. P domain is ~36kDa (only 62% of VP1) and can also form multimeric “P” particles

   “P” particles have surface P2 subdomains and are antigenically related to HuNoV VLPs



VLP (VP1) **P particle (P domain)**





P2 subdomain; facilitates norovirus interaction with host cells and is a likely target of protective antibodies

Prasad BV et al (1999) Science

Tan M et al (2008) Virology

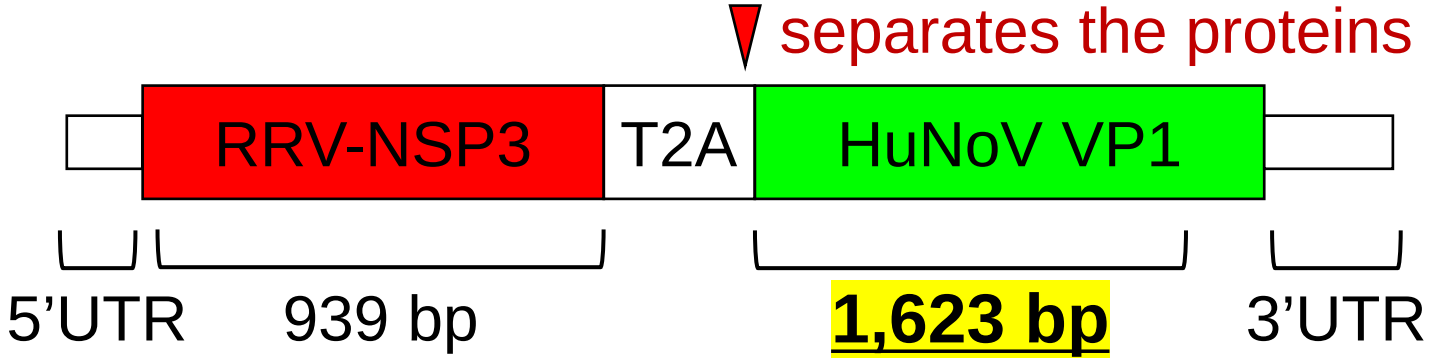
Constructions for expressing either HuNoV VP1 or P particle encoding sequences linked to the RRV NSP3 gene

Original RV NSP3 gene

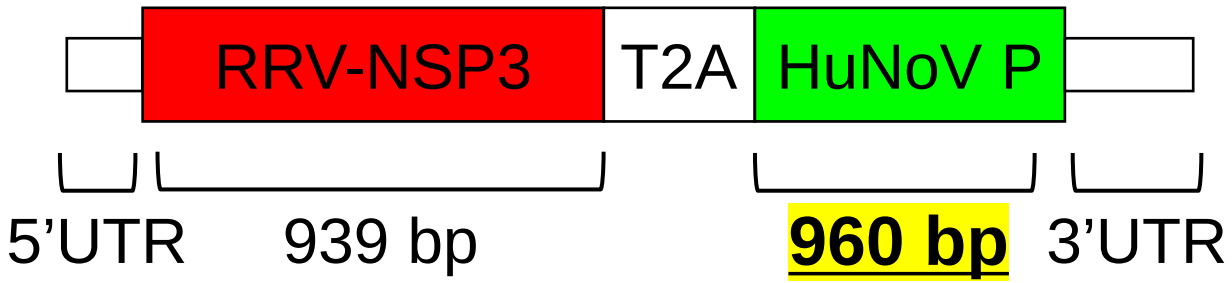
insert exogenous gene

Kanai Y et al (2017) PNAS and Philip AA et al (2019) J Virol

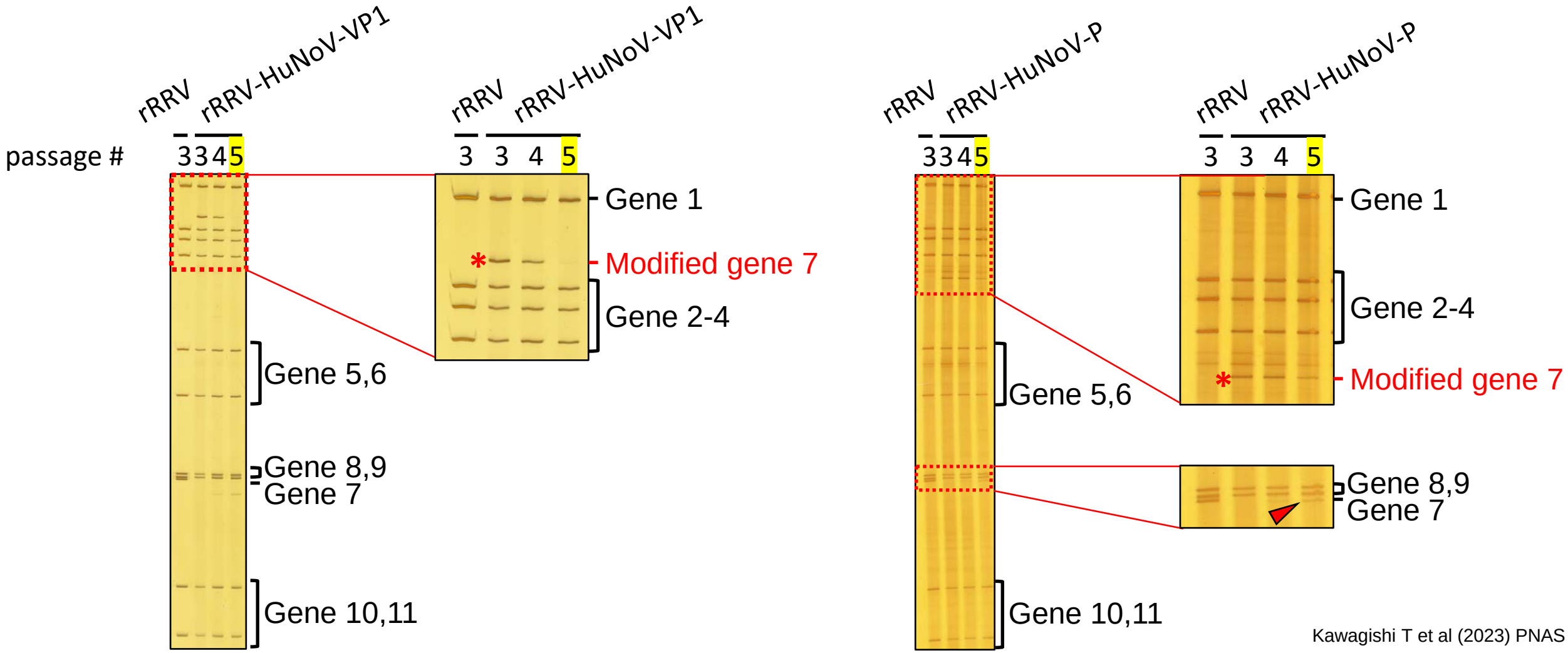
Modified RV gene7
with HuNoV VP1 insert



Modified RV gene7
with HuNoV P domain insert



RNA-PAGE analysis of rRRV expressing HuNoV-VP1 or -P

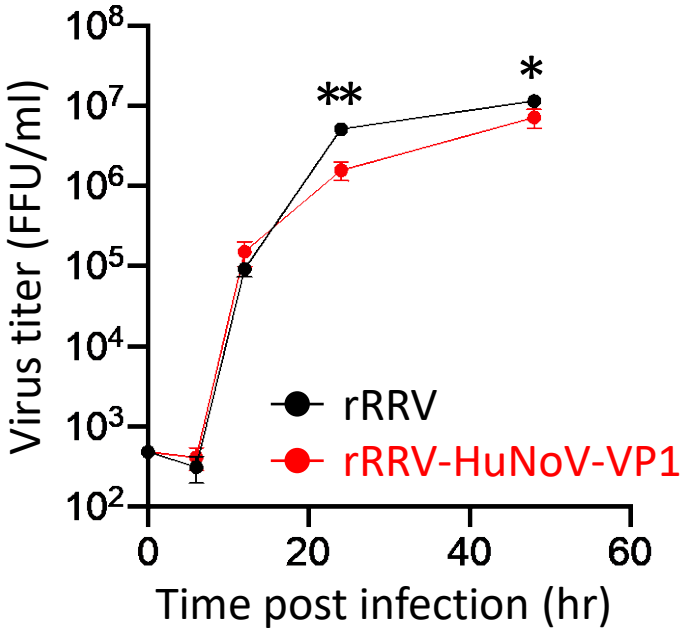


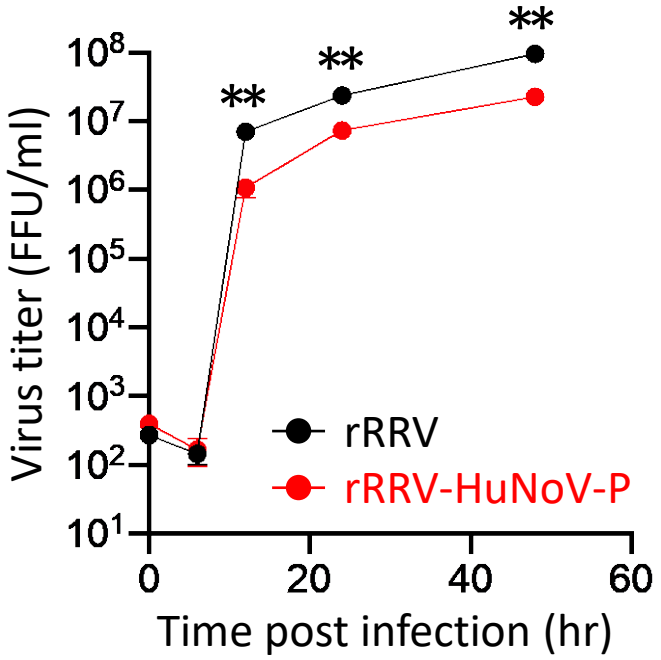
Kawagishi T et al (2023) PNAS

- **The rescued viruses, rRRV-HuNoV-VP1 and rRRV-HuNoV-P, both harbored modified gene 7s.**
- **rRRV-HuNoV-VP1 lost the modified gene 7 in passage five.**
- **rRRV-HuNoV-P harbored the modified gene 7 in passage 5 but also began to show a wild-type gene 7 band in passage 5.**

→Therefore, we used passage 3 stock in the following immunization experiment.

Viral replication of rRRVs expressing either HuNoV-VP1 or –P in cultured cells





MOI 0.01 FFU/cell (MA104 cell)

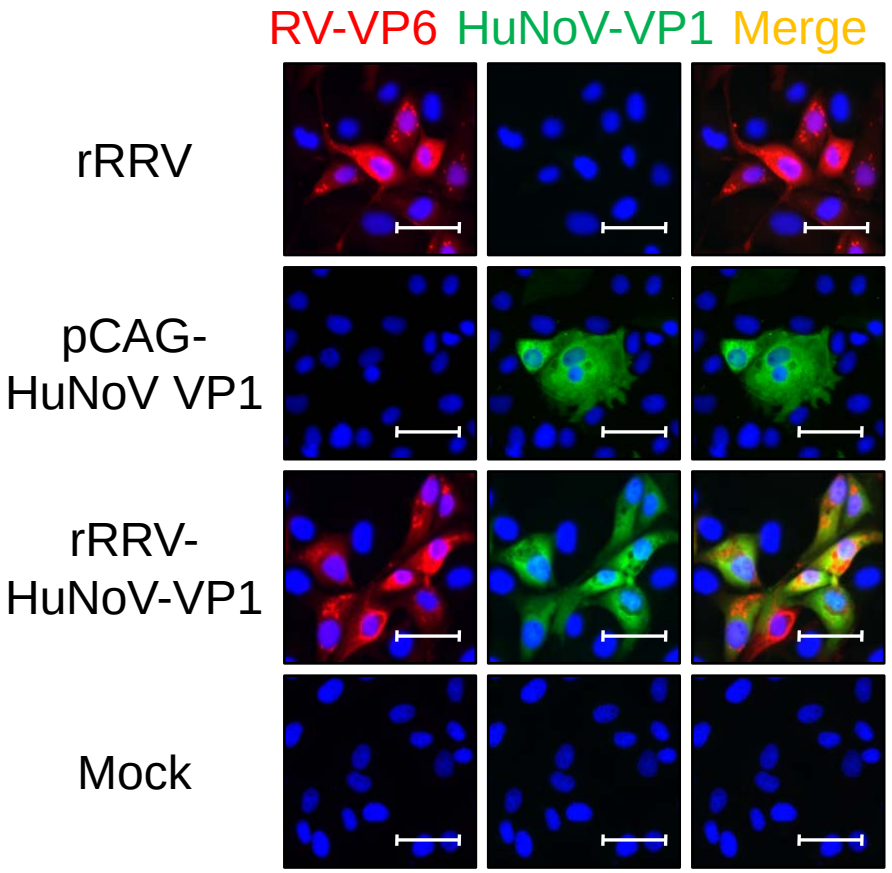
Kawagishi T et al (2023) PNAS

The 2 rescued vaccine candidates (rRRV-NoV-VP1 & rRRV-HuNoV-P) replicated well but significantly less well than their parental counterpart (RRV) in MA104 cells.

rRRV expression of HuNoV VP1 or P domain in MA104 cells

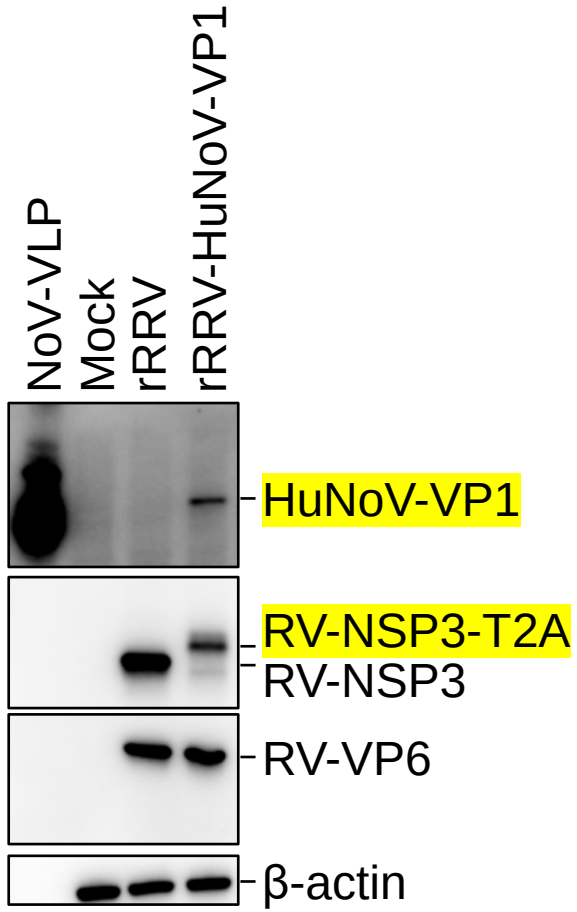
rRRV-HuNoV-VP1 infected cells

Immunostain with anti-



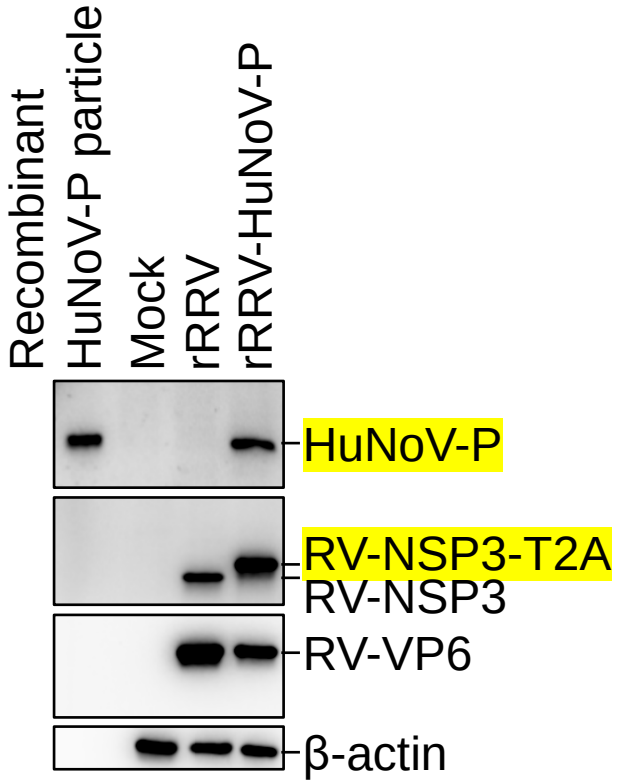
The scale bars show 50μm.

Western blot



rRRV-HuNoV-P infected cells

Western blot

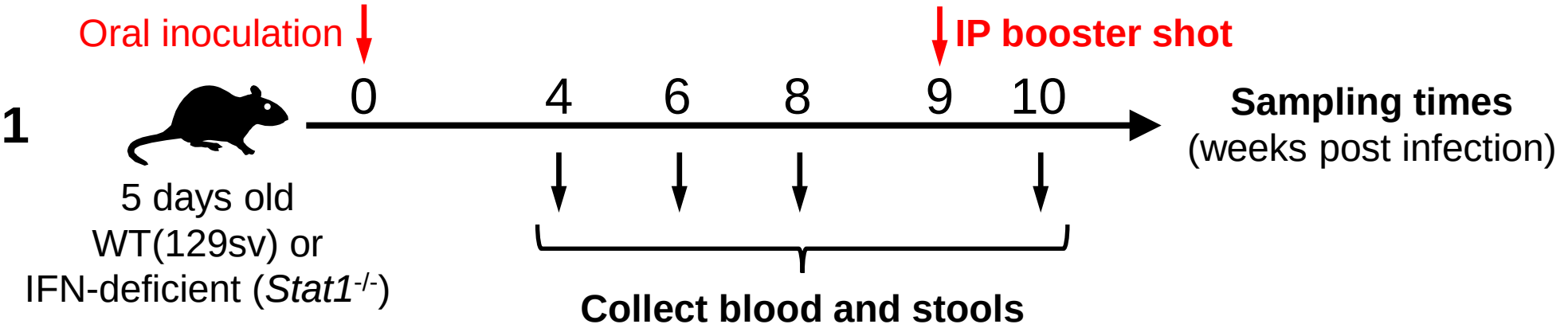


rRRV can express both the HuNoV-VP1 or P protein in infected MA104 cells

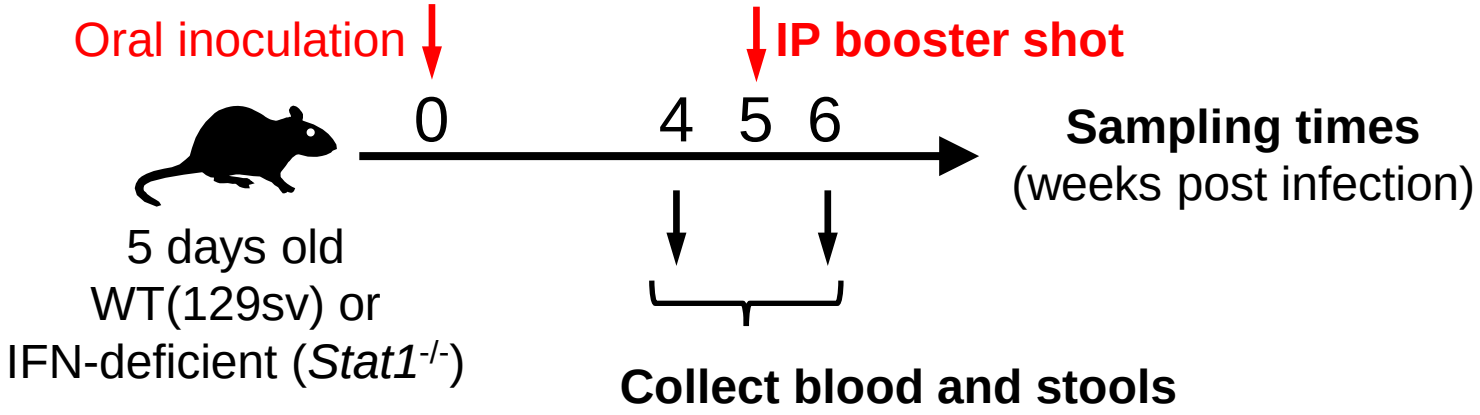
Approximately 99% of cells expressing RV VP6 also expressed NV VP1 Kawagishi T et al (2023) PNAS

Immunization schemes for the suckling mice infected with rRRV expressing either HuNoV VP1 or P proteins

rRRV-HuNoV-VP1



rRRV-HuNoV-P



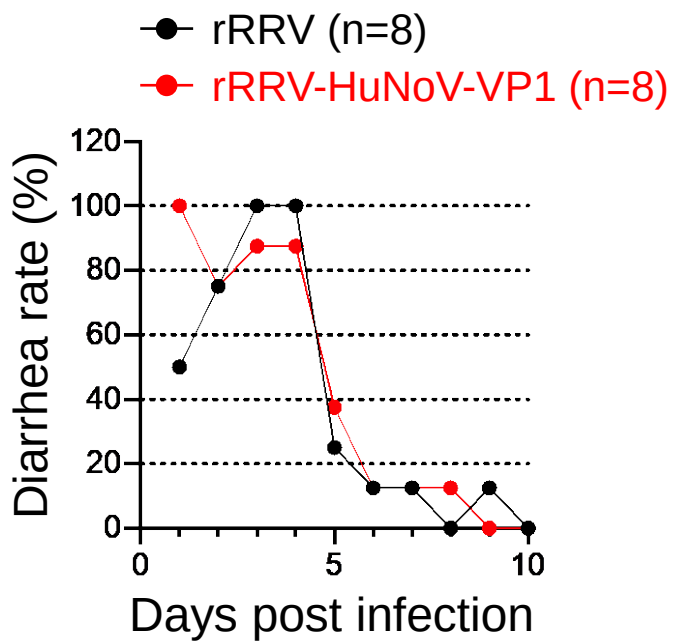
Serum & stool samples tested by immunostaining, ELISA, and neut. assays to detect IgG & IgA binding and neutralizing Abs against HuNoV and RV

Rationale for using both 129 (wt) and Stat 1 KO mice and a parenteral booster immunization for the *in vivo* "proof of principle" RV immunization studies

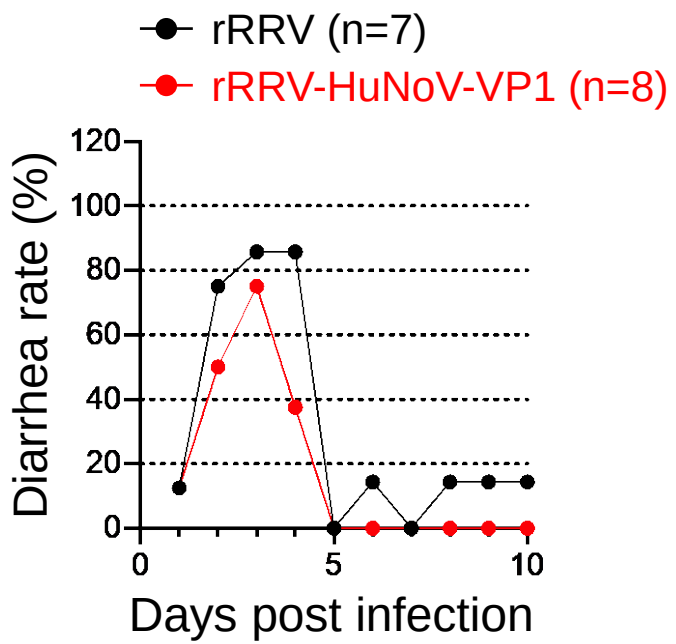
- Prior studies demonstrated that RRV replicates very poorly in suckling 129 & other mouse strains. It replicates better in several other heterologous species such as rats and humans.
- Prior studies showed that in murine strains with KO'd IFN responses (Stat 1& various IFNR KOs) RRV and other heterologous RV strains replicated more efficiently.
- Multiple immunization studies in various animal species demonstrated that parenteral boosting following mucosal priming enhances systemic/local? immune responses.
- This initial immunization study was designed to maximize chances for observing a positive neutralizing response in infant mice with the hope that if such studies proved positive, future studies in more RV permissive hosts (rats/pigs), using a more acceptable RV construct (a human RV vaccine), might be justifiable and further pursued.

Diarrheal responses following oral administration of rRRV-HuNoV-VP1 to wild type or Stat1 KO suckling mice

129sv (immunocompetent)



***Stat1*$^{-/-}$ (immunodeficient)**



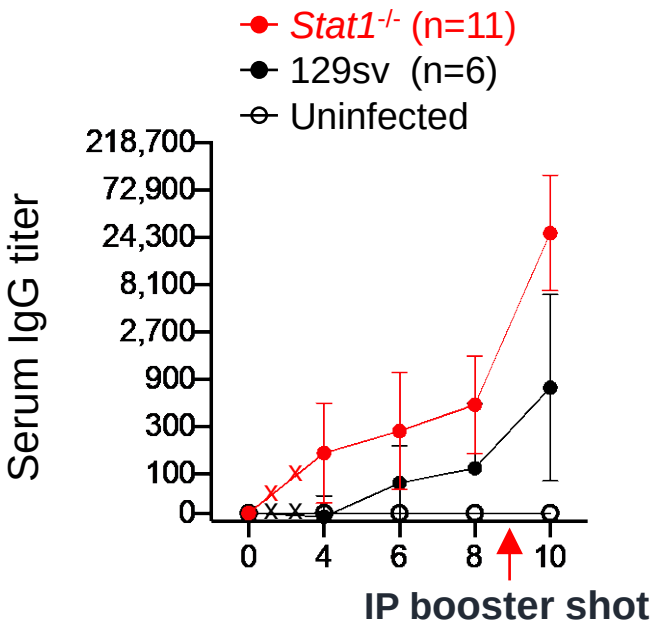
rRRV & RRV-HuNoV-VP1 at 4x10^{5} ^{FFU} induced similar rates and durations of diarrhea in both 129sv and *Stat1*$^{-/-}$ pups

Kawagishi T et al (2023) PNAS

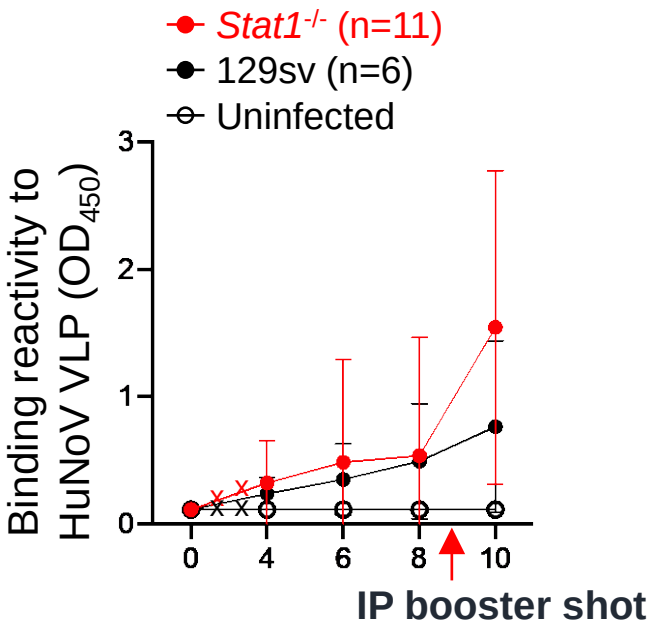
Serum and fecal ELISA responses to HuNoV VLPs or recombinant P particles following rRRV-HuNoV-VP1 or rRRV-HuNoV-P oral administration

rRRV-HuNoV-VP1 immunized mice

Serum IgG to HuNoV VLP

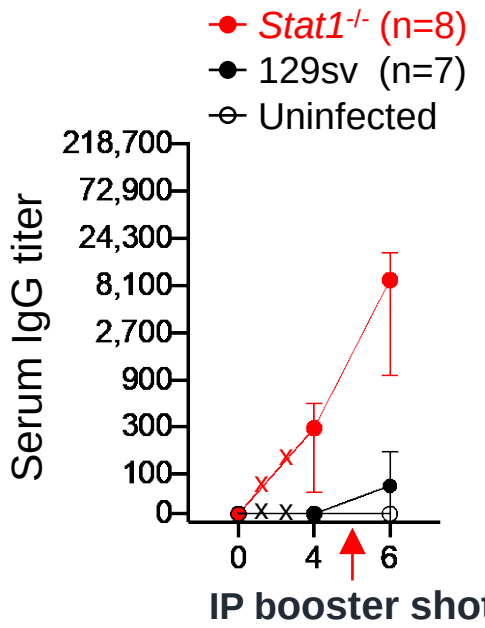


Fecal IgA to HuNoV VLP

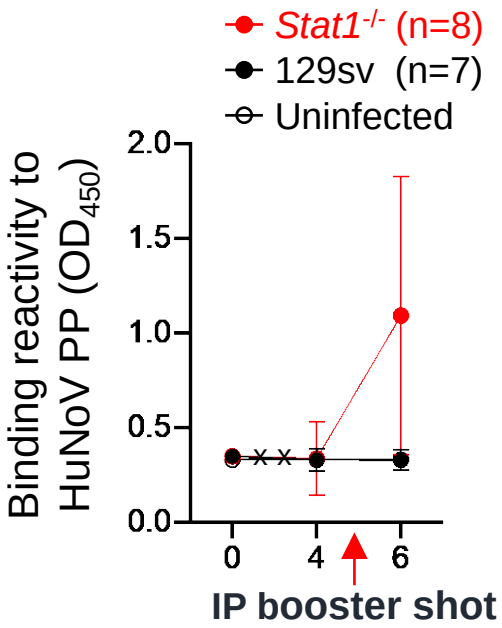


rRRV-HuNoV-P immunized mice

Serum IgG to HuNoV PP



Fecal IgA to HuNoV PP



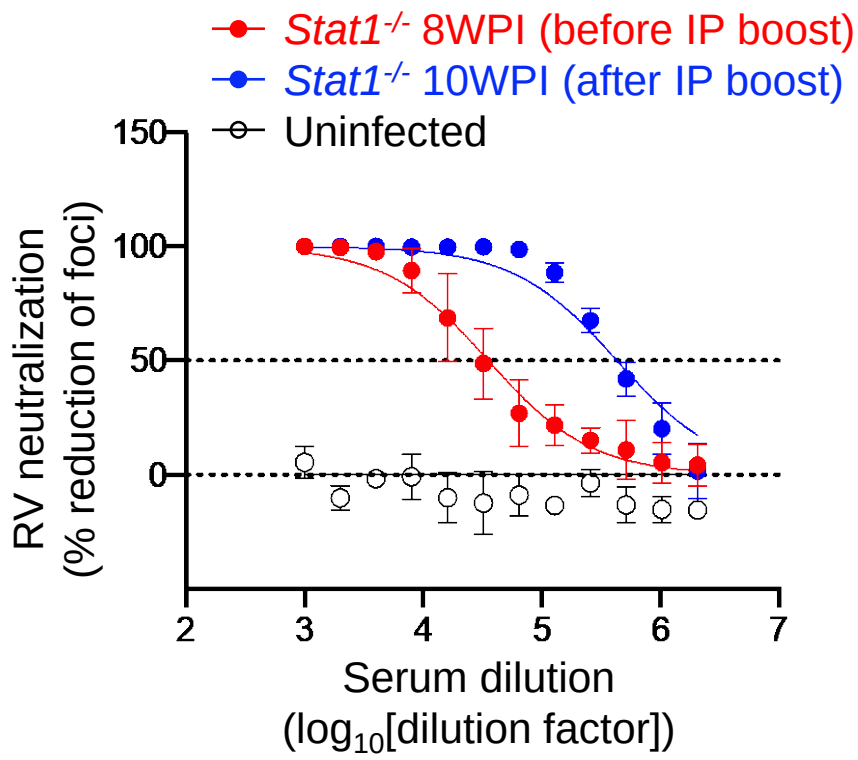
Note: time 0 antibody levels were derived from separate studies

Kawagishi T et al (2023) PNAS

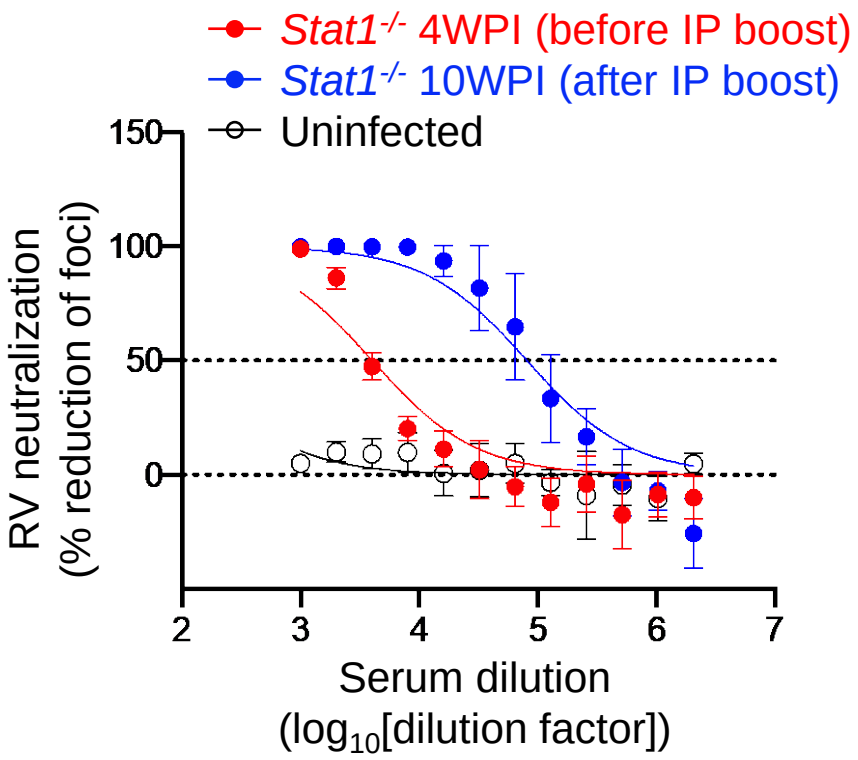
- **Serum IgG and fecal IgA vs. HuNoV VLPs or P particles increased after rRRV-HuNoV-VP1 or rRRV-HuNoV-P inoculation (wk 4-8 or wk 4) and were enhanced after intraperitoneal (IP) boosting.**
- **Systemic & local Ab responses to NV/ HuNoV-P in *Stat1*$^{-/-}$ mice are greater than in 129sv mice.**

Representative serum neutralizing responses against RRV in mice orally immunized with rRRV expressing either HuNoV VP1 or P particle

rRRV-HuNoV-VP1 immunized mice sera



rRRV-HuNoV-P immunized mice sera



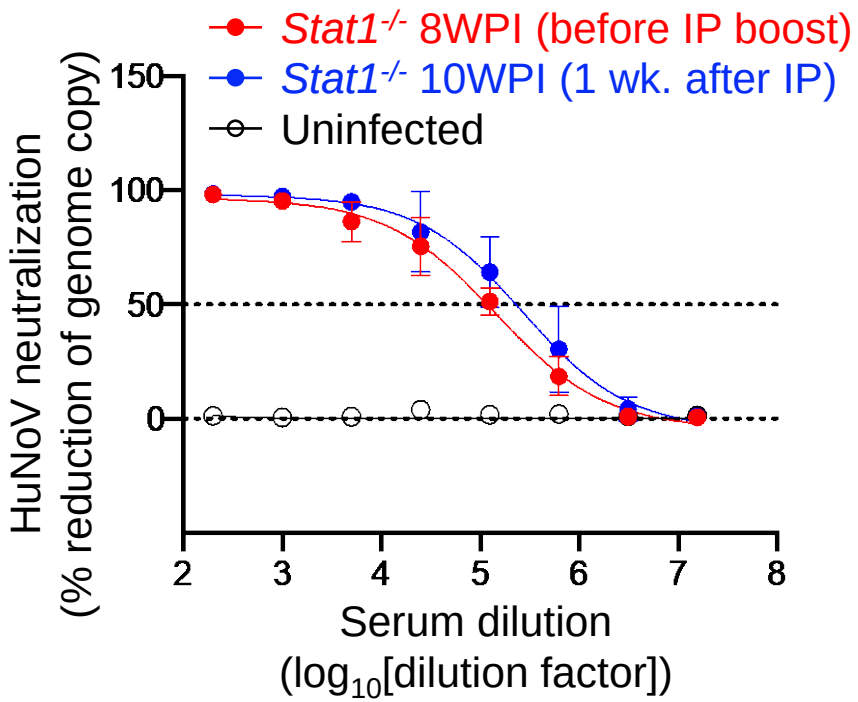
Kawagishi T et al (2023) PNAS

rRRV-HuNoV-VP1 and rRRV-HuNoV-P induced serum neutralizing antibodies against RV in immunized mice. IP boost substantially increased titers

Representative serum neutralization/blocking responses to HuNoV or PP in mice orally immunized with rRRV expressing either HuNoV VP1 or PP

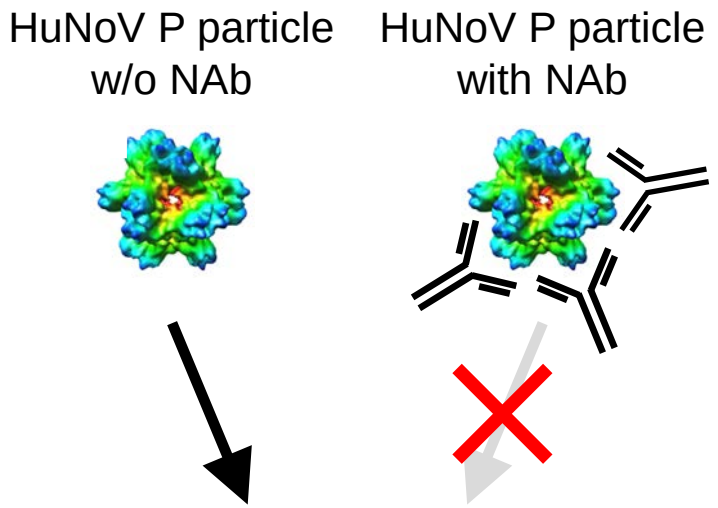
HuNoV neutralization in HIOs with sera from rRRV-HuNoV-VP1 immunized mice

In collaboration with Dr. Jan Vinje

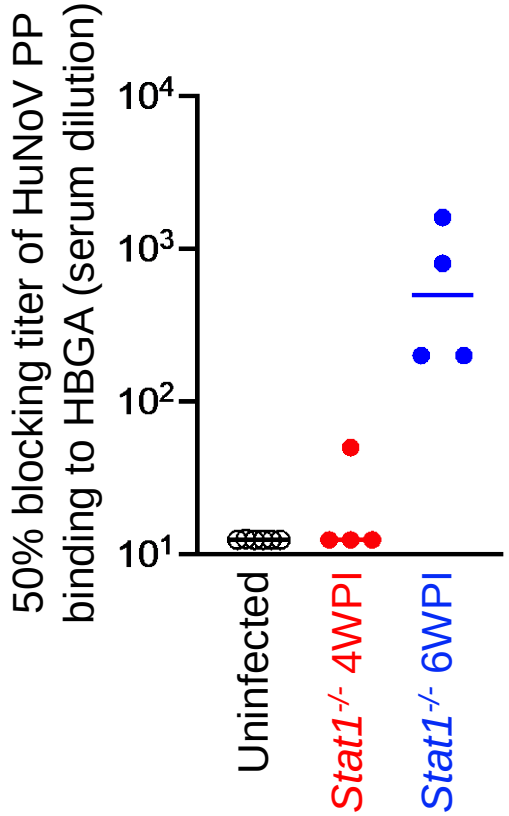


Blockade of HuNoV P particle binding to HBGA by sera from rRRV-HuNoV-P immunized mice

In collaboration with Dr. Ming Tan



Ming T et al (2008) Virology

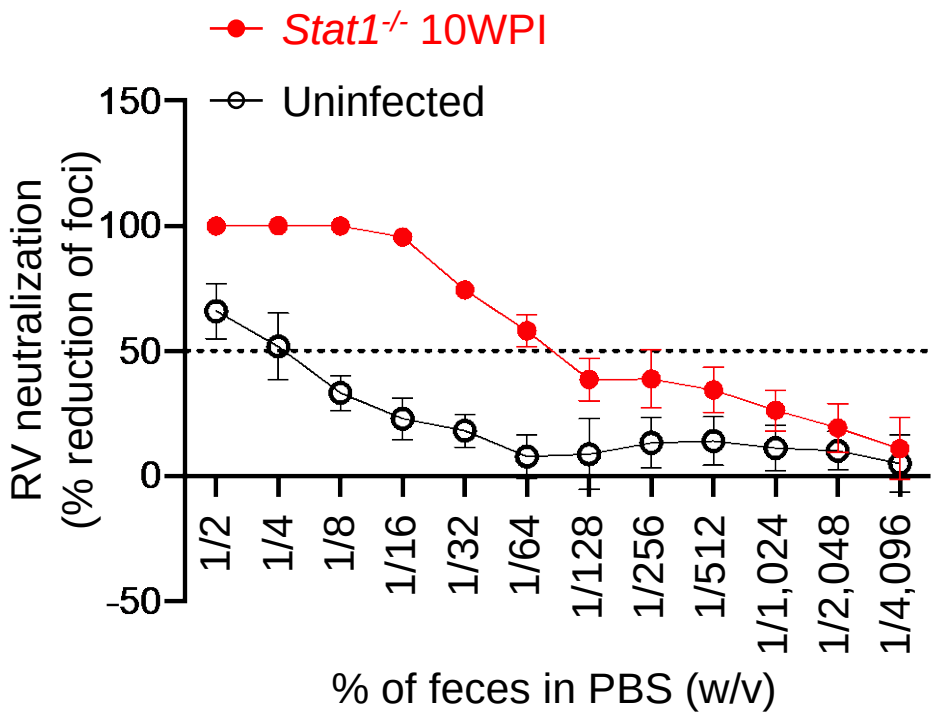


rRRV-HuNoV-VP1 and rRRV-HuNoV-P induced serum neutralizing/binding-blocking antibodies against both HuNoV and HuNoV PP in immunized mice.

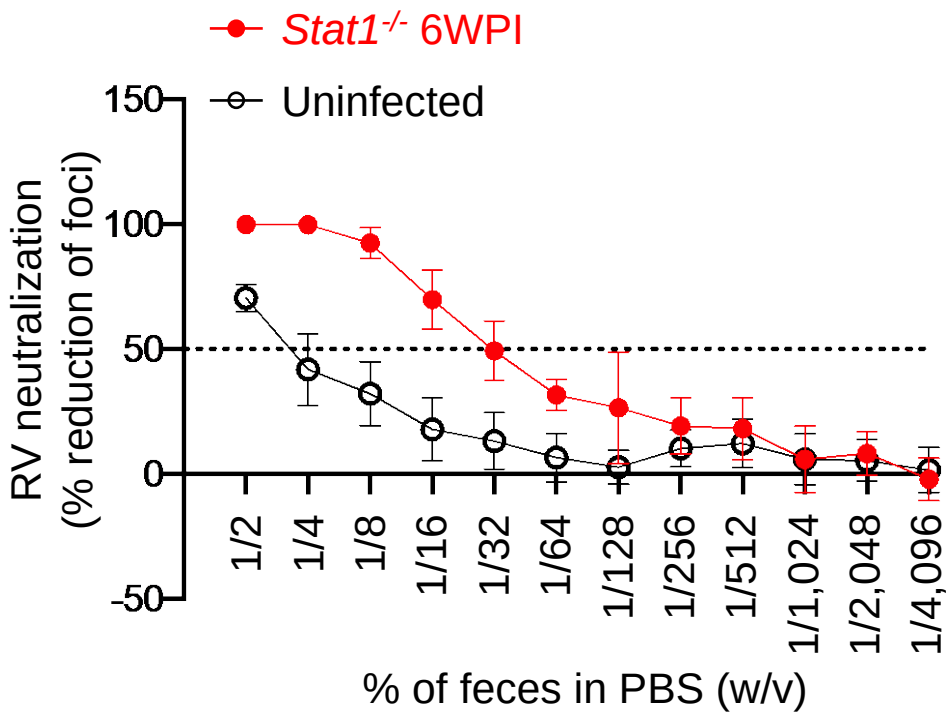
Kawagishi T et al (2023) PNAS

Representative fecal suspension neutralizing responses against RRV in mice orally immunized with rRRV expressing either HuNoV VP1 or P particle

rRRV-HuNoV-VP1 immunized mice fecal suspension



rRRV-HuNoV-P immunized mice fecal suspension

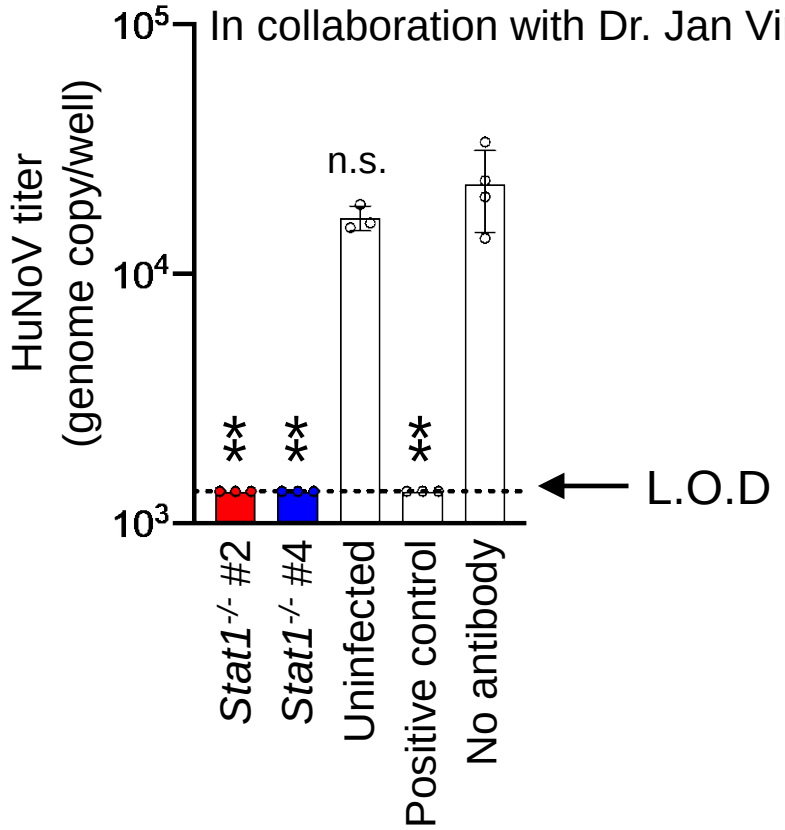


Kawagishi T et al (2023) PNAS

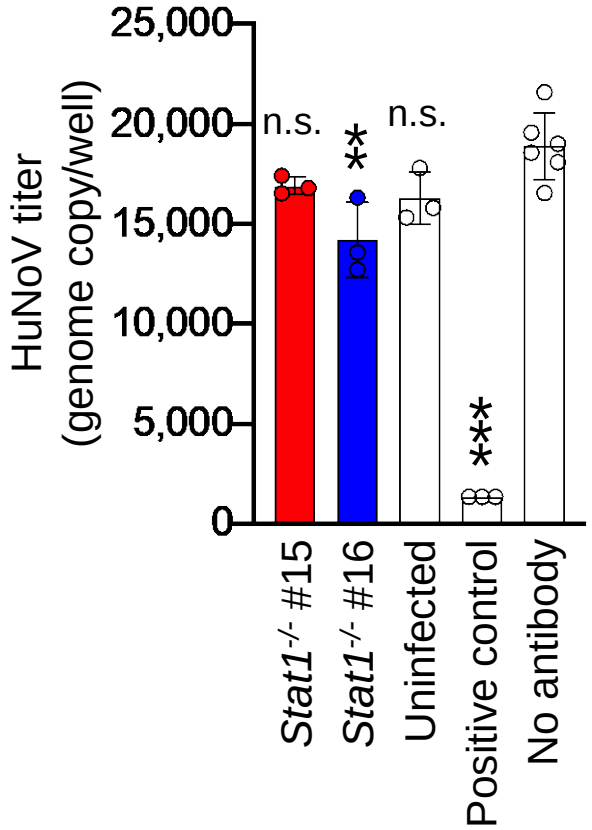
rRRV-HuNoV-VP1 and rRRV-HuNoV-P induced RRV neutralizing antibodies in the feces of indicated immunized mice.

Representative fecal neutralization responses post boost versus HuNoV in HIOs from mice orally immunized with rRRV expressing either HuNoV VP1 or PP

HuNoV (Sydney strain) neutralization in HIOs with 1% (w/v) fecal suspension from rRRV-HuNoV-VP1 immunized mice



HuNoV (Sydney strain) neutralization in HIOs with 1% (w/v) fecal suspension from rRRV-HuNoV-P immunized mice



Kawagishi T et al (2023) PNAS

*Note: HuNoV-P gene was cloned from **VA387 strain.***

- **rRRV-HuNoV-VP1 induced enteric neutralizing antibodies against HuNoV in immunized mice.**
- **Fecal suspensions from rRRV-HuNoV-P inoculated mice neutralized HuNoV Sydney strain very poorly.**

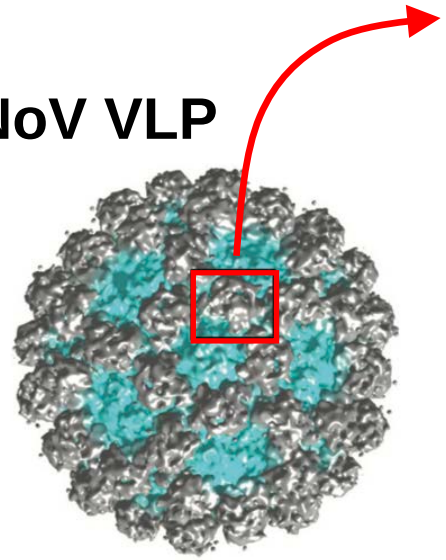
Possible cause for poor neutralization of HuNoV Sydney strain by feces from mice immunized with rRRV-HuNoV-P (VA387 strain)

HuNoV P2 dimer

Epitope A

Epitope D

HuNoV VLP



Epitope E

Debbink K et al (2012) J Infect Dis

Amino acid difference in blockade epitopes between VA387 and Sydney strains

Epitope A	294	296	297	298	368	372
VA387	A	S	H	D	T	N
Sydney	T	S	R	N	E	D

Epitope D	393	394	395
VA387	N	N	-
Sydney	G	T	T

Epitope E	407	412	413
VA387	N	T	G
Sydney	S	N	T

VA387 and Sydney strains don't share critical amino acids in previously characterized neut. epitopes.

Ideally, fecal neutralizing activity in HIOs should be tested versus a homologous NV strain.

Conclusions/Opinions

- Recombinant RRV can express HuNoV VP1 or HuNoV P protein *in vitro* and *in vivo*

•rRRV-HuNoV-VP1 and Noro P induce diarrhea and both systemic and local intestinal antibody binding responses vs. RV, HuNoV, and HuNoV P after enteric immunization of suckling mice.

•Serum and stool suspension from rRRV-HuNoV-VP1 immunized mice neutralized both RV and HuNoV.

•Stool suspensions from rRRV-HuNoV-P immunized mice neutralized RV, but poorly neutralized HuNoV. Matching “serotype” of insert with NV used for neut. assay is likely important .

•Additional immunization studies in a more replication permissive species (e.g. infant rat) seem warranted.

•To date, it was not possible for us to make highly stable RV/NV VP1/P constructs in RRV. The degree of genetic stability for VP1 or PP in RVs needs to be enhanced

• RRV is not an ideal candidate for human vaccination studies. The most viable candidates to study would likely be derived from existing and already licensed human RV vaccine strains.

Acknowledgements

- Stanford University
  - ***Takahiro Kawagishi***
  - ***Ningguo Feng***
  - ***Liliana Sánchez-Tacuba***
  - ***Zemin Wang***

- Washington University
  - ***Siyuan Ding* and Ding Lab**

- UNAM, Mexico
  - Susana Lopez and Lopez Lab

- CDC
  - Jan Vinjé
  - Veronica P. Costantini
  - Baoming Jiang
  - Theresa K. Resch

- National Institute of Health
  - Kim Y. Green

- EUKARYS
  - Philipe H. Jaïs

- University Cincinnati Med. School
  - Ming Tan
  - Xi Jiang

- University of Nottingham
  - Kenneth H. Mellits
  - Nathan J. Meade