PI: **Troemel, Emily R**	Title: Innate immunity against viral infection in intestinal epithelial cells of C. elegans	
Received: 08/09/2022	Opportunity: PA-20-185	Council: 01/2023
Notice of Special Interest: NOT-AI-21-066		
Competition ID: FORMS-G	FOA Title: NIH Research Project Grant (Parent R01 Clinical Trial Not Allowed)	
1R01AI176639-01	Dual:	Accession Number: 4740543
IPF: 577507	Organization: UNIVERSITY OF CALIFORNIA, SAN DIEGO	
Former Number:	Department:	
IRG/SRG: ZRG1 IIDA-M (90)	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A) Year 1: [redacted] Year 2: [redacted] Year 3: [redacted] Year 4: [redacted] Year 5: [redacted]	Animals: N Humans: N Clinical Trial: N Current HS Code: 10 HESC: N HFT: N	New Investigator: N Early Stage Investigator: N
Senior/Key Personnel:	*Organization:*	*Role Category:*
Emily Troemel	UNIVERSITY OF CALIFORNIA, SAN DIEGO	PD/PI
Alistair Russell	The Regents of the Univ. of Calif., U.C. San Diego	Faculty

OMB Number: 4040-0001
Expiration Date: 12/31/2022

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

3. DATE RECEIVED BY STATE	State Application Identifier

1. TYPE OF SUBMISSION*

❍ Pre-application ● Application ❍ Changed/Corrected Application

4.a. Federal Identifier

b. Agency Routing Number
NOT-AI-21-066

2. DATE SUBMITTED	Application Identifier	c. Previous Grants.gov Tracking Number

5. APPLICANT INFORMATION **UEI*:** [redacted]

Legal Name*: UNIVERSITY OF CALIFORNIA, SAN DIEGO
Department: [redacted]
Division: General Campus
Street1*: [redacted]
Street2: [redacted]
City*: LA JOLLA
County: San Diego
State*: CA: California
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: [redacted]

Person to be contacted on matters involving this application
Prefix: Mr. First Name*: Michael Middle Name: Last Name*: Kusiak Suffix:
Position/Title: Contract and Grant Officer
Street1*: [redacted]
Street2: [redacted]
City*: La Jolla
County: San Diego
State*: CA: California
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: [redacted]
Phone Number*: [redacted] Fax Number: Email: [redacted]

6. EMPLOYER IDENTIFICATION NUMBER *(EIN) or (TIN)** [redacted]

7. TYPE OF APPLICANT* H: Public/State Controlled Institution of Higher Education

Other (Specify):
Small Business Organization Type ❍ Women Owned ❍ Socially and Economically Disadvantaged

8. TYPE OF APPLICATION*

● New ❍ Resubmission
❍ Renewal ❍ Continuation ❍ Revision

If Revision, mark appropriate box(es).
❍ A. Increase Award ❍ B. Decrease Award ❍ C. Increase Duration
❍ D. Decrease Duration ❍ E. Other *(specify)* :

Is this application being submitted to other agencies?* ❍Yes ●No What other Agencies?

9. NAME OF FEDERAL AGENCY*	10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER
National Institutes of Health	TITLE:

11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT*
Innate immunity against viral infection in intestinal epithelial cells of C. elegans

12. PROPOSED PROJECT		13. CONGRESSIONAL DISTRICTS OF APPLICANT
Start Date*	Ending Date*	CA-049
01/01/2023	12/31/2027	

SF 424 (R&R) APPLICATION FOR FEDERAL ASSISTANCE

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: First Name*: Emily Middle Name: R Last Name*: Troemel Suffix:
Position/Title: Professor
Organization Name*: UNIVERSITY OF CALIFORNIA, SAN DIEGO
Department:
Division:
Street1*: [redacted]
Street2: [redacted]
City*: La Jolla
County:
State*: CA: California
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: [redacted]
Phone Number*: [redacted] Fax Number: Email*: [redacted]

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested* $[redacted]
b. Total Non-Federal Funds* $[redacted]
c. Total Federal & Non-Federal Funds* $[redacted]
d. Estimated Program Income* $[redacted]

16.IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?*

a. YES ○ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:
DATE:
b. NO ● PROGRAM IS NOT COVERED BY E.O. 12372; OR
○ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

● I agree*

** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.*

18. SFLLL or OTHER EXPLANATORY DOCUMENTATION File Name:

19. AUTHORIZED REPRESENTATIVE

Prefix: Ms. First Name*: America Middle Name: Last Name*: Vega Suffix:
Position/Title*: [redacted]
Organization Name*: University of California San Diego
Department: [redacted]
Division:
Street1*: [redacted]
Street2: [redacted]
City*: La Jolla
County: San Diego
State*: CA: California
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: [redacted]
Phone Number*: [redacted] Fax Number: Email*: [redacted]

Signature of Authorized Representative* [redacted]

Date Signed* 08/09/2022

20. PRE-APPLICATION File Name:

21. COVER LETTER ATTACHMENT File Name:Cover_Letter.pdf

424 R&R and PHS-398 Specific
Table Of Contents

OMB Number: 4040-0010
Expiration Date: 12/31/2022

Project/Performance Site Location(s)

Project/Performance Site Primary Location

❍ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name:	UNIVERSITY OF CALIFORNIA, SAN DIEGO
UEI:	[redacted]
Street1*:	UNIVERSITY OF CALIFORNIA SAN DIEGO
Street2:	OFFICE OF CONTRACT & GRANT ADMIN, 0934
City*:	LA JOLLA
County:	San Diego
State*:	CA: California
Province:	
Country*:	USA: UNITED STATES
Zip / Postal Code*:	920930621
Project/Performance Site Congressional District*:	CA-049

Additional Location(s) File Name:

Funding Opportunity Number: PA-20-185. Received Date: 2022-08-09T12:28:18.000-04:00

OMB Number: 4040-0010
Expiration Date: 12/31/2022

RESEARCH & RELATED Other Project Information

1. Are Human Subjects Involved?* ❍ **Yes** ● **No**

1.a. If YES to Human Subjects

Is the Project Exempt from Federal regulations? ❍ Yes ❍ No

If YES, check appropriate exemption number: _ 1 _ 2 _ 3 _ 4 _ 5 _ 6 _ 7 _ 8

If NO, is the IRB review Pending? ❍ **Yes** ❍ **No**

IRB Approval Date:

Human Subject Assurance Number

2. Are Vertebrate Animals Used?* ❍ **Yes** ● **No**

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ❍ Yes ❍ No

IACUC Approval Date:

Animal Welfare Assurance Number

3. Is proprietary/privileged information included in the application?* ❍ **Yes** ● **No**

4.a. Does this project have an actual or potential impact - positive or negative - on the environment?* ❍ **Yes** ● **No**

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ❍ Yes ❍ No

4.d. If yes, please explain:

5. Is the research performance site designated, or eligible to be designated, as a historic place?* ❍ **Yes** ● **No**

5.a. If yes, please explain:

6. Does this project involve activities outside the United States or partnership with international collaborators?* ❍ **Yes** ● **No**

6.a. If yes, identify countries:

6.b. Optional Explanation:

	Filename
7. Project Summary/Abstract*	Project_Summary.pdf
8. Project Narrative*	Project_Narrative.pdf
9. Bibliography & References Cited	References.pdf
10.Facilities & Other Resources	Facilities_and_Other_Resources_Final.pdf
11.Equipment	Equipment.pdf

Funding Opportunity Number: PA-20-185. Received Date: 2022-08-09T12:28:18.000-04:00

Project Summary/Abstract

RNA viruses have had an immense impact on human health. SARS-CoV-2 is only the most recent of many RNA viral zoonoses, and, even disregarding pandemics, the health burden of endemic RNA viruses, particularly in vulnerable populations, is substantial. Epithelial cells, abundant and exposed at mucosal surfaces, are often the first to be infected by RNA viruses, and are therefore often the first cell type to detect and respond to viral infection. However, unlike circulating immune cells, their *in vivo* behaviors cannot be measured from blood draws, and their behavior *ex vivo* may poorly correlate with *in vivo* dynamics. Our *long-term goal* is to understand how epithelial cells coordinate anti-viral responses in a whole-animal setting.

Our previous work demonstrated that the RIG-I-like receptor (RLR) DRH-1 in the nematode *C. elegans* activates an anti-viral transcriptional response in intestinal epithelial cells that we named the Intracellular Pathogen Response (IPR), which protects against infections by viruses and other intracellular pathogens. We found that DRH-1 responds to infection with Orsay virus–a single-stranded, positive-sense RNA virus that naturally infects *C. elegans* intestinal epithelial cells.

The *objective of this proposal* is to determine where and how DRH-1 triggers resistance to Orsay virus infection, and investigate whether in *C. elegans*, which lacks identified homologs of interferons, there is a role for bystander cells in mounting an immune response. The *central hypothesis* is that upon Orsay virus infection, DRH-1 in intestinal epithelial cells detects viral replication and induces the IPR, signaling to neighboring cells through an as-yet undescribed pathway. *The rationale* is based on our genetic analysis of DRH-1 and its role in anti-viral responses, and our visualization of IPR gene expression and DRH-1 localization dynamics in the context of infection. Our work is *innovative* because we are pursuing the IPR, which shares similarity with the type-I interferon (IFN-I) response in humans, but excitingly, appears to signal through novel factors, as homologs of MAVS, IRF3, NFkB, TNF-alpha and IFN-I itself are absent from the *C. elegans* genome.

We will test our hypothesis with three specific aims: Aim 1) Where and how does DRH-1/RLR promote anti-viral defense in *C. elegans*? Aim 2) What signaling pathway is activated downstream of DRH-1/RLR in *C. elegans*? Aim 3) Which host cells mount an anti-viral immune response in C. elegans? The *expected outcomes* are to establish the signaling cascade used by DRH-1/RLR to trigger the protective IPR immune response in intestinal epithelial cells of *C. elegans*, and to identify the components of a systemic defense system. The proposed research is *significant*, because it could lead to new treatments for infections by RNA viruses, as well as a better understanding of epithelial immune defense and inflammatory diseases.

Project Narrative

The proposed research is relevant to public health because it will characterize a novel form of immune defense against RNA virus infection in epithelial cells. By describing how the RIG-I-like receptor DRH-1 activates anti-viral defense in the nematode *C. elegans*, we may uncover novel forms of cell-intrinsic as well as systemic immune responses driven by epithelial cells. Thus, the proposed research is relevant to the NIH's mission to seek fundamental knowledge about living systems in order to reduce the burden of disease.

Facilities and Other Resources - Troemel Laboratory – University of California, San Diego

Scientific Environment – Contribution to Success

The facilities and other resources available to Professor Troemel include everything needed to undertake and complete the proposed research project successfully. [redacted] and all needed equipment has been purchased and is available. The lab is in a vibrant environment in [redacted] where 3 other *C. elegans* labs are also housed and interact regularly with the Troemel lab. In addition, The Troemel Lab participates in a weekly [redacted] with four other labs on campus studying host-virus or host-bacterial interactions (labs of co-Investigator [redacted]). We also have monthly [redacted] joint lab meeting with ~30 other Cell Biology labs here at UC San Diego, as well as monthly [redacted] meetings with 10 other labs here in the San Diego area. Thus, there are ample facilities and a rich intellectual environment for the proposed research plan.

The Troemel and Russell labs are in buildings only about [redacted] on the UC San Diego campus, and it is easy to walk back and forth and meet in common outdoor spaces for coffee, lunch, etc.

Laboratory:

The Troemel laboratory consists of several rooms totaling over 1500 sq. ft of laboratory space. The main lab consists of 900 sq. ft, which is used for molecular and genetic experiments. Connected to this room is a 200 sq. ft. dark room housed with four fluorescence microscopes, with additional room for other microscopes. In addition, there are two 135 sq ft. rooms that serve as chemical/weighing rooms and equipment rooms.

Biohazard Plan:

The Troemel lab has a Biosafety Use Authorization (BUA) that has been reviewed and approved by the Institutional Biosafety Committee (IBC) at UC San Diego (BUA number R1200). This BUA covers several BSL2 pathogens, and describes procedures for handling and disposal of these pathogens, as well as training of personnel, who wear personal protective equipment (gloves and laboratory coats). However, the Orsay virus, which is proposed for work in this grant is BSL1, and requires no special handling.

Computer:

The Troemel laboratory has two iMac computers with 2.8 GHz Intel Core 2 Duo processors, 2Gb 800 MHz RAM and 300 Gb hard drives. These are backed-up by a 4 Tb external hard drive and also synced on Dropbox. In addition the laboratory has several PC-based computers – one for running the software and imaging on the Zeiss fluorescence wide-field microscope, one for the Zeiss confocal microscope, one as an imaging workstation, one for running the BioRad CFX connect real-time PCR machine, and one for the GelDoc system and Nanodrop spectrophotometer. The lab has two Network-Associated Storage systems, including a recently purchased Drobo system with 50 Tb of storage, which is attached to the Molecular Devices ImageXpress Nano high-content, high throughput plate reader. In addition there is 2 Tb of UCSD server space for backing up lab data.

Office:

The laboratory has a 235 sq. ft room that serves as office/break room for researchers, as well as another 142 sq. ft room with additional office space. There also are 6 desks in the main laboratory. Prof. Troemel has a 142 sq ft office.

UCSD Biomolecular and Proteomics Mass Spectrometry Facility

We have access to the UCSD Biomolecular and Proteomics Mass Spectrometry Facility run by core [redacted]– see letter of support. The facility has an LC-MS set-up composed of Thermo nano-uPLC in tandem with LUMOS Orbitrap MS for proteomics analysis on a fee-for-service basis.

UCSD School of Medicine Institute for Genomics Core
We have access to the IGM Genomics Facility at the University of California, San Diego, which is a core research facility located just down the street in the [redacted] building. The IGM Genomics Facility provides a variety of services, including sequencing library preparation and sequencing on Illumina's HiSeq2500/2000 and MiSeq platforms as well as on the PacBio RS II Sequencing System. The mission of the IGM Genomics Facility is to provide UCSD researchers, as well as researchers in the greater UC community, high quality standard, cutting-edge, and custom genomics services as well as consultation on experimental design and training/education about genomic methods.

UCSD School of Medicine - Light Microscopy Facility:
In addition to our own confocal microscope and other confocal microscopes downstairs as well as a super-resolution scope next door, we have access to the UCSD Medical School Imaging Core on a recharge basis. This core provides a variety of services and has microscopes with various features and capabilities including in vivo imaging (e.g., two photon or total internal reflection microscopy).

Facilities and Other Resources - Russell Lab – University of California, San Diego

Laboratory
The Russell laboratory is located in the newly constructed [redacted] and consists of two 20’x5’ benches in a shared laboratory space and an adjoining tissue culture room reserved for my laboratory’s exclusive use. Each lab bench is equipped with pipettes, a vortexer, and a bunsen burner. Facilities shared with neighboring labs include a 37 degree warm room, a 4 degree cold room, autoclave, a chemical room (with fume hood), and a liquid nitrogen storage tank.

Office
The Russell lab has an office of 120 sq. ft, occupied by Professor Russell, and shares an open 1203 sq ft office in which eight desks are permanently assigned to the Russell Laboratory.

Research Environment
The School of Biological Sciences at UCSD is a highly collaborative and thriving research community. The Molecular Biology Department alone consists of ~30 laboratories working across many aspects of basic biology. Notably, this includes a number of biologists working on basic problems of immunology and RNA biology, providing a broader community in which the Russell laboratory will be working. There is ample opportunity for both the PI and trainees to obtain feedback from a diverse set of scientists; the Russell lab attends several subject-specific group meetings. Currently members of Russell lab attend and present at a general immunology meeting alongside [redacted] labs, a general biology meeting with [redacted] labs. They also participate in a weekly [redacted] club with four other labs on campus studying host-virus or host-bacterial interactions (labs of co-

PI Emily Troemel, [redacted]). This is in addition to training-grant specific training seminars for graduate students. The PI has a specific faculty mentor, [redacted], who has been providing guidance in navigating their first years as a faculty member. The shared lab space in [redacted] has also been a fantastic boon to the Russell lab, providing a forum for informal collaborations. Moreover, the presence of two laboratories starting immediately prior to Russell lab, [redacted] labs, has provided significant guidance to the first steps of the Russell laboratory, and the presence of a more senior colleague, [redacted], means that the Russell lab is also receiving frequent, informal, guidance on how to run an established, successful, research program.

In addition to our immediate division members and colleagues, the broader San Diego community is an incredible environment for biological sciences, with shared research opportunities and seminars with the [redacted], the La Jolla Institute for [redacted] and the [redacted].

Logistical support

The Russell lab was provided a generous startup package. Committee service is kept to a minimum for junior faculty. The department has provided, and will continue to provide, significant administrative support in managing grants, personnel, and purchasing. As part of Biological Sciences, the Russell Lab may also benefit from significant financial support to graduate students in the form of training grants, and is available to those students for rotations in their first year.

Equipment – Troemel Lab – University of California, San Diego

Microscopy and *C. elegans* work
The Troemel laboratory is currently equipped with a Zeiss LSM700 scanning confocal microscope with four lasers and motorized stage, a Zeiss AxioImager M1 fluorescence compound microscope with an Axiocam digital imaging system, a Zeiss AxioObserver A1 inverted fluorescent microscope with Femtoject microinjection system for generating transgenic *C. elegans* strains, and a Zeiss V8 fluorescence dissecting microscope with 5 fluorescent filters. We have a Union Biometrica Copas Biosort 'worm sorter' with red and green lasers, which allows bright-field and fluorescence-based analysis and sorting of live *C. elegans* nematodes. We have a Molecular Devices ImageXpress Nano high content imaging system with 2X, 4X, 20X and 40X objectives and DAPI, GFP, Cy3 filters for high throughput imaging of *C. elegans* in 96-well format. We have a BioRAD PDS 1000-HE Biolistic particle delivery system for ballistic transformation of *C. elegans*. We have eight dissecting stereomicroscopes for *C. elegans* strain maintenance. In the neighboring *C. elegans* lab downstairs we have access to spinning disk confocal microscopy (Andrew Chisholm lab), and have access to a Deltavision OMX SR through our next-door lab neighbor (Andreas Ernst lab). We also have an Integra Mediajet and Mediaclave system for automated plate pouring of agar plates for *C. elegans* growth, which we keep in a recently renovated clean room down the hall from the lab.

Molecular biology and protein work
For nucleic acid work we have a BioRad CFX Connect quantitative PCR machine, 2 BioRad regular PCR machines, a Nanodrop spectrophotometer, a BioRad ChemiDoc system for DNA imaging and bioluminescent imaging, and several DNA agarose gel boxes. For protein work we have an Innova 44R refrigerated shaking incubator for large-scale growth of *C. elegans* liquid culture, a Balch homogenizer for disrupting *C. elegans*, as well as access to sonicator and French press. We also have standard equipment for protein analysis including gels and Western blots. Downstairs we have access to a shared Li-Cor Odyssey Fc system for the digitial scanning of Western blots, including for ECL/chemiluminescence, but also for quantitative fluorescence detection.

Other equipment and resources
In addition, we have two -80°C freezers, two -20C freezers, 4C refrigerator, as well as 2 large benches of space in a 4C room, with access to sonicator and other protein biochemistry equipment. We have 3 large Forma refrigerated incubators for keeping *C. elegans* stocks, including one with humidity control. We also have 3 smaller refrigerated incubators for similar use, including 2 programmable incubators. We have a laminar flow hood for clean work. We also have a chemical hood, and access to a biosafety cabinet and full tissue culture facilities in a recently renovated room just down the hall from the lab. We also have a 37C incubator and access to a full room at 37C, as well as access to Sorvall Lynx 6000 centrifuge, a Beckman ultracentrifuge, several shaking incubators and dark room with X-ray film processor.

Proteomics work
In the lab of colleague Eric Bennett, we have access to a Thermo Q-Exactive-Orbitrap mass spectrometer with a high pressure nanoflow liquid chromatography HPLC for in-line peptide separation, which is owned and operated by the Bennett lab. They also have a computational cluster setup to facilitate data processing and analysis. The instrument is housed in the biochemistry proteomics core facility but is operated by members of the Bennett lab and is not be a core utilized instrument. Nano-capillary columns are manufactured and packed with solid-phase separation materials using shared resources with Steve Briggs' laboratory. All data processing is accomplished in house using proprietary MS search algorithms and analysis

software that we have licensed from Harvard Medical School. This software has been installed on a centralized server (8Tb, 12 Core processor) that is used to process and analyze all mass spectrometry data. This can be expanded to include more storage space as needed. The Bennett lab also has an akta-FPLC system that will be used for the protein purification and an Agilent 1100 HPLC for peptide purification.

Major equipment – Russell lab

The Russell lab is equipped as a modern molecular virology lab. The laboratory possesses standard molecular biology equipment, including several thermocyclers, -20 degree freezers, a -80 degree freezer, gel electrophoresis equipment, a nanodrop, several benchtop centrifuges, and access to a shared gel documentation system. The laboratory also possesses more specialized equipment for the validation of deep-sequencing libraries, including a BioRad qPCR thermocycler, and a Qubit fluorometer. We also possess standard equipment for the growth and propagation of human cells and safe handling of viruses, including centrifuges with aerosol control, two BSL-2 certified biosafety cabinets, an Echo Revolve epifluorescent microscope, and standard tissue culture incubators.

Shared Resources

Shared equipment on the floor include a BioRad ZE5 cell analyzer (5 laser loadout, 355/405/488/561/640nm, detection of up to 27 colors), several high-speed centrifuges, an Illumina iSeq benchtop sequencer and a lyophilizer. Additionally, we have access to shared equipment in neighboring buildings, including FACS-Calibur analyzers (dual-laser for 4-color capability), an ABI7700 Real-time PCR machine, a Nikon E800 fluorescence microscope with digital data acquisition and image processing, Luminometer, film processing, beta and gamma counters, and an ELISA reader. The Russell lab also has access within the School of Biology facilities to an ARIA FACS-sorter (adapted to use infectious BSL-2 material), and deconvolution and confocal microscopes.

Russell laboratory has access to the IGM Genomics Center at UCSD for sequencing, including access to a Bioanalyzer Tapestation, Illumina HiSeq 2500 and 4000, Miseq, and special pricing on an Illumina NovaSeq 6000 at UCSF, a PacBio Sequel, and a 10x Chromium controller for single cell analysis. Core facilities operate on a nonprofit basis for investigators based at UCSD, and have a low turn-around time.

Computational Resources

The Russell lab has priority on a dedicated node on the Triton shared compute cluster with 18 cores and 192 GB of RAM, and access to shared nodes for more computationally intensive processes. Individual lab members will be provided machines capable of modest analyses, an example being a mac mini consisting of 6 cores at 3 Ghz, 32 GB RAM, and at least 1 TB solid state storage.

All software for this project will be managed by GitHub, the modern standard for source code management. The Russell lab has access to institutional pricing for GitHub memberships for the lab members and a group account covering the entire lab. This platform will also serve as a means of publicly distributing code for analysis upon publication.

OMB Number: 4040-0001
Expiration Date: 12/31/2022

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: First Name*: Emily Middle Name R Last Name*: Troemel Suffix:

Position/Title*: Professor
Organization Name*: UNIVERSITY OF CALIFORNIA, SAN DIEGO
Department:
Division:
Street1*: [redacted]
Street2: [redacted]
City*: La Jolla
County:
State*: CA: California
Province:
Country*: USA: UNITED STATES
Zip / Postal Code*: [redacted]

Phone Number*: [redacted] Fax Number:

E-Mail*: [redacted]

Credential, e.g., agency login: [redacted]

Project Role*: PD/PI Other Project Role Category:

Degree Type: PHD,BS Degree Year: 1999,1992

Attach Biographical Sketch*: File Name: Troemel_Biosketch.pdf

Attach Current & Pending Support: File Name:

Tracking Number: GRANT13694683 Funding Opportunity Number: PA-20-185 . Received Date: 2022-08-09T12:28:18.000-04:00

PROFILE - Senior/Key Person

Prefix: First Name*: Alistair Middle Name B Last Name*: Russell Suffix:

Position/Title*: Assistant Professor
Organization Name*: The Regents of the Univ. of Calif., U.C. San Diego
Department:
Division:
Street1*: [redacted]
Street2:
City*: San Diego
County:
State*: CA: California
Province:
Country*: USA: UNITED STATES
Zip / Postal Code*: [redacted]

Phone Number*: [redacted] Fax Number:

E-Mail*: [redacted]

Credential, e.g., agency login: [redacted]

Project Role*: Faculty Other Project Role Category:

Degree Type: PHD,BA Degree Year: 2014,2009

Attach Biographical Sketch*: File Name: Russell_Biosketch.pdf

Attach Current & Pending Support: File Name:

OMB No. 0925-0001 and 0925-0002 (Rev. 12/2020 Approved Through 02/28/2023)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Emily Troemel

eRA COMMONS USER NAME (credential, e.g., agency login): [redacted]

POSITION TITLE: Professor in Biology, Department of Cell and Developmental Biology, UC San Diego

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
University of Wisconsin, Madison	B.S.	06/1992	Biochemistry
University of California, San Francisco	Ph.D.	06/1999	Cell Biology
University of California, San Diego		12/2000	Research Fellow
Mass. General Hospital/Harvard Medical School		07/2008	Research Fellow

A. Personal Statement

The overall goals of our research are to dissect host/pathogen interactions in intestinal epithelial cells and to define the unique physiology of the host response to intracellular infection. As a graduate student I identified and characterized the first chemosensory receptors in the nematode *C. elegans*. Next, after a brief postdoctoral fellowship, I helped launch a start-up biotech company where I studied questions of neuronal identity, neuroinflammation and performed drug screening. After this company went public, I returned to academic research to do a postdoctoral fellowship, where I identified the first natural pathogen of *C. elegans*. I named this pathogen *Nematocida parisii*, and it defines a new genus and species of microsporidia, which are priority pathogens of medical and agricultural significance. Here in my own lab at UC San Diego, we are using this natural host/pathogen system to investigate how microsporidia cause disease. We have defined intestinal cell restructuring and pathogen exit strategies in *N. parisii* infections, as well as host defense against infection. To provide more resources for studying microsporidia, I organized the Microsporidian Genomes Consortium, and we found shared strategies for pathogenesis among several microsporidia species. Following on this work, we have used localized proteomics to define microsporidia 'host-exposed' proteins. In our most recent work, we are studying how the *C. elegans* transcriptional response to microsporidia is surprisingly similar to the response to the Orsay virus, which is another natural intracellular pathogen of the *C. elegans* intestine. We have named this response the Intracellular Pathogen Response (IPR), and have found that Orsay virus infection will trigger this response via DRH-1, which is a *C. elegans* homolog of RIG-I receptor in mammals. IPR genes include ubiquitin ligase components, which we have found promote increased proteostasis capacity. We have also found regulation of the IPR by purine metabolism enzymes. I am committed to pre-doctoral and post-doctoral training. During my 13 years as I faculty member I have trained five pre-doctoral fellows who have all gone onto research positions, including one who went straight to a faculty position at SDSU, and another who recently started a lab at SF BioHub, as well as ten post-doctoral fellows who have gone onto independent positions in academia, government and industry. I was awarded the 2018 IRACDA mentoring award, and I provide training in the areas of infection and immunity, genetics, cell biology and biochemistry. I am committed to training, mentoring, and providing a supportive, safe and inclusive research environment.

Ongoing and recently completed projects that I would like to highlight include:

Troemel (PI), Bhabha (Co-I)
2R01GM114139
08/01/2019- 07/31/2023
The Intracellular Pathogen Response triggers defense against co-evolved pathogens

Troemel (PI), Ekiert (Co-I)
2R01AG052622
07/15/2022- 03/31/2027
Probing organismal proteostasis through the response to intracellular infection

Citations:

1. Tecle E, Chhan CB, Franklin L, Underwood RS, Hanna-Rose W, **Troemel ER**. The purine nucleoside phosphorylase pnp-1 regulates epithelial cell resistance to infection in C. elegans. PLoS Pathog. 2021 Apr 20;17(4):e1009350. doi: 10.1371/journal.ppat.1009350. eCollection 2021 Apr. PMID: 33878133
2. Sowa JN, Jiang H, Somasundaram L, Tecle E, Xu G, Wang D, **Troemel ER**. The Caenorhabditis elegans RIG-I Homolog DRH-1 Mediates the Intracellular Pathogen Response upon Viral Infection. J Virol. 2020 Jan 6;94(2). 2020 Jan 6. PMID:31619561. PMCID: PMC6955277
3. Reddy KC, Dror T, Underwood RS, Osman GA, Elder CR, Desjardins CA, Cuomo CA, Barkoulas M, **Troemel ER**. Antagonistic paralogs control a switch between growth and pathogen resistance in C. elegans. PLoS Pathog. 2019 Jan 14;15(1):e1007528. PMCID: PMC6347328.
4. Lažetić V, Wu F, Cohen LB, Reddy KC, Chang YT, Gang SS, Bhabha G, **Troemel ER**. The transcription factor ZIP-1 promotes resistance to intracellular infection in Caenorhabditis elegans. Nat Commun. 2022 Jan 10;13(1):17.

B. Positions, Scientific Appointments, and Honors

Positions and Scientific Appointments

2016	Sabbatical at the Broad Institute (MIT/Harvard)
2015-	Professor in Biology, University of California, San Diego, CA
2013-2015	Associate Professor in Biology, University of California, San Diego, CA
2008-2013	Assistant Professor in Biology, University of California, San Diego, CA
2005-2008	Research Fellow, Department of Genetics, Harvard Medical School; Assistant, Department of Molecular Biology, Massachusetts General Hospital (Advisor: Prof. Frederick Ausubel)
2000-2004	Scientist I (2000-2002), II (2002-2004), Renovis, Inc. South San Francisco, CA, (Advisors: Tito Serafini, Michael Ellis); 2001-2002 Project Leader, Obesity and Type II diabetes program; 2003-2004 Project Leader, Neurodegenerative Diseases II program
1999-2000	Postdoctoral Fellow, University of California, San Diego, CA (Advisor: Prof. Charles Zuker)

Professional Memberships

2015- pres.	Member, Center for Microbiome Innovation
2013- pres.	Member, UCSD Health Sciences Center for Immunity, Infection & Inflammation
2009- pres.	Member, UCSD Moores Cancer Center
2009- pres.	Member, UCSD Center for AIDS Research

Honors and Other Professional Activities

2020-2022	Organizer of *C. elegans*/virus/pathogen joint Zoom meetings with labs from 7 countries
2010-present	Instructor of Genetics BICD100 Spring quarters (~140-400 students each quarter)
2015-present	Instructor of Innate Immunity BGGN232 Spring quarters (seminar class with ~20 students)
2018-2019	UC San Diego Lattimer Faculty Research Fellowship
2018	UC San Diego IRACDA Postdoctoral Mentor Award
2017-	Standing Member, NIH Study Section for Innate Immunity and Inflammation (III)
2010-2014	Instructor of Molecular Biology, BGGN220 Fall quarters (~50 PhD students each quarter)
2013-2018	Burroughs Wellcome Fund Award, Investigators in the Pathogenesis of Infectious Disease
2013	Organizing committee, International *C. elegans* meeting UCLA
2013	Merck Irving S. Sigal Memorial Award – American Society of Microbiology
2012	Kavli Fellow; National Academy of Sciences meeting
2012	Co-organizer, *C. elegans* aging, pathogenesis, stress meeting in Madison, WI
2010	Organizing committee, *C. elegans* aging, pathogenesis, stress meeting in Madison, WI

2010-2015	David and Lucile Packard Foundation Fellowship
2010-2013	Ray Thomas Edwards Foundation Award
2010-2013	Searle Scholars Program Award
2009	Co-Organizer Microsporidian Genomes Consortium
2009-2010	Hellman Fellow, UCSD
2006-2008	Leukemia/Lymphoma Society Special Fellow
2006	Irvington Institute for Immunological Research Fellow
2000	UC President's Postdoctoral Fellow (declined NIH NRSA and Jane Coffin Childs fellowships)
1998-1999	UCSF Graduate Division Chancellor's Fellowship
1995	Richard Feinberg Memorial Teaching Award
1995-1998	National Science Foundation Fellowship

C. Contributions to Science

Note: From 2000-2004 I was employed as a Scientist at the start-up biotech company Renovis, Inc. I returned to academic research in 2005.

1. Described the shared host response to microsporidia and viral infection as a novel proteostasis pathway.

Microsporidia are common pathogens of humans and other hosts, but little is known about the host response and defense pathways used against them. We found a striking similarity in the host transcriptional response to microsporidia and viral infection in *C. elegans*, which we named the Intracellular Pathogen Response. Given that microsporidia and the Orsay virus are molecularly distinct, this shared response may be due to the effects of infection, rather than molecular cues. Indeed, we showed that IPR genes could be induced by perturbation of the proteasome. These findings are in keeping with the theme of surveillance immunity used for *C. elegans* defense against *P. aeruginosa* infection described in Section 2. Interestingly however, induction of the IPR can also be induced independently by the RIG-I homolog DRH-1 likely sensing viral RNA replication products, demonstrating that there are multiple pathways that regulate IPR gene expression. Through forward genetic screens, we have defined positive and negative regulators of the IPR, which indicate that the IPR promotes defense against virus and microsporidia infection. In addition, the IPR appears to improve proteostasis capacity in *C. elegans*, acting independently of canonical proteostasis pathways. This phenotype is dependent on a cullin-ring ubiquitin ligase complex that we defined through a combination of genetics and biochemistry.

a. Reddy KC, Dror T, Sowa JN, Panek J, Chen K, Lim ES, Wang D, **Troemel ER**. An Intracellular Pathogen Response Pathway Promotes Proteostasis in *C. elegans*. Curr Biol. 2017 Nov 20;27(22):3544-3553.e5. Epub 2017 Nov 2. PMCID: PMC5698132
b. Reddy KC, Dror T, Underwood RS, Osman GA, Elder CR, Desjardins CA, Cuomo CA, Barkoulas M, **Troemel ER**. Antagonistic paralogs control a switch between growth and pathogen resistance in C. elegans. PLoS Pathog. 2019 Jan 14;15(1):e1007528. PMCID: PMC6347328.
c. Sowa JN, Jiang H, Somasundaram L, Tecle E, Xu G, Wang D, **Troemel ER**. The Caenorhabditis elegans RIG-I Homolog DRH-1 Mediates the Intracellular Pathogen Response upon Viral Infection. J Virol. 2020 Jan 6;94(2). 2020 Jan 6. PMID:31619561. PMCID: PMC6955277
d. Tecle E, Chhan CB, Franklin L, Underwood RS, Hanna-Rose W, **Troemel ER**. The purine nucleoside phosphorylase pnp-1 regulates epithelial cell resistance to infection in C. elegans. PLoS Pathog. 2021 Apr 20;17(4):e1009350. doi: 10.1371/journal.ppat.1009350. eCollection 2021 Apr. PMID: 33878133

2. Identified 'effector-triggered' or 'surveillance' immunity in *C. elegans* response to bacterial infection

Very little was known about how *C. elegans* detects microbial infection when I started my postdoc in Fred Ausubel's lab. Using the foundation of work from my postdoc, here in my own lab we have shown how *C. elegans* uses effector-triggered or surveillance immunity. First, during my postdoc I used microarrays to define the repertoire of genes induced by *P. aeruginosa* and showed how this response is primarily a response to pathogenicity. I demonstrated that the p38 MAPK pathway mediates some of this transcriptional induction, but most was independent of known signaling pathways. Then with GFP reporters for p38 MAPK-independent genes we comprehensively screened transcription factors to identify the ZIP-2 bZIP transcription factor as a mediator of this response. Again, using the power of *C. elegans* genetics, my lab showed that this ZIP-2 response is due to translational block by *P. aeruginosa*-derived Exotoxin A, which is delivered into host cells to block mRNA translation specifically in the intestine. Furthermore, we showed that this block in general mRNA translation leads to an intriguing upregulation of ZIP-2 protein expression, which we show is controlled by an

upstream open reading frame (uORF) in ZIP-2. We also showed how this ZIP-2 pathway is activated by perturbation of other core processes, and most recently how it acts together with CEBP-2, the *C. elegans* orthologs of mammalian C/EBP-gamma. These studies provided important insight to the question of how hosts discriminate pathogens from innocuous microbes, by detecting the effects of pathogen-derived toxins, rather than the shape of these toxins. This immunity is referred to as 'effector-triggered' or 'surveillance' immunity.

a. **Troemel ER**, Chu SW, Reinke V, Lee SS, Ausubel FM, Kim DH. p38 MAPK regulates expression of immune response genes and contributes to longevity in *C. elegans*. PLoS Genet. 2006 Nov 10;2(11):e183. Epub 2006 Sep 11. PMCID: PMC1635533
b. Estes KA, Dunbar TL, Powell JR, Ausubel FM, **Troemel ER.** bZIP transcription factor *zip-2* mediates an early response to *Pseudomonas aeruginosa* infection in *Caenorhabditis elegans*. Proc Natl Acad Sci U S A. 2010 Feb 2;107(5):2153-8. Epub 2010 Jan 21. PMCID: PMC2836710
c. Dunbar TL, Yan Z, Balla KM, Smelkinson MG, **Troemel ER**. *C. elegans* Detects Pathogen-Induced Translational Inhibition to Activate Immune Signaling. Cell Host Microbe. 2012 Apr 19;11(4):375-86. PMCID: PMC3334869
d. Reddy KC, Dunbar TL, Nargund AM, Haynes CM, **Troemel ER.** The *C. elegans* CCAAT-Enhancer-Binding Protein Gamma Is Required for Surveillance Immunity. Cell Rep. 2016 Feb 23;14(7):1581-9. Epub 2016 Feb 11. PMID:26876169. PMCID: PMC4767654

3. Provided evolutionary insight into the Microsporidia phylum, which are ubiquitous fungal-related pathogens
As a postdoctoral fellow, I discovered the first natural pathogen of *C. elegans* that I named *Nematocida parisii*, which defined a new genus and species of microsporidia. Microsporidia comprise a phylum of over 1400 obligate intracellular pathogens that can infect all animals. In particular they are emerging eukaryotic pathogens of humans and can cause deadly disease in immunocompromised patients, but few effective treatments exist. To develop new tools and resources for microsporidia research, I organized the Microsporidian Genomes Consortium, which brought together 12 microsporidian researchers to perform genomic studies with the Broad Institute. We sequenced four *Nematocida* genomes (two strains each of two species), and performed phylogenomic comparisons to provide key insight into evolutionary events that enabled these pathogens to emerge as a large and successful phylum. In particular we found cancer-like strategies that microsporidia appear to use to rapidly replicate exclusively inside of host cells. Since this time we have identified and sequenced additional species of *Nematocida*, and used localized "APX" proteomics to determine which proteins interface with the host environment.

a. **Troemel ER***, Félix M-A, Barrière A, Whiteman NK, Ausubel FM. Microsporidia are natural intracellular parasites of the nematode *C. elegans*. PLoS Biol 2008 6(12): e309. * corresponding author. PMCID: PMC2596862
b. Cuomo CA, Desjardins CA, Bakowski MA, Goldberg J, Ma AT, Becnel JJ, Didier ES, Fan L, Heiman DI, Levin JZ, Young S, Zeng Q, **Troemel ER**. Microsporidian genome analysis reveals evolutionary strategies for obligate intracellular growth. Genome Research 2012 Dec;22(12):2478-88. PMCID: PMC3514677
c. Luallen RJ, Reinke AW, Tong L, Botts MR, Félix MA, **Troemel ER**. Discovery of a Natural Microsporidian Pathogen with a Broad Tissue Tropism in Caenorhabditis elegans. PLoS Pathog. 2016 Jun 30;12(6):e1005724. doi: 10.1371/journal.ppat.1005724. eCollection 2016 Jun. PMID: 27362540
d. Reinke AW, Balla, KM, Bennett EJ, **Troemel ER**. Identification of microsporidia host-exposed proteins reveals a repertoire of rapidly evolving proteins. Nature Communications. 2017 8:14023. PMCID: PMC5423893.

4. Defined how microsporidia restructure host cells and escape from intestinal cells *in vivo*
Microsporidia are intracellular pathogens that can infect virtually all animals and have been studied since the time of Louis Pasteur. Although these pathogens can cause significant disease in both medically and agriculturally relevant hosts, very little was known about the cell biological response to them. In my lab we have used the convenient *C. elegans/N. parisii* model to describe how microsporidia restructure host cells. Microsporidia commonly invade intestinal cells, go through elaborate stages of development, and then escape these cells to infect new

genetic screen we identified the small GTPase RAB-11 as a critical host factor required for spore fusion, spore exit and host contagiousness, demonstrating that *N. parisii* hijacks host recycling endocytosis for non-lytic, directional exit. These studies are the first to describe an exit strategy for microsporidia, and the first to show the major mode of exit for any intracellular pathogen *in vivo*. We have also found a role for actin in forming "coats" around compartments containing exocytosing *N. parisii* spores. Most recently we have shown that early during infection *N. parisii* causes the host intestine to form a syncytium, by fusing host cells together, while a related species *N. displodere* causes the muscle to become a syncytium.

a. Estes KA, Szumowski SC and **Troemel ER.** Non-Lytic, Actin-Based Exit of Intracellular Parasites from *C. elegans* Intestinal Cells. PLoS Pathogens 2011 Sept 15;7:e1002227 PMCID: PMC3174248
b. Szumowski SC, Botts MR, Popovich JJ, Smelkinson MG, **Troemel ER**. The small GTPase RAB-11 directs polarized exocytosis of the intracellular pathogen *N. parisii* for fecal-oral transmission from *C. elegans*. Proc Natl Acad Sci U S A. 2014 May 19. PMCID: PMC4050618
c. Szumowski SC, Estes KA, Popovich JJ, Botts MR, Sek G, **Troemel ER**. Small GTPases promote actin coat formation on microsporidian pathogens traversing the apical membrane of Caenorhabditis elegans intestinal cells. Cell Microbiol. 2015 18(1):30-45. PMCID: PMC5522806.
d. Balla KM, Luallen RJ, Bakowski MA, **Troemel ER**. 2016 Cell-to-cell spread of microsporidia causes Caenorhabditis elegans organs to form syncytia. Nat. Microbiol. 1(11):16144. PMCID: PMC5094362.

5. Deciphered the regulatory logic of chemosensory receptor signaling in *C. elegans*

My doctoral work in the laboratory of Dr. Cori Bargmann focused on questions of neuronal signaling, information encoding, and neuronal identity in the *C. elegans* chemosensory system. First, I identified a superfamily of candidate chemosensory receptors in *C. elegans*. Next I used these receptors to decipher the logic of chemosensory coding in *C. elegans*. By misexpressing the ODR-10 diacetyl receptor, I was able to completely reprogram *C. elegans* behavior toward the odorant diacetyl. Wild-type animals are normally attracted to diacetyl, but our transgenic animals, where ODR-10 was expressed in a different cell type, were repelled by diacetyl. These experiments indicated that the quality of an odor is encoded by the sensory neuron in which the receptor is expressed. Years later, this same coding logic has since been demonstrated in other chemosensory systems, such as the mammalian taste system. Later in my thesis research, I turned to questions of neuronal diversity. I discovered that the STR-2 chemosensory receptor had asymmetric expression in a bilateral pair of olfactory neurons. We identified a novel signaling pathway that regulates STR-2 asymmetric expression, which is now known to be important for *C. elegans* chemosensory discrimination.

a. **Troemel ER***, Chou JH*, Dwyer ND*, Colbert HA*, Bargmann CI. Divergent seven transmembrane receptors are candidate chemosensory receptors in *C. elegans*. Cell. 1995 Oct 20; 83(2): 207-18.
b. **Troemel ER**, Kimmel BE, Bargmann CI. Reprogramming chemotaxis responses: sensory neurons define olfactory preferences in *C. elegans*. Cell. 1997 Oct 17; 91(2): 161-9.
c. Dwyer ND, **Troemel ER**, Sengupta P, Bargmann CI. Odorant receptor localization to olfactory cilia is mediated by ODR-4, a novel membrane-associated protein. Cell. 1998 May 1; 93(3): 455-66.
d. **Troemel ER***, Sagasti A*, Bargmann CI. Lateral signaling mediated by axon contact and calcium entry regulates asymmetric odorant receptor expression in *C. elegans*. Cell. 1999 Nov 12; 99(4): 387-98.

* co-authors

Complete List of Published Works (>50 publications):
https://www.ncbi.nlm.nih.gov/myncbi/emily.troemel.1/bibliography/public/

OMB No. 0925-0001 and 0925-0002 (Rev. 03/2020 Approved Through 02/28/2023)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Alistair Russell

eRA COMMONS USER NAME (credential, e.g., agency login): [redacted]

POSITION TITLE: Assistant Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Cornell University, Ithaca, NY	B.A.	07/2009	Biology
University of Washington, Seattle, WA	PhD	08/2014	Bacteriology
Fred Hutchinson Cancer Research Center, Seattle, WA	Postdoc	09/2014 - 07/2019	Virology
University of California, San Diego	Asst. Professor	07/2019-current	Virology

A. Personal Statement

I possess the training and temperament to successfully collaborate on the proposed research. My own background consists of early training as a classical bacterial geneticist in my undergraduate and PhD years, now married to an extensive training in computational biology, sequencing analysis, virology, and single-cell viral transcriptomics during my postdoctoral fellowship. By melding old and new, my group takes a somewhat unique approach to the application of modern technologies to answering longstanding questions in biology. We work 'bottom-up', carefully defining our questions and hypotheses using high-throughput methodologies with a targeted approach—taking advantage of the saturating power of high-dimensional analyses when restricted to a particular problem rather than a broad but shallow inquiry. We thus ensure we procure the answers to the questions we pose, and can further refine our models and ask new questions as we let the biology lead our inquiries. Curiously, my early background in bacteriology has also aided greatly in my work on viral systems. Bacteriologists have developed a number of models of social behavior that have informed evolutionary trajectories of interacting populations. These same models are applicable to virus-host interactions, and, with our library-based approaches we can appropriately disentangle biological heterogeneity and ascribe distinct infection outcomes to disparate elements of viral and host cell populations.

My most recent work has provided my laboratory the full suite of genetic and bioinformatic tools required to collaborate on the proposed research program. We possess expertise in virology, a full suite of molecular biology methods adapted to the study of viral populations and host innate immunity, and sophisticated pipelines for single-cell analysis of viral infections. Critically, despite the breadth of knowledge required to execute this research program, we are in a unique position wherein we possess a complete capacity to effectively embark on, and see to completion, this work. As evidence of our expertise, we have recently published a sole-effort describing the impact of certain viral variants on innate immunity, and have collaborated on an in vivo study using single-cell RNASeq on a mouse model of influenza infection with the Heaton lab.

1. **Russell, A.B.** Elshina, E., Kowalsky, J.R., te Velthuis, AJW, Bloom, J.D., (2019) Single-cell

virus sequencing of influenza infections that trigger innate immunity. J. Virology 00500-19
2. Mendes, M., **Russell, A. B.** (2021) Library-based analysis reveals segment and length dependent characteristics of defective influenza genomes. PLoS Pathogens 17(12): e1010125

B. Positions and Honors

Positions and Employment

2020-current	Serving as a mentor in the Biology Undergraduate and Master's Mentorship Program connecting mentors to undergraduate students from underrepresented backgrounds.
2020-current	Member of the Society for Molecular Biology and Evolution
2020	Volunteer reviewer for abstracts at the Annual Biomedical Research Conference for Minority Students (ABRCMS)
2019-current	Assistant Professor, School of Biological Sciences, UCSD, San Diego, CA.
2019-current	Member of the American Society of Virology
2014-2019	Postdoctoral Fellow. Fred Hutchinson Cancer Center, Seattle, WA. Bloom laboratory.
2010-2014	PhD student. University of Washington, Seattle, WA. Mougous laboratory
2006-2009	Undergraduate researcher, Cornell University, Ithaca, NY. Collmer laboratory.

Honors

2022	R35 from NIGMS
2018	Dale Frey Award for Breakthrough Researchers
2018	K22 award from NIAID
2015-2019	Damon Runyon Merck Research Fellow
2014	Harold M. Weintraub Graduate Student Award
2013-2014	Helen Riaboff Whiteley Fellowship
2013-2014	Josephine De Karman Dissertation Fellowship
2010-2012	National Science Foundation Graduate Research Fellowship
2009	Top scholar award for incoming graduate student
2009	B.A. in Biological sciences, *summa cum laude*, distinction in all subjects

C. Contributions to Science

1. ***Establishing best-practices for single-cell virology.*** My research into the impact of viral heterogeneity on innate immune recognition of infection requires the use of single-cell transcriptomic approaches. As an initial effort, I, with help from my prior mentor, Dr. Jesse Bloom, and advice from Dr. Cole Trapnell at the University of Washington, developed a pipeline to analyze standing heterogeneity in Influenza A virus replication. This involved the development of novel internal controls permitting the appropriate thresholding of highly bimodal events (influenza infection and interferon production), the inclusion of controls for effective viral dose under a non-saturating infection regime, and the development of pipelines that consider the impact that viral transcripts have on the relative number of total host mRNAs. The robustness of my approach is underscored by the confirmation of earlier phenomena identified by flow cytometry studies. I confirmed that influenza A infections are marked by an incredible breadth of viral burden, partially explained by stochastic absence of gene products comprising the viral polymerase. I further confirmed reports that influenza A virus infections do very little to perturb the host transcriptome, and are relatively unrecognized by innate immune pathways. Crucially, I have developed basal observations and techniques that can now be applied to more complex models of infection, critical as we move towards understanding the relative rarity of interferon production when faced with viral challenge. In my independent laboratory I have continued

to apply these pipelines, and aided other groups, such as the Heaton group, in analysis of *in vivo* single-cell RNAseq data.

a. **Russell, A.B.**, Trapnell, C., Bloom, Bloom, J.D., (2018) Extreme heterogeneity of influenza virus infection in single cells. eLife. 7, e02020

b. Hamele, C.E., **Russell, A.B.**, Heaton, N.S., (2022) In Vivo Profiling of Individual Multiciliated Cells during Acute Influenza A Virus Infection. Journal of Virology, e00505-22

2. ***Development of single-cell viral genomics for the study of innate immune interactions***. Significant speculation predated my work on the nature of rare, interferon-precipitating events during influenza A virus infection, and whether they were linked to a single form of viral variation (particularly large internal deletions in the polymerase genes). To conclusively answer whether a single, deterministic, event precipitates interferon induction by influenza virus, I developed a readily-enrichable interferon reporter cell line, and coupled infection of this line to a modified protocol based on the commercial Drop-Seq platform developed by 10x genomics. My predominant modulation to the standard workflow was the inclusion of the circularization and amplification of influenza-specific cDNA, allowing me to use PacBio SMRT sequencing to jointly sequence the entire viral genome and associate that sequence with cell-specific barcodes; permitting the joint inference of the cellular transcriptome and the associated viral genome. My work also included molecular controls for PCR chimeras and an improved extracellular RNA contamination metric; useful for others who wish to expand my work to other models. Working with my mentor at the time, Jesse Bloom, we developed a pipeline for analyzing this new type of data. While the workflow itself is of significant use to the single-cell virology community, our data revealed quite a surprise on their own; no single event precipitates interferon induction by influenza A virus. Multiple forms of genetic variation contribute to, but are not necessary for, interferon induction. This work has motivated my continued efforts to understand the complexity of innate immune interactions with viral populations, and to develop comprehensive models to both describe and predict these interactions.

a. **Russell, A.B.** Elshina, E., Kowalsky, J.R., te Velthuis, AJW, Bloom, J.D., (2019) Single-cell virus sequencing of influenza infections that trigger innate immunity. J. Virology 00500-19

3. **Comprehensive analysis of defective influenza particle biology.** My independent laboratory has begun our efforts with an analysis of defective influenza A virus particles, replication-incompetent members of the viral population which generally bear large internal deletions in the three polymerase-encoding segments. We have, using a library-based approach, measured across hundreds of length polymorphisms represented by over a thousand potential junctions, comprehensively measured how defective species propagate, package, and induce an innate immune response. In doing so, we now know that the capacity of these parasitic species to arise to high levels in influenza populations hinges on their reduced length, which provides an advantage during genome replication. However, the smallest defective species are excluded at the stage of packaging. We find that interferon induction, while variable between different deletion junctions, is not a length-dependent process. Critically, interferon stimulation is highly sensitive to genome replication, which likely provides additional ligands to cellular surveillance. Combining these concepts, we find that the most potent stimulators of innate immunity would likely be deletions in non-polymerase segments, potentially explaining why this virus appears to exclude such species during growth and expansion.

a. Mendes, M., **Russell, A. B.** (2021) Library-based analysis reveals segment and length dependent characteristics of defective influenza genomes. *PLoS Pathogens* 17(12): e1010125

4. **Characterizing the enzymatic functions of diverse antibacterial effectors of the type VI secretion system(T6SS).** When I joined the lab of Dr. Joseph Mougous they had recently found that the T6SS, a bacterial secretion system, could deliver antagonistic effector proteins between bacteria. However, only a single antibacterial substrate of a single system in the bacterium *Pseudomonas*

aeruginosa had been identified, and its activity remained unclear although it was known to be toxic in the cytoplasm. My initial efforts in Dr. Mougous's lab found that a number of antibacterial effector proteins exert their activity in the periplasm, generating new paradigms of effector delivery. Furthermore, following informatic predictions, I identified the likely target of several of these effectors as the bacterial cell wall. Collaborating with the laboratory of Dr. Waldemar Vollmer in Newcastle, we confirmed these predictions and identified the precise molecular targets within the cell wall of these enzymes.

The other major breakthrough I was able to provide to the field of T6SS effector enzymology was the discovery and characterization of diverse antibacterial phospholipases. I found that at least five phyletically distinct families of phospholipases are associated with T6SSs, two of which I confirmed to have antibacterial activity. A third was confirmed by our collaborators in Dr. Sun Nyunt Wai's lab in Umeå. I further characterized the enzymatic specificities of all three of these families, which includes the first instance of an antibacterial phospholipase D enzyme. These findings overturned prior assumptions, as, in general, secretion system associated phospholipases were thought to solely target eukaryotic cells.

a. **Russell, A.B.**, Hood, R.D., Bui, N.K., LeRoux, M., Vollmer, W. and Mougous, J.D. (2011) Type VI secretion delivers lytic effectors to the periplasm of target cells. *Nature* 475, 343-347

b. **Russell, A.B.**, LeRoux, M., Hathazi, K., Agnello, D.M., Ishikawa, T., Wiggins, P.A., Wai, S.W., Mougous, J.D. (2013) Diverse type VI secretion phospholipases are functionally plastic antibacterial effectors. *Nature*. 496, 508-512

c. Chou, S., Bui, N.K., **Russell, A.B.**, Lexa, K.W., Gardiner, T.E., LeRoux, M., Vollmer, W., Mougous, J.D., 2012. Structure of a peptidoglycan amidase effector targeted to Gram-negative bacteria by the type VI secretion system. Cell reports 1, 656–64.

d. Whitney, J.C., Chou, S., **Russell, A.B.**, Biboy, J., Gardiner, T.E., Ferrin, M.A., Brittnacher, M., Vollmer, W., Mougous, J.D., 2013. Identification, structure, and function of a novel type VI secretion peptidoglycan glycoside hydrolase effector-immunity pair. The Journal of biological chemistry 288, 26616–24.

5. *In silico* identification and genetic characterization of T6SS effectors and divergent systems across bacteria. As T6SSs and their effectors are subject to considerable horizontal gene transfer, it makes little sense to study any given gene in isolation without considering its presence (or absence) across all Gram-negative bacteria. To expand upon the impact of my work as indicated in #3, I used available bioinformatic tools to find divergent examples of effectors and T6SSs across bacteria. This began with an early effort, with a bioinformatic collaborator, to identify amidase-type effector proteins using a heuristic pipeline. With the use of heuristics, we were able to identify, in a homology independent fashion, broad groups of cell-wall degrading T6SS effector proteins, and through subsequent *in vitro* and *in vivo* assays confirm our predications. My own contributions to these efforts were in curation of bioinformatic hits, generation of assays, and genetic analyses. Having come to understand the power of broad observations, I thereafter used new, HMM-based homology tools to identify divergent T6SSs in the Bacteroidetes, a deeply branching phylum of Gram-negative bacteria. Bacteroidetes are critical components of the gut microbiota, and their interactions with other microbes has profound implications for human health. By identifying a divergent, yet still antibacterial, T6SS in this phylum I extended the study of this system to organisms whose interactions are critical for maintaining gut homeostasis.

a. **Russell, A.B.**, Singh, P., Brittnacher, M., Bui, N.K., Hood, R.D., Carl, M.A., Agnello, D.M., Schwarz, S., Goodlett, D.R., Vollmer, W., Mougous, J.D., 2012. A widespread bacterial type VI secretion effector superfamily identified using a heuristic approach. Cell Host Microbe 11, 538–49.

b. **Russell, A.B.**, Wexler, A.G., Harding, B.N., Whitney, J.C., Bohn, A.J., Goo, Y.A., Tran, B.Q., Barry, N.A., Zheng, H., Peterson, S.B., Chou, S., Gonen, T., Goodlett, D.R., Goodman, A.L., Mougous, J.D.,

2014. A Type VI Secretion-Related Pathway in Bacteroidetes Mediates Interbacterial Antagonism. Cell Host Microbe 16, 227–236.

c. Wexler, A.G., Bao, Y., Whitney, J.C., Bobay, L.-M.M., Xavier, J.B., Schofield, W.B., Barry, N.A., **Russell, A.B.**, Tran, B.Q., Goo, Y.A., Goodlett, D.R., Ochman, H., Mougous, J.D., Goodman, A.L., 2016. Human symbionts inject and neutralize antibacterial toxins to persist in the gut. Proceedings of the National Academy of Sciences of the United States of America 113, 3639–44.

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PHS 398 Cover Page Supplement

OMB Number: 0925-0001

Expiration Date: 09/30/2024

1. Vertebrate Animals Section

Are vertebrate animals euthanized? ○ Yes ● No

If "Yes" to euthanasia

Is the method consistent with American Veterinary Medical Association (AVMA) guidelines?

○ Yes ○ No

If "No" to AVMA guidelines, describe method and provide scientific justification

2. *Program Income Section

*Is program income anticipated during the periods for which the grant support is requested?

○ Yes ● No

If you checked "yes" above (indicating that program income is anticipated), then use the format below to reflect the amount and source(s). Otherwise, leave this section blank.

*Budget Period	*Anticipated Amount ($)	*Source(s)

PHS 398 Cover Page Supplement

3. Human Embryonic Stem Cells Section

*Does the proposed project involve human embryonic stem cells? ❍ Yes ● No

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://grants.nih.gov/stem_cells/registry/current.htm. Or, if a specific stem cell line cannot be referenced at this time, check the box indicating that one from the registry will be used:

❑ Specific stem cell line cannot be referenced at this time. One from the registry will be used.

Cell Line(s) (Example: 0004):

4. Human Fetal Tissue Section

*Does the proposed project involve human fetal tissue obtained from elective abortions? ❍ Yes ● No

If "yes" then provide the HFT Compliance Assurance

If "yes" then provide the HFT Sample IRB Consent Form

5. Inventions and Patents Section (Renewal applications)

*Inventions and Patents: ❍ Yes ● No

If the answer is "Yes" then please answer the following:

*Previously Reported: ❍ Yes ❍ No

6. Change of Investigator/Change of Institution Section

❑ Change of Project Director/Principal Investigator

Name of former Project Director/Principal Investigator

Prefix:

*First Name:

Middle Name:

*Last Name:

Suffix:

❑ Change of Grantee Institution

*Name of former institution:

PHS 398 Modular Budget

OMB Number: 0925-0001
Expiration Date: 09/30/2024

Budget Period: 1

Start Date: 01/01/2023 End Date: 12/31/2023

A. Direct Costs

	Funds Requested ($)
Direct Cost less Consortium Indirect (F&A)*	[redacted]
Consortium Indirect (F&A)	
Total Direct Costs*	[redacted]

B. Indirect (F&A) Costs

	Indirect (F&A) Type	Indirect (F&A) Rate (%)	Indirect (F&A) Base ($)	Funds Requested ($)
1.	MTDC	[redacted]	[redacted]	[redacted]
2.				
3.				
4.				

Cognizant Agency
(Agency Name, POC Name and Phone Number) [redacted] Email:

Indirect (F&A) Rate Agreement Date 05/23/2018

Total Indirect (F&A) Costs [redacted]

C. Total Direct and Indirect (F&A) Costs (A + B)

Funds Requested ($) [redacted]

Funding Opportunity Number: PA-20-185. Received Date: 2022-08-09T12:28:18.000-04:00

PHS 398 Modular Budget

Budget Period: 2

Start Date: 01/01/2024 End Date: 12/31/2024

A. Direct Costs

Funds Requested ($)

Direct Cost less Consortium Indirect (F&A)* [redacted]

Consortium Indirect (F&A)

Total Direct Costs* [redacted]

B. Indirect (F&A) Costs

	Indirect (F&A) Type	Indirect (F&A) Rate (%)	Indirect (F&A) Base ($)	Funds Requested ($)
1.	MTDC	[redacted]	[redacted]	[redacted]
2.				
3.				
4.				

Cognizant Agency
(Agency Name, POC Name and Phone Number) [redacted] Email:

Indirect (F&A) Rate Agreement Date 05/23/2018

Total Indirect (F&A) Costs [redacted]

C. Total Direct and Indirect (F&A) Costs (A + B)

Funds Requested ($) [redacted]

Funding Opportunity Number: PA-20-185. Received Date: 2022-08-09T12:28:18.000-04:00

PHS 398 Modular Budget

Budget Period: 3

Start Date: 01/01/2025 End Date: 12/31/2025

A. Direct Costs

	Funds Requested ($)
Direct Cost less Consortium Indirect (F&A)*	[redacted]
Consortium Indirect (F&A)	
Total Direct Costs*	[redacted]

B. Indirect (F&A) Costs

	Indirect (F&A) Type	Indirect (F&A) Rate (%)	Indirect (F&A) Base ($)	Funds Requested ($)
1.	MTDC	58.00	[redacted]	[redacted]
2.				
3.				
4.				

Cognizant Agency
(Agency Name, POC Name and Phone Number) [redacted] Email:

Indirect (F&A) Rate Agreement Date 05/23/2018

Total Indirect (F&A) Costs [redacted]

C. Total Direct and Indirect (F&A) Costs (A + B) Funds Requested ($) [redacted]

Funding Opportunity Number: PA-20-185. Received Date: 2022-08-09T12:28:18.000-04:00

PHS 398 Modular Budget

Budget Period: 4

Start Date: 01/01/2026 End Date: 12/31/2026

A. Direct Costs

	Funds Requested ($)
Direct Cost less Consortium Indirect (F&A)*	[redacted]
Consortium Indirect (F&A)	
Total Direct Costs*	[redacted]

B. Indirect (F&A) Costs

	Indirect (F&A) Type	Indirect (F&A) Rate (%)	Indirect (F&A) Base ($)	Funds Requested ($)
1.	MTDC	58.00	[redacted]	[redacted]
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number) [redacted] Email:

Indirect (F&A) Rate Agreement Date 05/23/2018

Total Indirect (F&A) Costs [redacted]

C. Total Direct and Indirect (F&A) Costs (A + B) Funds Requested ($) [redacted]

Funding Opportunity Number: PA-20-185. Received Date: 2022-08-09T12:28:18.000-04:00

PHS 398 Modular Budget

Budget Period: 5

Start Date: 01/01/2027 End Date: 12/31/2027

A. Direct Costs

	Funds Requested ($)
Direct Cost less Consortium Indirect (F&A)*	[redacted]
Consortium Indirect (F&A)	
Total Direct Costs*	[redacted]

B. Indirect (F&A) Costs

	Indirect (F&A) Type	Indirect (F&A) Rate (%)	Indirect (F&A) Base ($)	Funds Requested ($)
1.	MTDC	[redacted]	[redacted]	[redacted]
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): Arif Karim Phone # (415) 437-7859 Email: Arif.karim@psc.hhs.gov

Indirect (F&A) Rate Agreement Date: 05/23/2018

Total Indirect (F&A) Costs: [redacted]

C. Total Direct and Indirect (F&A) Costs (A + B) Funds Requested ($): [redacted]

Funding Opportunity Number: PA-20-185. Received Date: 2022-08-09T12:28:18.000-04:00

PHS 398 Modular Budget

Cumulative Budget Information

1. Total Costs, Entire Project Period

Section A, Total Direct Cost less Consortium Indirect (F&A) for Entire Project Period ($)	[redacted]
Section A, Total Consortium Indirect (F&A) for Entire Project Period ($)	[redacted]
Section A, Total Direct Costs for Entire Project Period ($)	[redacted]
Section B, Total Indirect (F&A) Costs for Entire Project Period ($)	[redacted]
Section C, Total Direct and Indirect (F&A) Costs (A+B) for Entire Project Period ($)	[redacted]

2. Budget Justifications

Personnel Justification	Personnel_Justification_Final.pdf
Consortium Justification	
Additional Narrative Justification	

Tracking Number: GRANT13694683 Funding Opportunity Number: PA-20-185. Received Date: 2022-08-09T12:28:18.000-04:00

Personnel Justification:

Emily Troemel, PhD, Principal Investigator (effort = 1.5 months summer) will be responsible for the general supervision of the project. This includes meetings several times a week with all the personnel involved in the project, mentoring, designing experiments, analyzing data and interpreting results. Emily will also take part in the research and experimental procedures as necessary.

Lakshmi Batachari, PhD student in Troemel lab (effort = 12 months calendar), will be responsible for performing analysis of DRH-1 expression and localization as described in Aim 1, as well as the characterization of DRH-1 signaling as described in Aim 2. She performed the preliminary analysis of DRH-1 localization (Fig. 3), DRH-1 structural prediction (Fig. 5) and DRH-1 N-terminal signaling, as shown in the grant (Fig. 6). She also will work on some experiments related to analysis of DRH-1 in Aim 3B. She joined the Troemel lab in July 2021, after spending her 1st year of graduate school doing rotations.

Mario Bardan Sarmiento, Staff Research Associate I (effort = 12 months calendar) will be aiding in preparation of the large number of *C. elegans* required for co-IP/MS of DRH-1 (Aim 2B), as well as helping with screening and characterization of factors acting downstream of DRH-1 (Aim 2C). He also will be preparing samples for scRNAseq in Aim 3A, and working on smFISH for Aim 3B. Mr. Sarmiento has generated all viral preps used in the lab in the past couple years, and has analyzed several mutants for viral resistance using qRT-PCR and FISH staining. He started his work in the Troemel lab in October 2019.

Yingyin (Katie) Li, Undergrad Research Assistant (effort = 3 months calendar) will be responsible for routine laboratory tasks, including preparation of media and plates for worm growth. She also will assist with experiments as needed, such as *C. elegans* genotyping, viral infections and quantification of FISH staining.

Alistair Russell, PhD, Co-investigator (effort = 0.45 months) will provide the expertise and oversight of scRNAseq studies described in Aim 3. Alistair has performed analysis of anti-viral responses in mammalian cells infected by influenza A virus, developing new pipelines for the analysis of these data. Alistair will also take part in the analyses and experimental procedures as necessary.

Postdoctoral Research in the Russell Lab, TBD (effort = 6 months calendar), with assistance and oversight of Dr. Russell, will adapt the pre-existing Russell lab scRNAseq protocols, including bioinformatic analysis, to the analysis of *C. elegans* Orsay infection data as described in 3A.

PHS 398 Research Plan

OMB Number: 0925-0001
Expiration Date: 09/30/2024

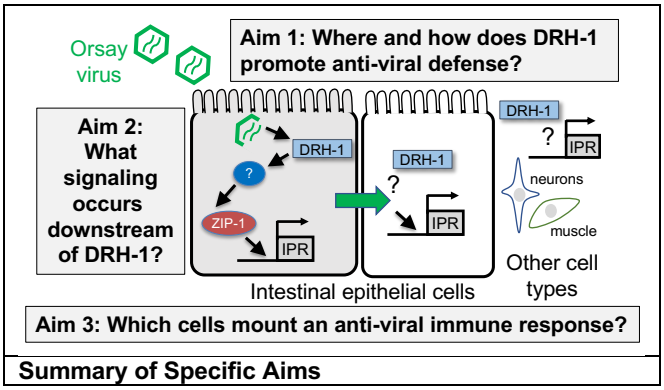
Introduction	
1. Introduction to Application (for Resubmission and Revision applications)	
Research Plan Section	
2. Specific Aims	Specific_Aims.pdf
3. Research Strategy*	Research_Strategy.pdf
4. Progress Report Publication List	
Other Research Plan Section	
5. Vertebrate Animals	
6. Select Agent Research	
7. Multiple PD/PI Leadership Plan	
8. Consortium/Contractual Arrangements	
9. Letters of Support	Letters_of_Support.pdf
10. Resource Sharing Plan(s)	Resource_Sharing_Plan.pdf
11. Authentication of Key Biological and/or Chemical Resources	Authentication_of_Key_Bio_Resources.pdf
Appendix	
12. Appendix	

Funding Opportunity Number: PA-20-185. Received Date: 2022-08-09T12:28:18.000-04:00

Specific Aims – Innate immunity against viral infection in intestinal epithelial cells of *C. elegans*

Nearly, if not every, animal on the planet serves as host to some virus. Four out of five animals are nematodes, and the Nematoda phylum originated over 400 million years ago. However, relatively little is known about viral infection and anti-viral immunity in these hosts, in part because natural, full-cycle viral infections of the model nematode *C. elegans* were only discovered as recently as 2011, with the identification of an RNA virus infecting intestinal cells of wild-caught *C. elegans* from Orsay, France. *C. elegans* provides a powerful opportunity to examine the dynamics of anti-viral innate immune responses in a transparent whole-animal host, with extensive tools available for microscopy, genetics, genomics, transcriptomics, and proteomics.

Work from my lab has identified a novel *C. elegans* immune response induced by both the Orsay virus and by microsporidia, fungal pathogens that are the only other known obligate intracellular pathogens of the *C. elegans* intestine. We named this pathway the Intracellular Pathogen Response (IPR). The IPR involves transcriptional up-regulation of about 80 genes and regulates defense against both viral and microsporidia infection. Mutant screens have identified several regulators of the IPR, including "Dicer-Related Helicase" DRH-1, a homolog of RIG-I-like receptors (RLRs) in mammals. RLRs are cytosolic receptors that sense aberrant RNA species, such as viral products containing 5' triphosphates and double-stranded RNA (dsRNA). Our work indicates that the DRH-1 receptor senses Orsay virus replication products in *C. elegans* to trigger anti-viral defense. We found that DRH-1 triggers the IPR independently of RNA interference (RNAi) components such as Dicer, which have previously been shown to provide anti-viral defense in *C. elegans*. Several aspects of the IPR, including the upstream activation by DRH-1/RLR, have similarities with the Type-I Interferon (IFN-I) response in mammals. However, *C. elegans* lacks obvious homologs of the signaling factors that act downstream of RLRs in mammals, including MAVS, IRF3, NFkB and IFN-I itself. Therefore, here we will characterize how DRH-1 controls anti-viral IPR-regulated immunity in *C. elegans* with the following **Specific Aims**:



Summary of Specific Aims

Aim 1: Where and how does DRH-1/RLR promote anti-viral defense in *C. elegans*? Although work from my lab and others has demonstrated that DRH-1 is likely the receptor that triggers anti-viral defense in *C. elegans*, its endogenous expression pattern has not been described. It is also unknown where DRH-1 acts at either the tissue or subcellular level to regulate defense. Here we will determine endogenous expression of DRH-1 and use tissue-specific rescue and depletion to determine where it promotes defense. We will also build on our unpublished data that the DRH-1 protein forms puncta upon viral infection, and analyze DRH-1 subcellular co-localization with markers for Orsay virus, as well as organelles like the mitochondria, which serve as platforms for RLR signaling in mammals via MAVS. We will also examine which virus-induced IPR genes promote defense.

Aim 2: What signaling pathway is activated downstream of DRH-1/RLR in *C. elegans*?
We have found that expression of the N-terminal domain (NTD) alone of DRH-1 will trigger the IPR in the absence of infection (unpublished data), similar to RIG-I NTD overexpression triggering IFN-I expression in mammals. Here we will characterize the effects of DRH-1 NTD overexpression on downstream signaling. We will perform a suppressor screen in a DRH-1 NTD strain to look for loss of IPR reporter expression to identify signaling factors. As an orthogonal method to identify these factors, we will perform proteomic analysis to identify binding partners of DRH-1, and examine candidates we identify for regulation of the IPR.

Aim 3: Which host cells mount an anti-viral immune response in *C. elegans*?
The major cell type infected by Orsay virus is the *C. elegans* intestinal cell, which has structural and functional similarity to human intestinal epithelial cells. The *C. elegans* intestine comprises 20 non-renewable cells, and it is unknown whether immune responses are restricted to infected intestinal cells, or if bystander cells participate in defense. Here we will use a combination of GFP reporters, Fluorescence In Situ Hybridization (FISH) and scRNAseq to determine whether bystander intestinal cells, or any other cells types in *C. elegans,* mount an immune response to viral infection, indicating there is intercellular and/or inter-tissue anti-viral signaling.

Overview: Through our analysis of responses in *C. elegans*, from the whole-animal to the molecular level, we will determine how this simple animal fights off natural viral infection of intestinal epithelial cells. Factors and mechanisms we identify may provide insight into anti-viral defense in humans, as well as inflammatory diseases.

Research Strategy - Innate immunity against viral infection in intestinal epithelial cells of *C. elegans*

A. SIGNIFICANCE

Viral infections are a major cause of death worldwide. RNA viruses in particular have been responsible for many pandemics, including those caused by influenzas and coronaviruses. These RNA viruses have been responsible for 10,000's (influenza) to 100,000's (SARS-CoV-2) of deaths in the U.S. per year. RIG-I-like receptors (RLRs) are an evolutionarily conserved class of receptors that detect viral RNA replication products in the cytosol and trigger downstream type-I interferon (IFN-I) signaling to promote anti-viral defense (1, 2). In mammals, RIG-I and MDA5 are two RIG-I-like receptors (RLRs) that promote defense, and they both have a protein structure composed of: 1) an N-terminal signaling domain that has two Caspase Activation and Recruitment Domains (2XCARD), 2) a central DEAD/H-box Helicase core, and 3) a C-terminal-binding domain or CTD (Fig. 1). The central helicase core and the CTD bind to RNA ligands characteristic of viral replication, with RIG-I binding short stretches of double-stranded RNA (dsRNA) with a 5'-triphosphate, and MDA5 binding longer stretches of dsRNA. RIG-I and MDA5 are both important players in detecting and promoting protection against a wide variety of RNA viruses, and they also have roles in inflammatory diseases. For example, mutations in the helicase domain of both RIG-I and MDA5 have been found in patients that have inflammatory diseases associated with aberrant levels of IFN-I, including systemic lupus erythematosus, Aicardi-Goutieres syndrome, and Singleton-Merten syndrome (3, 4). One proposed explanation is that mutations in the helicase domain of RLRs cause inappropriate recognition of self RNA, leading to upregulation of IFN-I signaling and inflammation (5). Thus, understanding RLR signaling is relevant to anti-viral immunity, as well as inflammatory diseases. As a sign of its significance, this proposal is submitted in response to *Notice of Special Interest (NOSI, NOT-AI-21-066): Understanding the Immune Functions of DEAD/H-box Helicases*, which include DRH-1 and other RLRs.

In the absence of ligand binding, the RLRs RIG-I and MDA-5 are held in an inactive state by their CARD domains binding to their helicase and CTD domains (6). Upon helicase/CTD binding to RNA, the 2XCARDs are released and available to interact with the CARD of the downstream signaling factor mitochondrial antiviral-signaling protein (MAVS). Oligomerization of RLRs/MAVS serves as a signaling platform that activates the kinase TBK-1, which phosphorylates the transcription factor IRF3 that enters the nucleus and induces expression of IFN-I ligands and downstream IFN-I signaling. RLR/MAVS also signal through the IKK kinase complex, which activates the NF-kB transcription factor for cytokine-mediated defense. In addition, RLRs have been proposed to act with more direct, 'effector-like' functions through binding viral RNA. Indeed, there are likely additional modes yet to be discovered by which these key viral sensors trigger protective immunity.

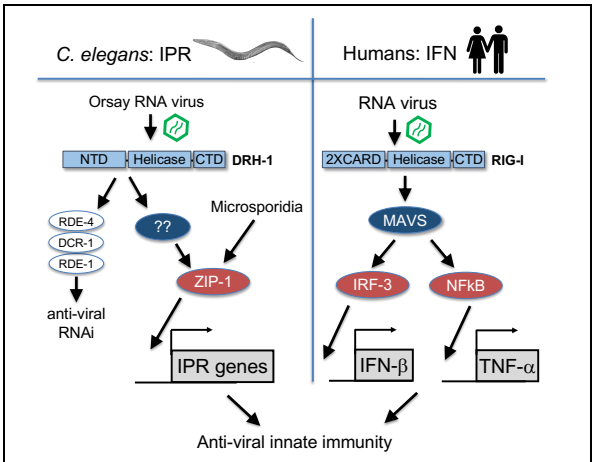


Fig. 1. Comparison of the DRH-1/IPR in *C. elegans* to the RLR/IFN-I response in humans.

There are three RLR homologs in the genome of the nematode *C. elegans*: Dicer-Related Helicase proteins DRH-1, DRH-2 and DRH-3 (7). Studies with both experimental and natural viral infection of *C. elegans* have demonstrated that DRH-1 serves as a sensor for RNA viruses, acting together with RNA interference (RNAi) factors like Dicer (DCR-1), dsRNA-binding protein RDE-4, and Argonaute RDE-1 to promote anti-viral RNAi (8-10). Work from my lab has revealed that, separate from its role in regulating RNAi, DRH-1 regulates a transcriptional response to viral infection that promotes defense (11). We named this transcriptional response the Intracellular Pathogen Response (IPR) and it has similarities with the IFN-I response (Fig. 1) (12, 13). The IPR is triggered by intracellular pathogens of the intestine, including the Orsay virus, a single-stranded, positive-sense RNA virus, as well as species in the microsporidia phylum, which are obligate intracellular fungal pathogens (14). The Orsay virus has been isolated from wild-caught *C. elegans* from Orsay and other regions of France (15, 16). This virus, together with several microsporidia species, are the only known natural intracellular pathogens of the *C elegans* intestine, and activation of the IPR induces defense against both the Orsay virus and microsporidia (13, 17, 18). Although DRH-1 has high similarity with mammalian RLRs in the helicase and CTD, and the human and *C. elegans* helicase and CTD are in fact functionally interchangeable, the DRH-1 NTD does not have primary sequence similarity with RLRs, and has no annotated CARD domains (7) (although we have found structural similarity with CARDs – see Aim 2). Furthermore, there are no obvious homologs of downstream signaling factors MAVS, IRF3, NFkB or IFN-I in *C. elegans* (19). Little is known about what factors serve these roles in *C. elegans*,

although we have defined the bZIP transcription factor ZIP-1 as acting downstream of DRH-1 to induce a subset of IPR genes upon viral infection (Fig. 1) (18). In this grant we propose to use a combination of molecular genetics, transcriptomics and proteomics to identify the factors acting downstream of DRH-1 in *C. elegans*. These factors will be different on the sequence level from what has previously been described to signal downstream of RLRs in mammals, as MAVS and other factors appear absent from the *C. elegans* genome. However, it is possible that we will find a factor with structural similarity to MAVS, despite lacking sequence similarity. Alternatively, the signaling factors we find in *C. elegans* may be completely different from the MAVS pathway, but may be similar to a pathway that is also important in mammalian hosts, providing an opportunity to discover new mechanisms of anti-viral signaling relevant to human health.

B. INNOVATION

C. elegans provides a convenient and powerful whole-animal system for studying anti-viral defense (20, 21). The lineage and precise location of every cell have been determined in this 959-somatic cell animal, which at the adult stage contains only terminally differentiated cells, other than the germline. Furthermore, microscopy is facilitated by a transparent body plan, enabling *in vivo* analysis without need for dissection. Extensive knowledge has been gained from gene-tagging in vivo, as *C. elegans* was the first animal in which transgenic GFP was deployed, and there are thousands of marker genes and strains (22). Historically *C. elegans* research has focused more on understanding cell-specificity of the nervous system, and almost nothing has been described about cell-specific functions and responses of intestinal cells. The *C. elegans* intestine is comprised of only 20 non-renewable cells that are present for the entire lifetime of the animal, and notably, *C. elegans* intestinal cells have substantial structural and functional similarity to intestinal epithelial cells in humans (23).

C. elegans anti-viral signaling occurs in the intestine. Similar to mammals, it is activated by an RLR called DRH-1, but appears to involve downstream signaling components distinct from mammals (11). Thus, innovative forms of anti-viral defense will likely be revealed through our proposed studies in a whole-animal model for response to RNA virus infection. Of note, revolutionary findings such as CRISPR/Cas9 and RNA interference have been discovered through studying anti-viral responses in non-mammalian hosts like bacteria and *C. elegans* (24, 25). Also innovative is that we propose to use a newly developed version of single-cell RNAseq (scRNAseq) (26). While scRNAseq is becoming standard in mammalian systems, it is still relatively rare in *C. elegans*. **Through developing this tool, we may have the unprecedented ability to monitor the anti-viral response in every cell and tissue-type of a whole animal host.**

C. APPROACH

Aim 1 Where and how does DRH-1/RLR promote anti-viral defense?

Background/Preliminary data

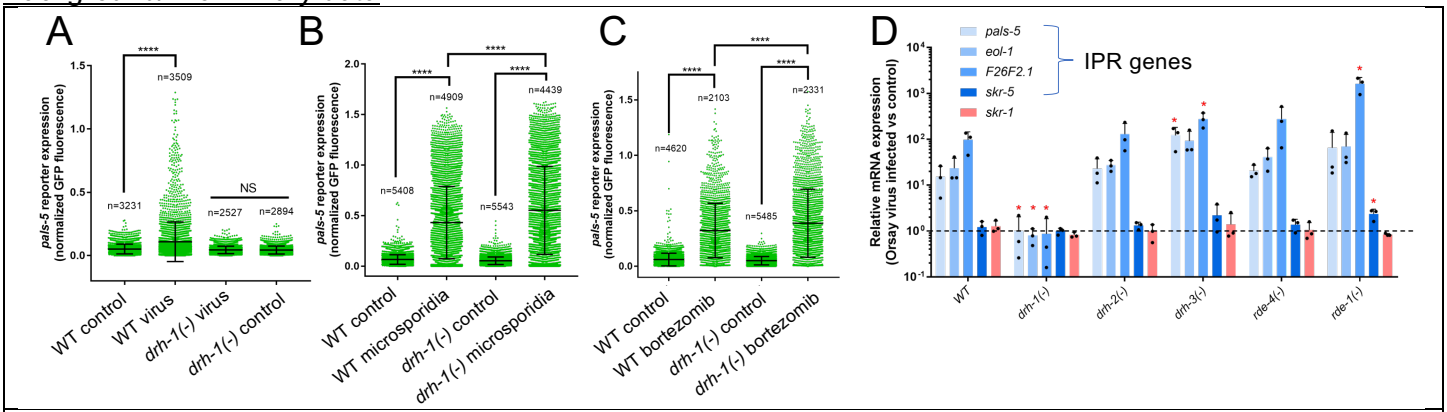


Fig. 2. DRH-1 is required for inducing the IPR upon viral infection. A) The Orsay virus induces *pals-5p::GFP* IPR reporter gene expression in wild-type (WT) animals, but not in *drh-1* mutants. B, C) *drh-1* mutants still induce *pals-5p::GFP* upon microsporidia infection and proteasome blockade by bortezomib. A-C) Each dot represents fluorescence of an animal as measured by the COPAS Biosort. Means were compared using One-way ANOVA with Bonferroni correction. NS = Not significant, ****p<0.0001. D) qRT-PCR indicates *drh-1* is required to induce mRNA expression of IPR genes; RLRs *drh-2* and *drh-3* are not required, nor are RNAi factors *rde-4* and *rde-1*. Mean of 3 independent experiments; each sample contains ~1000 animals; *p<0.05 v WT Adapted from Sowa et al 2020.

The IPR is a gene set induced by the Orsay virus and microsporidia infection (12, 27). The IPR is also induced by proteotoxic stress like proteasome blockade (of note, proteasome mutations in humans lead to induction of the IFN-I response through unknown mechanisms) (13, 27). There are about 80 IPR genes, and they include

ubiquitin ligase components, which we have found promote tolerance of proteotoxic stress. The IPR genes also include *pals* (protein containing ALS2cr12 signature) genes (13), that form an expanded gene family in *C. elegans* (39 members) compared to a single *pals* ortholog each in mice and humans of unknown function (28). The *pals* genes are defined by a divergent protein signature, and 26/39 of the pals genes are strongly upregulated as part of the IPR, including the gene *pals-5*. While this gene is of unknown function, a *pals-5* promoter-GFP reporter provides a robust and convenient read-out for IPR activation (27). Using this reporter, together with qRT-PCR and RNA-seq, we found that virus-mediated upregulation of the IPR requires the RLR homolog DRH-1. However, this receptor is not required for induction of the IPR by microsporidia or proteotoxic stress, indicating there are distinct receptors for these triggers (Figs 1, 2). Furthermore, we found that anti-viral RNAi factors like *rde-1* and *rde-4* are not required for inducing the IPR, indicating DRH-1 is acting separately from its previously described role in RNAi (Figs 1, 2) (11).

Despite the key role of DRH-1 in sensing RNA virus infection in *C. elegans* and triggering anti-viral defense, the endogenous expression of this RLR homolog has not been characterized. However, overexpression of DRH-1 driven by the ubiquitous *rpl-28* promoter has enabled investigations into the subcellular localization of DRH-1 (29). In unpublished findings, we have used this *rpl-28p::mScarlet::drh-1* strain to show that, in contrast to its diffuse cytosolic distribution in intestinal cells in the absence of infection, mScarlet::DRH-1 protein forms discrete puncta in infected animals, localizing to cells that contain viral RNA (Fig. 3). In addition, we found that *rpl-28p::mScarlet::drh-1* rescues the *drh-1* mutant defect in IPR induction, indicating that it is functional (not shown). In this Aim we will determine where and how DRH-1 regulates antiviral immunity.

A) Determine the tissue expression and subcellular localization of DRH-1

Based on RNAseq studies summarized on Wormbase, *drh-1* mRNA has been found in intestinal, as well as muscle and neuronal tissue. *Here we will examine the endogenous tissue and subcellular expression of DRH-1 protein.* First, we will use CRISPR/Cas9 gene editing to insert a *C. elegans*-optimized version of mScarlet, called wrmScarlet (30), together with 3XFLAG tag, at the N-terminus of endogenous *drh-1*, in frame with the coding region, to generate an N-terminally tagged DRH-1 protein. Once we have generated the strain, we will confirm that it does not impair DRH-1 activity by performing viral infection and then using qRT-PCR to measure viral load as well as IPR gene expression. If this tag does not substantially impair DRH-1 function, we will examine its anatomical location. Here we will use our Zeiss LSM700 confocal microscope to determine whether, like the RNAseq results for *drh-1* mRNA, DRH-1 protein is found in the intestine, muscle and neurons, or if it has a different tissue distribution. In order to boost expression, we will infect with virus, which upregulates *drh-1* mRNA levels, and may also increase signal due to clustering of protein.

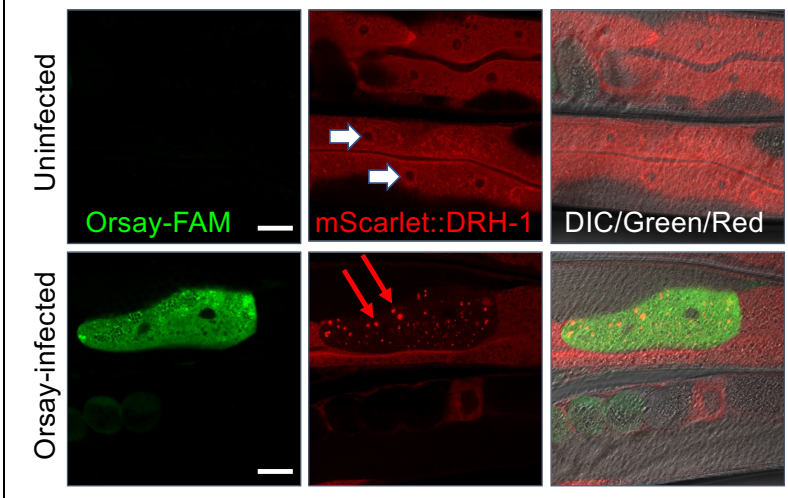


Fig. 3. wrmScarlet::DRH-1 protein forms puncta in Orsay virus-infected intestinal cells. Left: Green channel shows a FAM-labeled FISH probe for Orsay virus RNA; center: red channel shows *rpl-28p::mScarlet::DRH-1*. Right: overlay of red, green and DIC/bright-field. In uninfected animals (top row), no puncta were observed (0% or 0/34 animals). In infected animals (bottom row), puncta were observed in 100% of infected animals (39/39 animals). Red arrows indicate puncta; white block arrows indicate nuclei. Scale bar = 25μm.

Next, we will examine whether endogenously expressed wrmScarlet::DRH-1 forms puncta upon viral infection of the intestine, similar to what we have seen with the transgenic *rpl-28p::mScarlet::drh-1* strain (Fig. 3). If puncta are formed, we will assess where they are located by crossing into strains that have GFP-tagged organelles including mitochondria, the endoplasmic reticulum and the lysosome. Mammalian RLRs like RIG-I and MDA5 have been shown to localize to mitochondria upon activation by viral RNA ligands, and so we will explore this organelle first (31, 32). In addition to examining GFP-labeled mitochondria for co-localization to DRH-1, we will also use the Mitotracker dye. If there are negative results for localization with mitochondria with these two approaches, we will examine localization to the ER and lysosomes, using GFP markers and lysotracker dye (for the lysosome). If there are negative results with these studies, we will expand to other subcellular markers such as markers of the Golgi/endosome network.

To complement the analysis of DRH-1 protein, we will examine tissue distribution of *drh-1* transcription, by fusing the wrmScarlet::3XFLAG tag at the start codon of *drh-1*, together with a stop codon to generate soluble wrmScarlet protein under the control of *drh-1* regulatory regions. This approach will likely lead to higher levels of wrmScarlet expression than the tagged protein, and thus may better inform us about the tissue distribution of *drh-1*, which is relevant to Aim 1B

Potential outcomes, pitfalls and future directions

Here we expect that DRH-1 will be expressed in at least the intestine, and possibly other tissues like muscles and neurons, given previous mRNA profiling data. However, protein expression does not always reflect mRNA expression, and we may observe a distinct distribution. Given that we have used the CRISPR/Cas9 technique previously to tag other genes, such as the transcription factor *zip-1* that acts downstream of *drh-1 (18)*, we expect generating an endogenously tagged *drh-1* strain should be relatively straightforward and will not impair function. Indeed, the overexpressed N-terminal tag on DRH-1 above allowed it to be tracked and also to be functional. However if we are unable to insert the entire wrmScarlet::3xFLAG into the endogenous *drh-1* locus, or if the full tag impairs function, we can instead insert a smaller 'split wrmScarlet' tag, which is easier to insert and less likely to interfere with function. This strain can then be crossed with strains that express the other part of wrmScarlet under either a ubiquitous or a tissue-specific promoter. (We prefer to start with the full-length wrmScarlet because it is more well-established). In terms of subcellular localization, we expect that DRH-1 will form puncta localized to the mitochondria upon infection, similar to RLRs in mammalian cells. A major concern here is that expression will be low, given the low mRNA levels we see for *drh-1* in RNAseq studies. Therefore, if protein expression is visible but too dim for robust colocalization studies by confocal microscopy, we will use CRISPR/Cas9 to insert seven tandem copies (7X) of the split wrmScarlet tag at the *drh-1* locus, which has been successful in boosting expression in other studies (33, 34). If we still see very dim expression with this approach, or if we see no expression with the 'non-split' wrmScarlet approach described in the Aim, we will generate a *drh-1 promoter::drh-1 cDNA::unc-54 3'UTR* construct and insert this as single-copy into the genome, as well as in multi-copy as an extrachromosomal array. Adding an *unc-54* 3'UTR has been successful in boosting expression in many other studies. If these approaches are still not successful, we can use the existing ubiquitously expressed *rpl-28p::wrmScarlet* strain to image DRH-1 subcellular distribution.

In terms of assessing *drh-1* tissue distribution, if none of the methods above yields robust wrmScarlet expression, we will instead generate a transcriptional fusion containing 2 kb of the upstream region of the *drh-1* start codon (this is often more than enough regulatory region to drive rescue in *C. elegans*) and fuse it to GFP, and then examine animals containing a multi-copy extrachromosomal array of this transgene. These types of transcriptional reporters are almost always brighter than translational reporters or endogenous tagging, and allow for better visualization of the tissue distribution of genes with lower expression. Expression of this transcriptional reporter would then be used as the basis for tissue-specific analysis of DRH-1 described in Aim 1B below. A final back-up strategy would be to depend on existing RNAseq data for experiments in Aim 1B.

B) Tissue-specific rescue and depletion of DRH-1, to determine where it acts to regulate resistance

Next we will determine where drh-1 is required and sufficient to induce the IPR upon viral infection. First, we will use the well-characterized *vha-6* promoter to drive expression of *drh-1* specifically in the intestine. A *vha-6p::wrmScarlet-3XFLAG::drh-1* construct will be introduced as a single-copy into a *drh-1* mutant background, using the Cas-SCI (CRISPR/Cas-Single Copy Insertion) technique that we have used successfully in the past (17). Based on findings from Aim 1A, we will also perform rescue experiments that drive expression of *drh-1* in other tissues where endogenous expression is observed, using established promoters such as *myo-3* for muscle*, rab-3* for neurons, and *dpy-7* for epidermis. The wrmScarlet tag will enable confirmation of the tissue-specificity of each construct. These strains will be subjected to Orsay virus infection followed by measurement of viral load by qRT-PCR and IPR induction to assess the level of rescue. In addition to determining where *drh-1* is sufficient to rescue IPR gene induction by viral infection, we will also determine where it is required using the auxin-mediated protein degradation method (35). Here, we will tag endogenous *drh-1* with a GFP::AID degron tag and then cross this strain with existing *C. elegans* strains that have tissue-specific expression of the ubiquitin ligase adaptor TIR1 to cause degradation of DRH-1 in specific tissues. We and others have successfully used this technique in the past (36). Again, we will assess viral load and IPR gene expression using qRT-PCR.

Potential outcomes, pitfalls and future directions

Based on Orsay virus tropism for the intestine, and the previously described expression of *drh-1* in this tissue, we expect *drh-1* will be both required (as assessed by auxin degradation experiments) and sufficient (as

assessed by rescue experiments) in the intestine for antiviral response. However, it may be more complicated and there may be cross-tissue signaling. Indeed, there are several examples of cross-tissue immune signaling. For example, oomycetes, eukaryotic natural pathogens of the epidermis, appear to be sensed by neurons, which signal to the epidermis (37). Thus, although unlikely, it is possible that other tissues actually sense viral infection, and then send a cross-tissue signal to the intestine to trigger defense. If we are unable to achieve robust degradation of DRH-1 with the AID system, or if there are issues with basal degradation, we can turn to the newer AID2 system that includes improved versions of the ligand, ubiquitin ligase adaptor, and degron tag (38). Another option is to use tissue-specific RNAi, as there is a newer, improved version that has recently been described (39). However, we have experienced issues with tissue-specific RNAi techniques of *C. elegans* in the past, including lack of specificity and efficacy (18, 39), and so we prefer to first use auxin-mediated depletion. Furthermore, auxin-mediated depletion acts more quickly than RNAi, and thus we can more easily examine acute effects of losing *drh-1*. Further analysis of anti-viral signaling on a single-cell level is proposed in Aim 3.

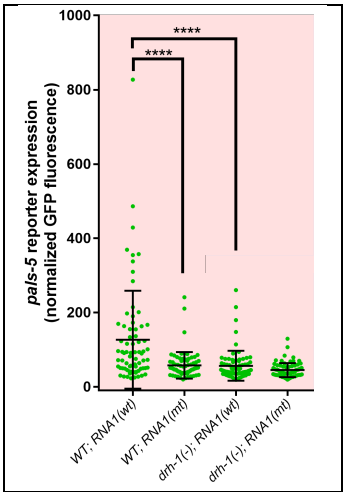


Fig. 4. Ectopic expression of Orsay RNA1 with RDRP(WT) induces *pals-5p::GFP* in WT *C. elegans*, but not in *drh-1* mutants. Expression of RNA1 with RDRP(mutant) fails to induce *pals-5p::GFP*. ****p<0.0001. From Sowa et al 2020.

C) Determine which IPR genes mediate antiviral defense downstream of *drh-1*

In an effort to understand what aspect of viral infection activates DRH-1, we previously analyzed the transcriptomic response in *C. elegans* strains that ectopically express the Orsay virus genome segment called RNA1 (Orsay virus has two genome segments, RNA1 and RNA2) (11). The only known open reading frame on RNA1 codes for an RNA-dependent RNA polymerase (RDRP), which is an enzyme that binds to a viral ssRNA template and catalyzes viral RNA replication, generating dsRNA and other viral replication products. We compared the host response to ectopic expression of RNA1 containing wild-type RDRP with the host response to catalytically inactive RDRP; wild-type RDRP catalyzes RNA1 replication, while mutant RDRP does not. In these studies, we found that most of the IPR genes were induced upon ectopic expression of WT compared to mutant RDRP, and this induction was dependent on the presence of wild-type *drh-1* (*pals-5* reporter expression shown in Fig. 4). Therefore, similar to RLRs in mammals, DRH-1 likely detects viral RNA replication products to induce the IPR. Of note, we performed RNAseq experiments in an RNAi-defective *rde-1* mutant background, further supporting the distinction of the IPR and RNAi. These studies identified 58 high-confidence genes that are: a) induced by Orsay virus infection, b) induced by WT RDRP expression, and also c) depend on wild-type *drh-1* for their induction. Here we will investigate the role of these genes in anti-viral defense. These genes include 22 *pals* genes including *pals-5*, as well as 8 cullin-ring ubiquitin ligase components including Skp-related protein *skr-4* and F-box protein *fbxa-75*, which we previously have shown to be important for thermotolerance associated with IPR upregulation (40). There are other genes of predicted biochemical function on this list such as the *eol-1* RNA de-capping enzyme (41), as well as genes of unknown function, such as *F26F2.1*, both of which are robustly induced as part of the IPR and discussed further in Aim 3. Here we will determine whether knock-down of any of these genes leads to increased viral load, by using RNAi knockdown and then measuring Orsay virus RNA1 levels with qRT-PCR. Our previous preliminary results with RNAi against the cullin-ring ubiquitin ligase components *cul-6, skr-3, skr-4, skr-5, fbxa-75* indicate they promote resistance (27), and thus we will investigate the phenotypes of mutants in these genes here. Any genes that show an effect with RNAi will be then analyzed through gene deletions, either by using existing strains from the knock-out consortium and publicly available from the *Caenorhabditis Genetics Center*, or by generating them in-house with CRISPR/Cas9 using established protocols.

Potential outcomes, pitfalls and future directions

While the RNAi experiments in a wild-type background may yield mostly negative results because of redundancy among the multiple upregulated genes, we will note that we have found several independent cullin-ring ubiquitin ligase components that are each completely required for increased thermotolerance associated with IPR activation, despite the extensive expansion of this gene family in *C. elegans*. Furthermore, we have found individual roles for several *pals* genes like *pals-22* and *pals-25*, which are not upregulated by infection but control expression of all IPR pals genes and regulate anti-viral resistance (13). We also have already made overexpression strains for several of these genes, such as *cul-6*, where overexpression drives increased thermotolerance, as well as *pals-5*, where overexpression reduces formation of protein aggregates (40, 42). If

we do not find any IPR genes that have a significant effect on viral load in a wild-type background either through their loss or overexpression, we will next test whether loss of any of these genes suppresses the increased viral resistance of *pals-22* mutants, which have >10X lower viral load than wild-type animals (13). Because these mutants constitutively express IPR genes prior to infection, it may be easier to see a role for IPR genes in this background. After our RNAi, mutant and overexpression studies are completed, we will take the top five genes with the greatest effect and investigate their expression and subcellular localization of encoded proteins to determine whether they may for example, be proteins that target viral components. Of particular interest may be ubiquitin ligase adaptors such as MATH proteins and F-box genes, which are greatly expanded in the *C. elegans* genome, perhaps as part of a cytosolic anti-viral defense strategy (43). Ubiquitin ligases like TRIM5 act as anti-viral restriction factors in primates (43).

Aim 2: What signaling pathway is activated downstream of DRH-1/RLR in *C. elegans*?

Background/Preliminary data

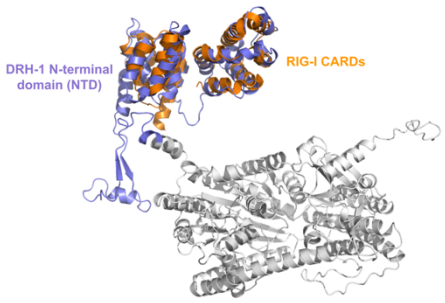


Fig. 5. AlphaFold prediction of DRH-1 (gray/purple). RIG-I CARDs (orange) are superimposed on the N-terminus (purple) of DRH-1. Unpublished data.

As mentioned in the Significance section, DRH-1 has high similarity to human RLRs in the helicase and CTD domains, and the human versions of these domains will functionally substitute for DRH-1 in *C. elegans*. In contrast, the NTD of DRH-1 does not have high primary sequence similarity to the NTD of human RLRs, which contains two CARDs. However, in unpublished data, we have used the Alpha Fold prediction program to analyze the DRH-1 NTD, and have found that it is predicted to have high 3-D structural similarity to human 2XCARD (Fig. 5). Thus, despite the sequence divergence, this region may have similar structure and function as human 2XCARDs, which are the signaling domains that trigger downstream signaling through CARD-CARD interactions with the CARD in the signaling factor MAVS. Early characterization of RLRs like RIG-I demonstrated that expression of just the 2XCARDs alone will trigger downstream signaling in the absence of viral infection (44). To determine whether DRH-1 may act similarly, we generated transgenic *C. elegans* strains that express just the DRH-1 NTD fused to wrmScarlet, under the control of the strong, intestinal-specific *vha-6* promoter. Excitingly, we found that these DRH-1-NTD::wrmScarlet strains express the *pals-5p::GFP* IPR reporter in the absence infection, suggesting that the NTD domain is capable of activating the IPR (Fig. 6). In this Aim we will further characterize these effects, and determine what signaling pathway is activated downstream of DRH-1.

A) Characterize downstream signaling caused by over-expression of DRH-1-NTD

The results described above and shown in Fig. 6 were performed by generating transgenic strains that contain extrachromosomal arrays, which have mosaic expression and are stochastically inherited each generation. In order to characterize the effects of DRH-1-NTD, we first will generate strains that have this transgene integrated into the genome, to enable the transgene to be transmitted at 100% frequency each generation and stabilize expression across the intestine. Using a standard and successful protocol for integration (27), we will take transgenic strains that have roughly 50% transmission frequency and treat them with UV/psoralen to generate double-stranded DNA breaks, then clone out F2 animals with 100% transmission efficiency of *DRH-1-NTD::wrmScarlet*, to identify strains where this transgene has integrated into the genome. Once we have isolated integrated strain(s), back-crossed them 3X and confirmed *pals-5p*::GFP expression, we will characterize these strains in several ways. (It can be helpful to isolate multiple integrated strains because they may show slightly different expression levels, and integration on different chromosomes can be useful when crossing with mutants or transgenes inserted in varying chromosomal locations). We will examine whether integrated strains are signaling similarly to the wild-type DRH-1 in three ways. First, we will ensure that DRH-1-NTD acts upstream of the transcription factor ZIP-1 (Fig. 1). Here, we will treat *DRH-1-NTD::wrmScarlet;pals-5p::gfp* animals with *zip-1* RNAi to determine whether this blocks *pals-5p::GFP* expression. Second, we will cross the integrated *DRH-1-NTD::wrmScarlet* into a *zip-1::gfp* strain, which exhibits nuclear ZIP-1::GFP expression upon viral infection in a manner dependent on *drh-1* (18), to determine whether DRH-1-NTD triggers ZIP-1::GFP expression in intestinal nuclei. Third, we will examine whether DRH-1-NTD expression in the intestine triggers IPR gene expression more broadly by performing RNAseq of this strain, and comparing expression to a non-transgenic strain. Fourth,

we will determine whether DRH-1-NTD expression triggers increased resistance to viral infection compared to wild-type animals, using our standard viral load assays.

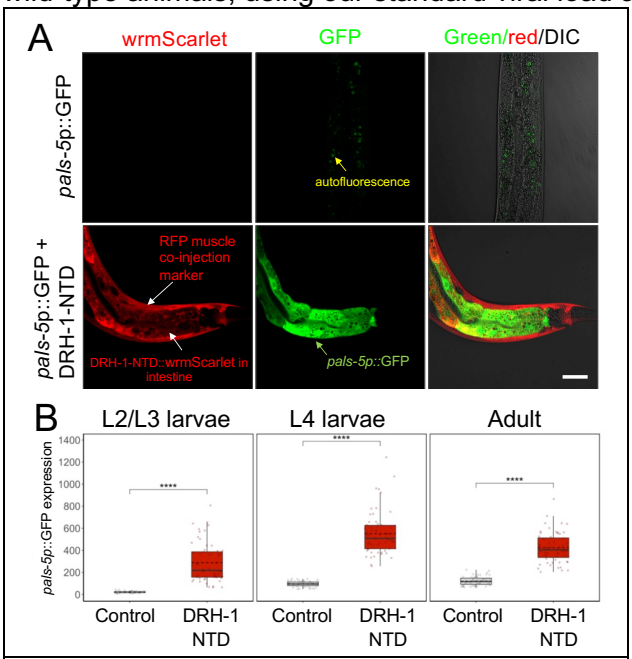


Fig. 6. Overexpression of DRH-1-NTD::wrmScarlet in the intestine induces *pals-5p::GFP* expression. A) Top is *C. elegans* with *pals-5p::GFP* reporter, bottom is *C. elegans* with *pals-5p::GFP* reporter and *vha-6p::DRH-1-NTD::wrmScarlet*, together with *myo-3p::mCherry* muscle co-injection marker. Scale bar 25 µm. B) DRH-1-NTD overexpression induces the IPR reporter across different life stages. Two independent experimental replicates were performed, and 60 animals were analyzed for each condition. The box reflects the 25th to 75th percentiles. The solid line represents the median and the dashed line represents the mean. A two-tailed t-test was used to determine significance, ****p<0.0001.

Potential outcomes, pitfalls and future directions

Given our preliminary results indicating the robust induction of *pals-5p::GFP* expression upon DRH-1-NTD expression from extrachromosomal arrays (Fig. 6), we expect that we will see endogenous *pals-5* upregulation, as well as other IPR gene expression with our RNA-seq analysis of an integrated version of this strain. We also expect that *zip-1* will be required for *pals-5p::GFP* expression and that this transcription factor will be visible in the nuclei of animals with integrated DRH-1-NTD expression. If for some reason we are unable to integrate the strain, we will perform our analyses on existing extrachromosomal arrays strains, using the COPAS Biosort to isolate transgenic animals for RNAseq, which is what we did for our RNAseq analysis of ectopic RNA1 expression (11). To cope with mosaic expression, we can use our FISH based assay to stain for viral RNA and assess infection level, only analyzing cells in which DRH-1-NTD is present. If we see that NTD DRH-1 drives expression of only a subset of genes induced by full-length DRH-1, this would indicate that the helicase and CTD may be involved in signaling in addition to the NTD, and would help sub-divide different downstream arms of DRH-1 signaling.

B) Perform Co-Immunoprecipitation/Mass Spectrometry (co-IP/MS) to find binding partners of DRH-1/RLR

The results mentioned above indicate that the NTD of DRH-1 is the signaling domain of this RLR, similar to mammalian RLRs like RIG-I and MDA5. Upon viral infection, the 2XCARDs of RIG-I and MDA5 are released to bind the CARD domain of the signaling factor MAVS and activate downstream signaling and IFN-I expression. Here we will explore whether there is a MAVS analog for DRH-1, or instead some very different binding partner that mediates DRH-1 signaling, by performing co-IP/MS to identify binding partners of DRH-1. Based on our previous experience with co-IP/MS studies, and the observation that *drh-1* mRNA is expressed at relatively low levels, we expect that we will need to use an overexpressed version of DRH-1 to identify a substantial number of binding partners with co-IP/MS. Therefore, we will use the strain described in Aim 1B, where we use an intestinal-specific *vha-6* promoter and drive expression of a wrmScarlet-3XFLAG-tagged version of DRH-1, and insert this as a single-copy using the CasSCI approach. We will also generate a *vha-6p::wrmScarlet-3XFLAG* single-copy strain for control comparison. These two strains will be infected with virus or control, and we will perform co-IP/MS studies with experimental replicates in triplicate, using 200,000 animals per sample, as per our previously successful co-IP/MS studies (40). We will perform MS at the UCSD Biomolecular and Proteomics Mass Spectroscopy Facility using their new, highly sensitive system, the UltiMate 3000 RSLCnano in tandem with the Orbitrap Fusion Lumos Tribrid Mass Spectrometer (see letter of support from Core Director Majid Ghassemian). Once we have identified proteins that show a significant interaction with wrmScarlet-3XFLAG-DRH-1 compared to wrmScarlet-3XFLAG control, we will explore whether these factors regulate the IPR using RNAi knock-down followed by viral infection, and measure IPR gene expression in both the presence and absence of infection, as well as viral load by qRT-PCR. We will perform RNAi against our highest confidence hits, and any genes which show an effect with RNAi will then be analyzed through gene deletions, as described in Aim 1C.

Potential outcomes, pitfalls and future directions

The workflow described above has been successful in identifying interactors for other proteins we have studied in the past, so we do not anticipate technical difficulties. However, each protein is different, and it is possible that protein-protein interactions with DRH-1 are too transient to be easily identified with this approach. In this case

we can use the TurboID method, which uses a promiscuous biotin ligase to biotinylate proximal proteins. We have expertise with this system, as we previously developed a related biotin ligase technique to identify effector proteins in *C. elegans* (45, 46). In addition to the strains proposed above, we can include integrated DRH-1-NTD strains if they are successfully made as described in Aim 2A, to perform co-IP and identify proteins that specifically interact with the N-terminal signaling domain of DRH-1. Depending on results with endogenous tagging of DRH-1 with wrmScarlet::3xFLAG as proposed in Aim 1, we can also use these strains, which could reduce the likelihood of false positive interactors that might be identified from analysis of overexpressed DRH-1.

One potential pitfall to our functional validation using RNAi knock-down is the possibility that we may have false negatives, i.e. miss a true hit because of insufficient knock-down of gene expression by RNAi. Thus, if we do not find positives with our highest confidence hits, we can expand the list, pending the number of significant hits from co-IP. We can also investigate the possibility of insufficient knock-downs by testing deletions of the top hits from co-IP/MS and then assessing their IPR induction and viral load. These strains will also be useful in the case there are redundant signaling pathways downstream of DRH-1, and we do not see effects with loss of just one gene. We will also prioritize these analyses based on characteristics of the hits, including known signaling domains, localization, homology with other genes, and whether genes are implicated in other immune responses. Of note, we have performed yeast two-hybrid (Y2H) studies with DRH-1 and identified several candidate interactors from that approach in unpublished data. However, upon follow-up analysis of the top 14 hits with RNAi knock-down, we did not find any candidates that had an effect on endogenous IPR gene expression. These negative results may have been because of redundancy. Fortunately, we now have this Y2H dataset that we can use for cross-reference with our co-IP/MS data. If there is a candidate from both co-IP/MS and Y2H datasets that does not have a phenotype on its own with RNAi, we can prioritize testing a deletion of that gene. In that same deletion mutant, we can also perform RNAi against other candidate interactors, which can reveal functional roles that are redundant in a wild-type context. For any proteins that function with DRH-1 to promote the IPR, we will perform co-localization studies in vivo with DRH-1, as described in Aim 1A, to identify proteins that may act as signaling factors downstream of DRH-1.

Ultimately, we expect to identify how DRH-1 signals. If we find a factor that directly binds DRH-1, and whose loss prevents IPR induction upon viral infection, this factor would represent an IPR activator, perhaps similar to MAVS. Alternatively, we may find a factor that binds DRH-1, and whose loss leads to IPR induction in the absence of infection. This factor would be a candidate IPR repressor that is de-repressed by DRH-1, in order to turn on the IPR upon viral infection. Such findings would make a novel contribution to our understanding of RLR signaling mechanisms, which are generally thought to involve a cascade of activators.

C) Forward genetic screen to identify signaling factors between DRH-1 and ZIP-1 transcription factor

As an orthogonal method to the proteomics approach described above, here we propose to identify which signaling factors act downstream of DRH-1 using a forward genetics approach. We will use the easy-to-screen phenotype of the constitutive *pals-5p::GFP* expression we have seen with the DRH-1-NTD overexpression described in Aim 2A. Using a standard EMS mutagenesis approach, we will identify F2 animals that lack *pals-5p::GFP* expression. As validation, we will test whether endogenous *pals-5* and other IPR gene expression is reduced in these mutants. Of mutants that pass this secondary screen, we will identify the causative mutations using whole-genome sequencing, looking for predicted loss-of-function SNP mutations in recessive mutants. Recent analyses in *C. elegans* have demonstrated that traditional characterization by backcrossing and complementation analysis is often not necessary to identify mutations where the same gene is isolated in more than one mutant. Instead, mutations are determined through comparison of SNP calls in several mutants (47). If however, we are unable to identify causative alleles for our mutant with this faster approach, we will perform back-crossing, mapping and complementation, as we have done successfully in prior genetic screens (12, 13, 17). Once candidate genes are identified, we will confirm the IPR phenotype with mutants as described above.

For any genes we identify that are required for DRH-1-NTD-mediated activation of *pals-5* gene expression, we will cross mutations in these genes into a wild-type background to confirm that they are required for viral-mediated induction of the IPR. We will also analyze whether they impact ZIP-1::GFP nuclear localization, which occurs downstream of viral-mediated DRH-1 activation (18). We will perform RNAseq to determine the full suite of genes that they control, both in a basal situation and in a viral-induced state. Previously we have found that ZIP-1 controls the expression of about 37% of the IPR genes, so we may find two classes of factors required for DRH-1-NTD activation of *pals-5p::GFP* expression; one gene class would affect all IPR genes, and the other would only affect the ZIP-1-controlled subset of IPR genes. We will also determine their expression through CRISPR-Cas9-mediated tagging of the endogenous gene, and analyses as described in Aim 1A.

In the unlikely scenario that our studies in Aim 2A with DRH-1-NTD activation do not robustly activate endogenous IPR genes, we can perform screens using *pals-5p::GFP* activation by either virus infection, or by heat-shock induction of RNA1, and perform similar follow-up analyses.

Potential outcomes, pitfalls and future directions

Like Aim 2B, a major goal of Aim 2C is to identify the downstream signaling component for RLR signaling in *C. elegans*. Unlike Aim 2B, the screen we propose in Aim 2C may also identify factors that do not directly interact with DRH-1, but rather act more proximal to the transcription factor ZIP-1, or perhaps act as a transcription factor together with ZIP-1. If the factor(s) that act downstream of DRH-1 are encoded by essential genes, then we may not identify them with this approach, although EMS screens regularly identify partial-loss-of-function mutations. However, if we are not successful with this mutagenesis approach, an alternative strategy is to perform a whole-genome RNAi screen, which we have performed in the past for several phenotypes (48).

Aim 3 Which host cells mount an anti-viral immune response in *C. elegans*?

Recent work from co-Investigator Russell together with his collaborators, has revealed the robust restriction of influenza A viral growth in responding bystander (uninfected) mammalian cells (49). Incredibly, at late timepoints, all epithelial cells in a mouse lung were responding to interferons. This response dramatically limits viral expansion. Despite the damaging impacts of interferons, this global response is nevertheless required for clearance of a viral infection, and is subject to subsequent negative feedback loops that, ideally, limit immunopathology once viral infection is cleared (50-53). Is a similar response observed in *C. elegans*? How much response is found in bystander (uninfected) cells in this host? Little is known about these questions, as previous genome-wide analyses of the *C. elegans* transcriptional response to viral infection have all relied on whole-animal RNAseq (11, 54, 55). These studies have helped to define the IPR genes, some of which we have analyzed for their tissue distribution using GFP reporters and smFISH to provide preliminary insight about bystander cells. These genes include cullin ring ubiquitin ligase components such as Skp-related proteins *skr-3* and *skr-5*, as well as the Cullin *cul-6*, all of which promote thermotolerance and preliminarily appear to regulate resistance to viral infection (27, 40). These genes are expressed in the intestine, as well as the pharynx. We have also analyzed tissue expression of *pals-5* and the RNA de-capping enzyme *eol-1*, which are expressed in the intestine as well as in the nervous system (12, 29, 41). Altogether, we have identified several IPR genes expressed in the intestine, which is likely due to the fact that much of the anti-viral response occurs in this tissue. However, this intestinal bias is also likely due to the nature of whole-animal RNAseq, which averages changes across the entire animal and is biased toward larger tissues such as the intestine, which comprises about 1/3 the entire mass of *C. elegans.*

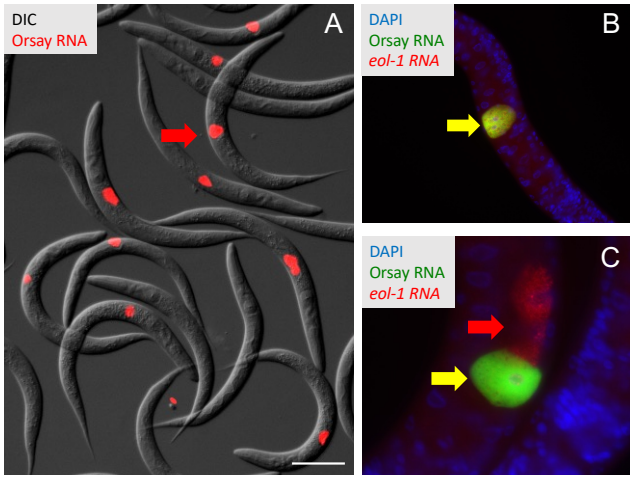


Fig. 7. FISH staining for viral RNA and IPR response. A) Orsay red FISH probe staining shows 1-3 infected intestinal cells in most worms, Scale bar = 100 µm, from Reddy et al 2019. B) Yellow arrow indicates a cell exhibiting colocalization of Orsay virus RNA (green) + *eol-1* RNA (red). C) Yellow arrow indicates cell with Orsay virus RNA (and low *eol-1* RNA) staining is adjacent to cell with *eol-1* RNA staining (red), but no virus staining, indicated with red arrow. B-C) Unpublished.

In order to analyze which cells respond to viral infection, we have performed colocalization in the intestine for FISH staining against viral RNA and smFISH staining against the IPR gene *eol-1*. Here we have found that at 11 hours post-inoculation (hpi), 100% of animals with detectable viral RNA express *eol-1* in the infected cells, while 34.5% of neighboring cells with undetectable viral RNA also express *eol-1* (Fig. 7). These results indicate there may be cell-to-cell signaling from infected to uninfected cells, and there may be a role for bystander cells in mounting an immune response. This result is intriguing, as while systemic RNAi has been observed in *C. elegans* (56), a cell-to-cell signaling system analogous to interferons has yet to be described in this host. Intercellular signaling is a major component of the vertebrate host antiviral response; a transcriptional response in an infected cell is a clear target of antagonism by the virus and, at least in acute models of infection with vertebrate viruses, is generally too slow to actually antagonize viral replication. While an intercellular innate immune signal can also be antagonized, rare, successful, detection of a viral pathogen in vertebrate systems generates a systemic

IFN-I antiviral response, which also “primes” other cells for enhanced viral detection, permitting a positive feedback loop that overcomes viral antagonism (57).

As further evidence that there is some level of intercellular signaling mediated by the IPR, the nervous system appears to play a role in *C. elegans* survival upon viral infection, as work from David Raizen’s lab has shown how animals defective in the neurons that control sleep exhibit decreased survival upon viral infection (see Letter of Support from Prof. Raizen). This result ties into several examples of neuro-immune signaling in *C. elegans* (58), although no inter-tissue signaling molecules have yet been found to regulate anti-viral defense. Of note, several virally induced genes are predicted to be secreted, one or more of which may play a signaling role and promote ‘inflammation’, similar to IFN-I and other cytokines in mammals. *C. elegans* lacks homologs not just of IFN-I, but also other canonical cytokines like TNF-alpha. Could DRH-1 activation of the IPR lead to a systemic response mediated by cytokine-like molecules? Or, alternatively, does the *C. elegans* IPR manage to control infection in the absence of a response from bystander, uninfected, cells? If so, what is the observed impact on viral replication at the cellular level in animals with, and without, intact IPR signaling?

Here we will use single-cell RNAseq (scRNAseq) and FISH staining to address the questions raised above. Specifically, we will investigate: a) which tissues mount a response to viral infection? b) what sort of response is mounted by uninfected intestinal cells? c) which virally induced genes have secretion signals and are intestinally expressed, and thus may act as cytokines? and d) what role does *drh-1* play in infected vs. uninfected intestinal cells?

A) Perform scRNAseq on virally infected animals to determine which cells mount a response to viral infection

Little is known about the role of cell-intrinsic defense vs systemic responses coordinated by bystander cells in *C. elegans*, as relatively few RNAseq studies have used methods other than whole-animal analysis for any question (59-62). Adult *C. elegans* are only 1 mm long, and a major challenge has been dissociating this tiny animal into separate tissues and cells because it has a very tough cuticle that can be difficult to disrupt. Nonetheless, a growing number of studies are reporting optimized dissociation techniques that have enabled both tissue-specific RNAseq, as well as scRNAseq. In particular, a recently reported method on bioRxiv from *C. elegans* pioneer Cynthia Kenyon and her group at Calico (see Letter of Support) describe an optimized method for cell recovery through methanol fixation, as well as optimized methods for increasing signal from somatic cells. This method produced a dataset of 47,423 cells quantifying 20,305 genes across a time-series of *C. elegans*, which provides a useful reference set for our studies (26). We will employ a related version of this method here to investigate response to viral infection on the single-cell level, comparing infected and uninfected cells.

We will first infect *drh-1* knockout mutants and control animals with Orsay virus, producing datasets wherein we may observe changes with, and without, intact sensing. Differences could thus be attributed, directly or indirectly, to the IPR. For our first set of experiments, we will use an empirically derived dose of Orsay virus at which we observe 100% of animals infected. Here we will use an *rde-1* mutant background, which leads to higher levels of infection due to deficiency in RNAi. Future experiments may use lower viral inoculum to test more natural transmission routes, but our initial efforts will benefit from a saturated system. We will thereafter harvest animals for collection at 8h, 16h, and 24h, to take measurements over the breadth of infection. Then, following the established protocol of Cynthia Kenyon’s group, we will prepare single-cell suspensions for reverse-phase emulsion generation in the 10x Genomics platform. One exception is that we will not sort cells based on DNA content, as the original protocol would exclude intestinal cells, which are over 4N due to endoduplication. The reason to sort based on DNA content is to exclude germ cells. To minimize this problem, we will use younger animals that have much less germ tissue, and infect at the first larval stage (L1).

Viral infection and the innate immune response are interesting problems to probe using scRNAseq, but they have a unique set of challenges. In both cases, we will want to call bimodal, or “on/off” events, requiring an empirically derived threshold for positivity. While “off” or “uninfected” cannot be definitively claimed, as a true negative is impossible to define, we may, with appropriate controls, call true positives. To do so we will need to correct for the contributions of extracellular RNA, a known issue during sample preparation and one that is highly idiosyncratic across samples. We will use a “barnyard” or cellular spike-in approach to correct for lysis-based contributions. Adding a small fraction of human cells, all *C. elegans* transcripts found in the “human” fraction may be considered contamination, and thus a threshold for their contribution towards observed *C. elegans* RNAseq data procured. While other, indirect, methods such as SoupX exist, this empirical method has the advantage of directly measuring, rather than inferring, the contaminating fraction (63, 64).

For Orsay virus specifically, an issue is that the viral genome is non-polyadenylated, and does not generate polyadenylated mRNAs (65). Therefore, viral dynamics will not be captured using standard oligo(dT)

priming, as is performed in the 10x Genomics approach. However, fortuitously, this family of viruses does possess a relatively small bi-partite genome (for Orsay virus, RNA1 is 3.6 kb, and RNA2 is 2.6 kb (15)), amenable to complete conversion to cDNA, and possesses a 5' methyl-cap. The methodology by which 10x Genomics generates full-length cDNA prior to fragmentation follows the SMARTSeq approach, utilizing template-switching at a 5' methyl-cap (66, 67). Additionally, while the original 10x pipeline appended a cellular barcode to the 3' end, they have more recently released a methodology appending the cell barcode to the 5' end in the template-switching oligonucleotide. Therefore, by simply adding Orsay-specific primers to the standard 10x Genomics run we can concurrently capture viral sequences as well as host. We will first validate the efficiency of this approach in bulk RNAseq, prior to using scRNAseq, and whether it is better to simply amplify the 5' end of the viral genome, or attempt to capture entire viral sequence during reverse transcription.

With these controls, we will be able to confidently identify definitively infected, and definitively responding, cells, and compare to those that are more likely bystanders to either event, delineating the total nature of the *C. elegans* transcriptional response to viral infection, and providing a wealth of data regarding the structure of that response (Fig. 8).

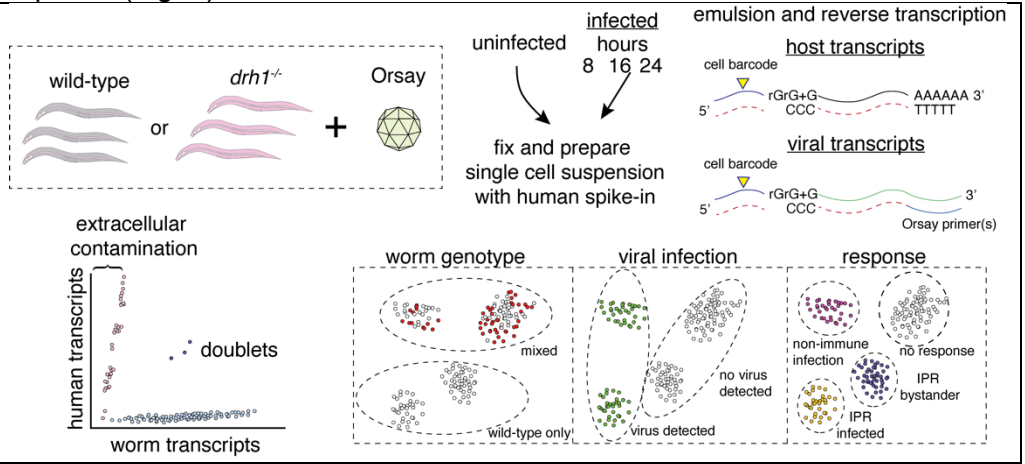


Fig. 8 Overview of work-flow for scRNAseq prep and analysis of virally infected *C. elegans*.

Potential outcomes, pitfalls and future directions

While protocols exist for scRNAseq of *C. elegans*, it may take time to achieve the quality of data necessary to answer our research questions. However, we may use whole-animal RNAseq with a slightly different experimental setup to still begin to develop an understanding of the *C. elegans* IPR and whether bystander cells may play a role. Comparing *drh-1* infections to wild-type will provide us with the whole suite of relevant transcriptional changes (we have done this comparison for RNA1 expression but now whole virus), even if we cannot parse in which cells such changes are occurring. If some signaling element exists, we may generate homogenates from infected worms, both wild-type and *drh-1,* and expose *drh-1* knockout mutants to these homogenates. Any transcriptional changes induced specifically by the wild-type homogenates would indicate a signaling element, which could thereafter be parsed using biochemical fractionation to narrow down the nature of that signal.

Alternatively, as hypothetical secreted and non-secreted IPR components are known, we may use smFISH, as described in Aim 3B, to explore whether these components exhibit distinct patterns of expression. If signaling exists, we would expect there to be more signal responders than signal producers, with perhaps producers also being responders due to autocrine signaling. If such patterns emerge, they may suggest the underlying network. We are fortunate to collaborate with David Wang, who used whole-genome sequencing to identify the first strain of the Orsay virus, and has subsequently identified several more strains by sequencing. The Wang lab has developed many tools for study of Orsay virus, including antibodies against viral proteins, which are available to us and will be useful for these studies (see Letter of Support) (16, 68, 69).

If we encounter unsurmountable difficulties with scRNAseq, we can also pivot toward comparing how intestinal and non-intestinal tissues respond to viral infection by performing tissue-specific RNAseq, using GFP markers to FACS sort out different cell types based on Coleen Murphy lab protocols (62), and then perform RNA extraction and RNAseq analysis on ~100,000 cells, as per published protocols. As a final back-up plan, we can simply build on our preliminary data from Fig. 7, and expand smFISH analysis in Aim 3B, to assess which cells are responding to viral infection, using FISH probes against known IPR and other virus-induced genes.

B) Assess the correlation between infection and response, and investigate the role of *drh-1* in bystander cells

We will use smFISH to orthogonally validate Aim 3A, and to provide additional spatial context for the bystander response. Although results from scRNAseq in 3A will likely provide new information about: a) which genes are induced in infected cells vs. bystander cells, b) which genes are induced in a *drh-1*-dependent manner, and, c) which genes are induced in the intestine vs other tissue, we already have insight into each of these questions.

In particular, based on existing information, we propose to investigate 10 genes, separate from any information gained from Aim 3A. We will focus on seven genes induced in a *drh-1*-dependent manner: *eol-1, pals-5, pals-14, pals-39, F26F2.1, skr-4, fbxa-75,* and three genes induced in a *drh-1*-independent manner: *zip-1*, *F26F2.2*, *cul-6*. These were chosen based on their being among the most highly induced genes during viral infection, and also being genes with known functional and/or biochemical activities (*eol-1, pals-5, skr-4, fbxa-75, zip-1, cul-6*), and for which we have GFP reporters that can be used for follow-up experiments (*eol-1, pals-5, F26F2.1, zip-1, F26F2.2* and *cul-6,* all of which show intestinal expression). We will test these genes two at a time, generating smFISH probes with red and far-red probes that are spectrally separate and which we have successfully used together in the past. We will stain Orsay virus with green FISH probes, and use DAPI to stain nuclei in blue to help with cellular localization. We will then infect wild-type or *drh-1* mutant animals, and harvest samples at 8h, 16h, and 24h, fix and stain with the FISH probes to co-localize and quantify which genes are induced specifically in virally infected cells, and which genes are induced in cells without detectable virus.

In addition to these studies, we will co-localize viral RNA with two other molecular events that occur during viral infection: DRH-1 puncta formation and ZIP-1::GFP nuclear localization. Here we will infect *rpl-28p::DRH-1::wrmScarlet* (or other DRH-1 strains generated in Aim 1), and ZIP-1::GFP strains, fix and stain with DAPI and either a red or green FISH probe for viral RNA, depending on the strain. Again, we will be investigating whether bystander cells also exhibit these responses, in the absence of detectable virus. To investigate the role of *drh-1* in bystander cells, we will take advantage of the mosaic nature of extrachromosomal transgenic arrays. Here, we will use existing DRH-1-NTD::wrmScarlet extrachromosomal transgenic strains (Aim 2) in which loss of the transgene can be monitored by loss of red fluorescence. We will stain this strain with the smFISH probes mentioned above including *eol-1*, to determine whether cells lacking the DRH-1-NTD::wrmScarlet transgene also express IPR genes, focusing in particular on cells neighboring the DRH-1-NTD containing cells, to determine whether IPR activation in one cell can induce the IPR in the neighboring cell if that cell lacks DRH-1-NTD. These experiments will be performed in the absence of infection and address concerns about whether 'uninfected' cells have actually cleared infection. We will similarly use a *vha-6p::DRH-1::wrmScarlet* construct to drive full-length DRH-1 and generate extrachromosomal array transgenic strains, infect these animals and perform smFISH. Here we will analyze animals in which a bystander cell exhibits *eol-1* expression like in Fig. 7, and then determine whether it also expresses the DRH-1 transgene to assess the role of *drh-1* in bystander cells.

Potential outcomes, pitfalls and future directions

We expect to divide IPR genes into at least three classes: 1) genes like *eol-1* that are expressed in infected cells and bystander cells, 2) genes that are expressed only in infected cells, and 3) genes that are expressed only in bystander cells. Given that DRH-1 appears to be a sensor for viral RNA, we do not expect that it will be required in bystander cells, although there are intriguing examples of RLRs detecting self RNA, and so it is possible that an uninfected cell may use self RNA to activate DRH-1 as a receptor that plays a role in bystander cells.

Statistics, Rigor and Reproducibility, Sex as a Biological Variable for all Aims

Experiments will include technical and biological replicates. For parametric data, Student's t-tests (2 groups) or ANOVA (>2 groups) will be performed. For ANOVA ($P < 0.05$), Tukey's test will be used to make individual comparisons. For non-parametric data, Mann-Whitney test or Kruskal–Wallis ANOVA will be used. Benjamini-Hochberg correction will be used to control false discovery rates. Because experiments involve *C. elegans*, the number of animals is generally not limiting and thus power analysis not required in many experiments; qRT-PCR, whole-animal RNA-seq, western and metabolomics analyses will be performed with 1000's of animals per condition, per experiment; virus quantification will be performed in 100 or more animals per condition; all assays will be performed in at least three independent experiments. To control for any problems with mutant strain background in *C. elegans*, we perform experiments with two independently isolated alleles and/or rescue strains. *C. elegans* hermaphrodites will be used for all experiments.

Overview

Altogether, we expect our studies to define a new mode of anti-viral signaling through our analysis of DRH-1 downstream signaling, as well as its tissue expression and subcellular localization. Our analysis of anti-viral responses on the single-cell level will also provide insight into the role of intercellular signaling and bystander cells in *C. elegans*. These findings may illuminate evolutionarily conserved or divergent principles about anti-viral responses, as well as principles about systemic innate immune signaling and inflammatory diseases.

PHS Human Subjects and Clinical Trials Information

OMB Number: 0925-0001

Expiration Date: 09/30/2024

Use of Human Specimens and/or Data

Does any of the proposed research in the application involve human specimens and/or data *	❍ Yes ● No
Provide an explanation for any use of human specimens and/or data not considered to be human subjects research.	
Are Human Subjects Involved	❍ Yes ● No
Is the Project Exempt from Federal regulations?	❍ Yes ❍ No
Exemption Number	❑ 1 ❑ 2 ❑ 3 ❑ 4 ❑ 5 ❑ 6 ❑ 7 ❑ 8
Other Requested Information	

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