PI: **Mir, Mohammad Ayoub**	Title: Preferential translation of host cell factors by hantavirus nucleocapsid protein	
Received: 06/07/2016	FOA: PA16-200	Council: 01/2017
Competition ID: FORMS-D	FOA Title: Academic Research Enhancement Award (Parent R15)	
1 R15 AI126395-01A1	Dual:	Accession Number: 3943416
IPF: 1387101	Organization: WESTERN UNIVERSITY OF HEALTH SCIENCES	
Former Number:	Department:	
IRG/SRG: ZRG1 IDM-S (81)A	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A) Year 1: [redacted]	Animals: N Humans: N Clinical Trial: N Current HS Code: 10 HESC: N	New Investigator: N Early Stage Investigator: N
Senior/Key Personnel:	*Organization:*	*Role Category:*
Mohammad Mir Ph.D	Mohammad Mir	PD/PI

OMB Number 4040-0001
Expiration Date 06/30/2016

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 [redacted]

b. Agency Routing Number

2. DATE SUBMITTED 2016-06-07

Application Identifier

c. Previous Grants.gov Tracking Number

5. APPLICANT INFORMATION

Organizational DUNS*: [redacted]

Legal Name*: Western University of Health Sciences
Department:
Division:
Street1*: [redacted]
Street2:
City*: [redacted]
County:
State*: [redacted]
Province:
Country*:
ZIP / Postal Code*: [redacted]

Person to be contacted on matters involving this application
Prefix: First Name*: Matthew Middle Name: Last Name*: Katz Suffix:
Position/Title: Assistant Vice President, Sponsored Programs
Street1*: [redacted]
Street2:
City*: [redacted]
County:
State*: [redacted]
Province:
Country*:
ZIP / Postal Code*: [redacted]
Phone Number*: [redacted] Fax Number: [redacted] Email: [redacted]

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

7. TYPE OF APPLICANT* O: Private 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*
National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER
TITLE:

11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT*
Preferential translation of host cell factors by hantavirus nucleocapsid protein

12. PROPOSED PROJECT
Start Date* 04/01/2017
Ending Date* 03/31/2020

13. CONGRESSIONAL DISTRICTS OF APPLICANT
CA-035

SF 424 (R&R) APPLICATION FOR FEDERAL ASSISTANCE

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: Dr. First Name*: Mohammad Middle Name: Last Name*: Mir Suffix: Ph.D
Position/Title: Associate Professor
Organization Name*: Mohammad Mir
Department:
Division: College of Veterinary Medicine
Street1*: [redacted]
Street2:
City*: [redacted]
County:
State*: [redacted]
Province:
Country*: [redacted]
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*	$0.00
c. Total Federal & Non-Federal Funds*	$[redacted]
d. Estimated Program Income*	$0.00

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: Dr. First Name*: Steven Middle Name: Last Name*: Henriksen Suffix: Ph.D
Position/Title*: Vice President
Organization Name*: Western University of Health Sciences
Department:
Division: Research and Biotechnology
Street1*: [redacted]
Street2:
City*: [redacted]
County:
State*: [redacted]
Province:
Country*: [redacted]
ZIP / Postal Code*: [redacted]
Phone Number*: [redacted] Fax Number: [redacted] Email*: [redacted]

Signature of Authorized Representative*
Matthew Katz

Date Signed*
06/07/2016

20. PRE-APPLICATION File Name:

21. COVER LETTER ATTACHMENT File Name:1235-Cover letter.pdf

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

	Page Numbers
SF 424 R&R Cover Page	1
Table of Contents	3
Performance Sites	4
Research & Related Other Project Information	5
Project Summary/Abstract(Description)	6
Project Narrative	7
Facilities & Other Resources	8
Equipment	13
Research & Related Senior/Key Person	14
Research & Related Budget Year - 1	20
Budget Justification	23
Research & Related Cumulative Budget	25
PHS398 Cover Page Supplement	26
PHS 398 Research Plan	28
Introduction to Application	29
Specific Aims	30
Research Strategy	31
Bibliography & References Cited	43
Letters of Support	45
Resource Sharing Plan(s)	52

OMB Number: 4040-0010
Expiration Date: 06/30/2016

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: Western University of Health Sciences
Duns Number: [redacted]
Street1*: [redacted]
Street2:
City*: [redacted]
County:
State*: [redacted]
Province:
Country*: [redacted]
Zip / Postal Code*: [redacted]
Project/Performance Site Congressional District*: CA-035

File Name

Additional Location(s)

Funding Opportunity Number: PA-16-200. Received Date: 2016-06-07T18:27:44.000-04:00

OMB Number: 4040-0001
Expiration Date: 06/30/2016

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

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*	1236-Abstract.pdf
8. Project Narrative*	1237-Project Narrative.pdf
9. Bibliography & References Cited	1238-Biblography.pdf
10.Facilities & Other Resources	1239-Facilities and Other Resources 1.pdf
11.Equipment	1240-Equipment.pdf

Funding Opportunity Number: PA-16-200. Received Date: 2016-06-07T18:27:44.000-04:00

Abstract: Hantaviruses, members of the *Bunyaviridae* family are enveloped negative strand RNA viruses and category A pathogens that are transmitted to humans through aerosolized excreta of infected rodent hosts. Hantaviruses have evolved a unique translation mechanism for the preferential translation of their mRNAs. This preferential translation is carried out by hantavirus nucleocapsid protein (N-protein) which specifically binds to the mRNA 5' cap and ribosomal protein S19 (RPS19), a structural component of the 40S ribosomal subunit. In addition, a trimeric N-protein molecule specifically binds to a highly conserved triplet repeat sequence (UAGUAGUAG) of the viral mRNA 5' UTR. Our results suggest that N-protein associated ribosomes are selectively loaded on viral mRNA 5' UTR to boost the translation of viral transcripts in the host cell cytoplasm where cellular transcripts are competing for the same translation machinery. Interestingly, our preliminary data shows that N-protein mediated translation strategy also favors the translation of certain host cell factors by an unknown mechanism. Using multifaceted experimental avenues we will determine the mechanism for the selective translation of certain host cell mRNAs by N-protein mediated translation strategy. We will determine whether preferential translation of host cell factors plays a role in virus replication. As antiviral response, host cells transiently shutdown the host translation machinery to create roadblocks for the synthesis of viral proteins. This antiviral response is triggered by the activation of protein kinase R (PKR), which phosphorylates its downstream target eIF2α, causing translational shutdown. Our preliminary results show that N-protein inhibits PKR activation in virus-infected cells to ensure continuous synthesis of viral proteins during the course of infection. We will test the hypothesis that N-protein requires the assistance from endogenous host cell factors to inhibit PKR antiviral response. These studies will reveal new targets for therapeutic intervention of hantavirus disease.

There is no treatment for hantavirus associated disease at present. The major goal of this application is to delineate the mechanism of hantavirus replication in cells. In addition, the proposed studies will help in the identification of new host targets for antiviral drug design.

Facilities and Other Resources:

Laboratory: Dr. Mir's research space is in the Rodney Wineberg Center for Research (RWC). The laboratory is 1540 sq.ft with an attached 120 sq.ft cell culture room. The cell culture room has a bio-safety cabinet (Forma Scientific Class II A), two inverted microscopes, table top refrigerated centrifuge and two CO_2 incubators. The main lab has all necessary equipment required for biochemical and molecular biology work, including protein and DNA electrophoresis equipments, 4 °C, -20 °C and - 80 °C storage, a liquid nitrogen tank, centrifuges, gel documentation system, NanoDrop, thermal cyclers, water baths, bacterial shaker incubators, power supplies, spectrophotometers, spectrofluorometer RF5301PC, BLITZ system (ForteBio), water purification system, hot plates, balances, ice machine, protein purification system and microplate reader. Other PIs in the college of veterinary medicine study other viruses, such as Feline immunodeficiency virus, avian influenza virus, infectious bronchitis virus, herpesviruses, vaccinia virus and poxviruses.
Clinical: N/A
Animal: N/A
Computer: The laboratory has a desktop Macintosh and three PC computers. The PCs are equipped with software for flow cytometry data analysis, SigmaPlot and Origin 6. Macintosh computers are equipped with DNA sequence and manipulation software such as MacVector. The laboratory is equipped with phone and Ethernet connections.
Office: PI's office space consists of a 230 sq. ft. room. Office spaces for other virologists in the college of veterinary medicine are located close to Dr. Mir. Faculty members of the college of veterinary medicine are supported by full-time administrative staff to assist with grant preparation, publications, travel arrangements, lab supply expenditure tracking and account reconciliation.
Other: Two common use BSL3 laboratories, one located in the Rodney Wineberg Center for Research (RWC) and other located in the Health Education Center (HEC) are available for this project for studies requiring BSL3 containment. The BSl3 labs are well equipped with biosafety cabinets, biohazard containment centrifuges, 4 °C, -20 °C and -80 °C storage and inverted microscopes. Support for the operation of these two core laboratories is provided by the Western University of Health Sciences and includes oversight by a Select Agent expert (Ms. Sheila Redjai). Western University provides monitoring of personnel training, health monitoring and equipment preventive maintenance within the facility.

PI has access to the common equipment maintained by the College of veterinary medicine, including ultracentrifuges, CO2 incubators, biohazard and laminar flow hoods, ice machines, ELISA readers, bacterial incubators, gel electrophoresis equipments, spectrophotometers, fluorescence microscopes, autoclaving room, darkrooms, dish washers, cold rooms, ultra centrifuges, flow cytometer (BD Accuri, C6), confocal microscope (Nikon TE2000-U, Storm 820 phosphorimage system and quantitative PCR machines.

Scientific Environment: The scientific environment in which the proposed work will be carried out is excellent. College of veterinary medicine (CVM) has five virologists including Dr. Mir, who work on other viruses, such as Feline immunodeficiency virus, avian influenza virus, infectious bronchitis virus, herpesviruses, vaccinia virus and poxviruses. The diverse research expertise promotes useful collaborations and intellectual support among virologists. Our students, post docs and faculty members participate in research seminar series that further promotes the intellectual rapport among scientists working in different areas of infectious diseases. CVM has great facilities for research in diverse areas of Biomedical Science. Other schools in the Western University of Health Sciences such as school of nursing, dentistry, basic biomedical sciences, Osteopathic Medicine, Optometry, Allied Health, Pediatric Medicine and Pharmacy are located within the short distance in the same campus. Their research programs in diverse areas of biology promote the opportunities for collaborative research and stimulate the overall research environment in the University.

The Western University of Health Sciences is committed to strengthen the research environment in the diverse fields of biomedical sciences. Over the past several years there are noticeable improvements in the research environment of the University. For example, the hiring of Dr. Henriksen from Scripps Research institute as Voice present of the Research and Biotechnology at the Western University of Health Sciences has promoted the scholarly activity by improving intramural research funding and development of interdepartmental graduate program. More recently, Dr. Baudry from the University of Southern California was recruited as dean of new Graduate College of Biomedical Sciences. His main responsibility is to initiate a PhD program in Biomedical Sciences, which may start its first batch in the fall of this year. The research infrastructure is improving, evident from the new BSl3 laboratory in the Health Education Center of the University. This new BSl3 lab space is equipped with modern animal and cell culture facilities to carry our cutting edge research

with highly infectious pathogens. The University office of Research and Biotechnology provides funding for core equipment facilities shared by the faculty, facilitates strong programs for interdisciplinary research, supports cooperative initiatives to enhance technology utilization and supports collaborative research programs between the colleges. While the University is actively improving its research infrastructure, it is still not the major recipient of NIH funding. The university is surrounded by other major academic institutions such as The California State Polytechnic University at Pomona, The Claremont Colleges, UCLA, UC-Irvine, UC-Riverside, Caltech, USC, Loma Linda University, City of Hope Cancer Institute. All these institutions are within 40 miles of radius from the Western University of Health Sciences.

Special characteristics of the college of veterinary medicine (CVM) at the Western University of Health Sciences (WUHS): WUHS, a graduate level institution, delivers 21 degrees in health sciences, and serves over 3,500 students across nine colleges. The CVM was established in 1998, it offers doctor of veterinary medicine (DVM) degree, and recruits 105 students in each class. In its current strategic plan, the college of veterinary medicine identified microbiology and infectious disease as emerging research program of potential significance to veterinary medicine that will receive additional attention and development. The CVM has felt the need to train veterinarians in frontier areas of virology and infectious disease research to generate the specialized work force who can safely handle the emerging infectious pathogens of zoonotic origin. The CVM is determined to improve its research infrastructure to provided cutting edge research opportunities in infectious disease research to veterinary students. Consistent with this plan, the University has established a state of the art BSL3 laboratory and CVM has put a thrust on the recruitment of infectious disease researchers. Last year two virologists (myself and Dr. Brennan) were recruited by CVM and one more position in infectious disease and antibiotic resistance has been requested. CVM has thus for assembled a team of six virologists (Myself, Dr. Brennan, Dr. Drechsler, Dr. Barr, Dr. Collison and Dr. Diniz) to provided opportunities for specialized training in virology to the students at the Western University of health Sciences. The college of veterinary medicine is investing in the renovation of laboratory spaces and core facilities for the infectious disease researchers at the Rodney Wineberg Center for Research (See letter of support from Dr. Griffon, Associate Dean for Research, CVM).

Student profile and research opportunities for students at CVM: The students entering for DVM program at CVM or for other programs in remaining eight colleges have completed their baccalaureate degrees prior to their enrolment at the WUHS. As WUHS serves over 3,500 students, a significant proportion of them remain interested to establish their careers in Biomedical research and are eagerly interested to receive training in biomedical research during their stay at Western University. Thus, the students from other colleges also remain available for proposed research in this R15 application (See the letter of support from Voice president of Research and Biotechnology, Dr. Henriksen).

The CVM encourages its students early on in their career towards Biomedical Research. During their 1st and 2nd year, the students of CVM are encouraged to participate in laboratory apprenticeship program, a paid research-training program in which students receive training in diverse areas of biomedical sciences. The students selected for this program receive a stipend up to 4 months while working on a research project under the supervision of the PI of the lab. Funding from this R15 grant will allow the PI to provide small stipend to the students, enabling them to work on the project for the entire academic year. The CVM students have the opportunity to participate in the summer research program. The students receive a stipend of $5000 to work on the research project in summer months under the supervision of the PI. The broad aim of this program is to foster interests in students to pursue biomedical research careers and remain scholarly active in their filed of interest. Funds from this R15 grant will again allow the PI to provide small stipend and thus extend the ability of the students to conduct research throughput the academic year. The students at CVM are encouraged to take CVM-7564 course (4 credit hour) and CVM 8095 course (8 credit hour). During these courses the students work on a research project under the close supervision of the PI. They are trained to review scientific literature, develop research hypothesis, and learn experimental design, data analysis and presentation. Students are required to participate in seminars, journals clubs and lab meetings. At the completion of the project, students are required to summarize their research project in the form of a short thesis, which then student defends in an informal setting. Funds from this R15 application will allow the PI to purchase the reagents and other supplies required for the completion of their research projects, proposed in this grant application.

Further more, the other institutions especially the California state polytechnic University at Pomona (Cal Poly) provide great opportunities to engage undergraduate students from diverse backgrounds in research. Cal Poly is located within 3-4 miles from the CVM. In my previous job at Kansas University Medical center, I have engaged students from diverse backgrounds for PhD program. I intend to continue this pattern of training, which is more possible and easy due to geographical location of the WUHS, as well as the demographics of

the Cal Poly. At Mir lab the students will receive hands-on training in diverse biomedical research techniques, such as gene cloning in prokaryotic and mammalian expression vectors, protein expression and purification in bacterial and mammalian expression systems, gene knockdown using siRNA and lentivirus delivery systems, western blotting, ELISA, FPLC, Biolayer interferometry, fluorescence spectroscope, viral, bacterial and mammalian cell culture techniques, confocal microscopy and immunopecepitation. In addition, students will receive training on theoretical aspects of basic virology, presentation of virology research articles in weekly lab journal club to improve oral communication skill and stay updated in the current virology research. The funds from this R15 application will help the PI to train next generation of virologists and other healthcare professionals at his lab.

Student recruitment plan*: The WUHS provides two short-term funding opportunities to students for summer research program. One of the funding opportunities is through CVM and another one is through the voice president of research and biotechnology (Dr. Henriksen). Both remain available to all the students of WUHS although the veterinary students from CVM are preferred for funding through CVM. Both the mechanisms provide a stipend of $5000 per student to work on a research project for 2-3 months in summer.*

Graduate Student recruitment through CVM*: The student recruitment at CVM for summer research program starts early in the winter. The CVM faculties are provided an opportunity to present their research program to students by one-hour oral presentation. The interested students contact the PI and prepare a joint summer research proposal, which is submitted to CVM in the early spring for funding. The PI must have active grant support to purchase reagents and other consumables for the summer research project, as the research funding from CVM pays only the student salary for 2-3 months. This year 15 students contacted the PI for summer research program. After careful review of their curriculum vitae and a personnel interview, PI selected two students (Brittany Voss and Sean Pador) for this year's summer research program. The PI is glad that both Brittany and Sean received stipend for summer research. Brittany and Sean are second year DVM students having a GPA of 4.0. They are highly interested in virology research and would like to have a career in veterinary virology. Both students would like to work on their projects throughout the academic year and will take CVM-7564 (4 credit hour) and CVM 8095 (8 eight credit hour) courses to strengthen their research skills. Due to limited summer research grant support the funding from this R15 application will allow the PI to provide stipend to both of these students to continue working on their projects. Thus the students through this mechanism will be annually recruited to the PI's lab.*

Graduate student recruitment through University wide summer Research program: *The student recruitment plan for the second summer research program through Dr. Henriksen's office is similar to the recruitment plan of CVM. The faculties are provided the opportunity to present their research program through oral presentation to the students. The students from all 9 colleges are encouraged to attend. Interested students are interviewed by the faculty member to prepare a summer research grant proposal. Again the PI's existing grant support significantly favors the application for funding (See letter of support from Dr. Henriksen). To recruit students through this mechanism, the PI will submit proposals in the spring. The support from this R15 grant will help the PI to secure funding and support students throughout the academic year to continue working on their projects.*

Recruitment of undergraduate students*: Based on the history of undergraduate volunteers from Cal Poly, the PI intends to recruit students from the College of Agricultural Sciences and College of Biological Sciences, Cal Poly for this research program. Cal Poly is a minority serving public institution enrolling over 22,000 students with the largest enrollment demographic. Cal poly is located at a distance of 3-4 miles (6 minutes by car) from WUHS. The PI will present his virology research program to the undergraduate students by one-hour oral presentation in both the colleges. The presentations will take place in the fall or early spring and will be coordinated by Dr. Lappin (Seminar coordinator at the College of Biological Sciences) and Dr. Holz-Clause (Dean College of Agricultural Sciences). Shortly after the oral presentation the availability of undergraduate research opportunity in the PI's lab will be communicated to the students through e-mail and printed notices posted on student bulletin boards. The coordinators will encourage the qualified student to apply for the available positions. Applications will be screened by the PI, followed by a personal interview of the shortlisted candidates. The selected students will join the lab in the summer. During the first year two graduate and two undergraduate students will be recruited in the program. Depending upon the number of available spots the recruitment will be repeated each year. Please see letters of recommendation from Dr. Holz-Clause and Dr. LaMunyon for further details.*

Past trainees from the Mir lab: In my previous job at Kansas University Medical center, I got significant experience in training post docs, graduate and undergraduate students. All the trainees who worked in my lab have published their work in good journals.

Post docs:

1. **Dr. Absarul Haque** (2009-2011). Dr. Haque received his PhD degree in bacteriology from JNU, New Delhi India. Dr. Haque joined the lab with no experience in virology. He finished his post-doctoral training with significant expertise in molecular virology and viral translation control. He authored **five manuscripts** in my lab. He is currently working as Assistant professor at King Abdulaziz University, Kingdom of Saudi Ara*bia.*
2. **Dr. Abdul Haseeb** (2009-2010). Dr. Hasseb received his PhD in Microbiology from National Institute of Nutrition, Hyderabad, India. Dr. Haseeb joined my lab with no experience in virology. During his short post-doctoral training, he got significant expertise in molecular virology and cap snatching mechanism of viral transcription initiation. He authored **two manuscripts** from my lab. He is currently working as research scientist at *Case Western Reserve University, Cleveland*
3. **Dr. Islam Hussein** (2009-2012). Dr. Hussein received his PhD in virology from Cambridge University, London. He finished his post-doctoral training with significant expertise in molecular virology and role of autophagy in virus replication. He authored **four manuscripts** in my lab. He is currently working as scientist at **MIT, Boston**.
4. **Dr. Nilshad Salim** (2012-2014). Dr. salim received his PhD in Chemistry from Wayne State University. Dr. Salim joined the lab with no experience in virology. He finished his post-doctoral training with significant expertise in molecular virology and therapeutic intervention of viral disease. He published **one manuscripts and one more is under review in J. virology**. He is currently working as scientist at Indiana University.
5. **Dr. Zekun Wang** (2012-2015). Dr. Wang did is PhD in virology from China. During his post doc he opened a new area of research on protein kinase R in my lab. He published **two manuscripts and one more is under review in J. virology**. He is currently working for his second post doc at Kansas University Medical Center.
6. **Dr. Kanchan Senha** (2012-2013). Dr. Sinha received her PhD in Molecular Biology from JNU, New Delhi, India. Dr. Senha joined the lab with no experience in virology. In her short post-doctoral training she got significant expertise in molecular virology and viral translation control. She is currently working as scientist at Indian Institute of Technology, Mumbai, India.

Graduate Students: I got the opportunity to train 4 PhD students in my lab at Kansas University Medical center. One of the students finished his degree and has joined the post doc at University of Pittsburg Medical Center. Three more students are still working on their PhD thesis and could not move with me due to lack of PhD program at Western University of Health Sciences.

1. **Erdong Cheng** (2009-2014). Erdong was the first graduate student who joined my lab with no past experience in virology. Erdong received his Bachelors degree in Biology from Newcastle University, United Kingdom. He finished his PhD with significant expertise in molecular virology and authored **six manuscripts** from my lab. He is currently working as post doc at the **University of Pittsburg Medical Center**.
2. **Mary Ashley Rimmer** [redacted]. Mary received her Bachelors degree in chemistry from the University of Mississippi. She joined the lab with no experience in virology. She authored **one manuscripts** from my lab and is about to complete her PhD from Kansa University Medical Center.
3. **Safder Syeed Ganaie** (2011-2014). Safder received his Masters degree in Biotechnology from the University of Kashmir, India. He joined the lab with no experience in virology. He authored **three manuscripts and one more is under review in J. virology** from my lab. Safder is finishing up his PhD thesis at Kansas University Medical Center
4. **Tania S Bunny** (2011-2012). Tania received her MS degree in Biology from the University of Dhaka, Bangladesh. She joined the lab with no experience in virology. She authored **one manuscripts** from my lab. Tania left the program because of her father's illness back home at Bangladesh. However, Tania returned back to US last year to peruse her PhD at the University of Florida.

Undergraduate students: I got the opportunity to train three undergraduate students from the University of Kansas. The students worked in my lab in summer months. They joined the lab with no prior experience in virology. They received significant training in virology. One student *authored a manuscript* and has joined the *MD-PhD program* at the University of Texas; another one received a *training grant* to work on the research project on hantavirus glycoprotein Gn.

1. **Alex little** (2010-2010): Alex joined my lab from the University of Kansas for a summer training program. He had no prior experience in virology. He received significant training in virology and

authored a *manuscript in J.Biol. Chem.* The training from my lab helped Alex to join *MD-PHD program* at the University of Texas.

2. **Albert Kim** (2013-2014): Albert joined my lab from the University of Kansas for a summer training program. He had no prior experience in virology. He received significant training in virology and got an internal *training grant of $10,000* to work on a project with me. He generated interesting data, which will be submitted for publication in future. Albert is preparing at the University of Kansas to join the MD-PhD program.
3. **Hasan Maz** (2013-2014). Hasan joined my lab from the University of Kansas for a summer training program. He had no prior experience in virology. He received significant training in virology and is preparing at the University of Kansas to join the MD program.

Graduate student thesis committees: I served in the PhD thesis committees of numerous PhD students at the University of Kansas. I was actively involved in monitoring their progress over the academic years. This required routine committee meetings, examination of student's research data, helping the students in data presentation, providing technical inputs in their research projects. Below is the list of students who I mentored while serving in their PhD thesis committees.

A. Li Chen (Baumann lab, Stowrs institute)
B. Yong Luo (Qiu lab, KUMC)
C. Rachel Olsen (Hardwidge lab, KUMC)
D. Alexander Dowdell (Zuckert lab, KUMC)
E. Weiran Shen (Qiu lab, KUMC)
F. Kellyann Jones (Weinman lab, KUMC)
G. Josiah Cox (Weinman lab, KUMC)
H. Saswati Biswas (Zuckert lab, KUMC)

Current trainees in Mir lab at Western University: Currently Mir lab at Western University of Health Science has one post doc and two DVM summer research students engaged in hantavirus research. The summer research students are supported by the limited summer research fellowship from the CVM. Both of these students have shown strong interest in research focused on emerging animal viruses. Funding from this R15 grant will help PI to provide stipend to the students to allow them work on their projects throughout the academic year.

1. **Jeeva Subbiah, PhD (Post doc)**, 2016-present: Dr. Subbiah did his PhD in virology from Pukyong National University, Busan, South Korea. Dr. Subbhia has started working on CCHF N protein.
2. **Brittany Voss (summer research student)**, 2016-present: Brittany is a second year DVM student at the college of veterinary medicine. Brittany has received her bachelors degree in Animal Sciences from Cal Poly before joining DVM program at Western U. After the completion of her DVM degree Brittany plans to join a PhD program in emerging RNA viruses. Brittany has shown a strong interest to work with hantaviruses and is currently working on hantavirus translation control.
3. **Sean Pador (summer research student), 2016-present:** Sean is also a second year DVM student at our college . Prior to joining the Western University, Sean has received a bachelors degree in Pharmacological Chemistry from UC-San Diego. Sean is highly interested to establish his career as independent investigator in emerging animal viruses. He has strong passion in antiviral drug development. Sean is currently working on a project focused on further characterization of a novel chemical scaffold as anti-hantaviral therapeutic targeting the N-protein.

Matching funds: if this R15 application is funded, the CVM will provide a matching fund of $5,000 to support the tuition, research supplies or stipend to the participating students**.**

As a faculty member at the University of Kansas, School of Medicine, I have established a research program in virology and trained post docs, graduate and undergraduate students. Most of the trainees have published their research findings in good journals and are moving ahead in their careers. With this multidisciplinary research and teaching expertise I joined the Western University of Health Sciences in July 2015. I am enthusiastic and feel obliged to train a next generation of virologists with a background in veterinary sciences at the College of veterinary medicine, Western University of Health Sciences. The veterinarians with research experiences in cutting edge virology will serve as specialized lead work force in the frontier areas of infectious disease research. This unique work force will be of potential significance to handle emerging viral infections of zoonotic origin during unpredictable outbreaks worldwide.

Equipment:

The PI's laboratory is well equipped with the necessary equipment required for this project. In addition Dr. Mir has access to the shared equipment of the College of Veterinary Medicine, including ultra centrifuges, BSL2 cabinets, visible and UV spectrophotometers, fluorescence microscopes, CO2 incubators, Real time PCR machines, Flow cytometry equipment, Confocal microscope, autoclave, Odyssey infrared Imaging System, Microplate readers, numerous -20 and -80 ℃ storages.

OMB Number: 4040-0001
Expiration Date: 06/30/2016

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: Dr. First Name*: Mohammad Middle Name Last Name*: Mir Suffix: Ph.D

Position/Title*: Associate Professor
Organization Name*: Mohammad Mir
Department:
Division: College of Veterinary Medicine
Street1*: [redacted]
Street2:
City*: [redacted]
County:
State*: [redacted]
Province:
Country*: [redacted]
Zip / Postal Code*:

Phone Number*: [redacted] Fax Number: E-Mail*: [redacted]

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

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

Degree Type: Ph.D. Degree Year: 2002

	File Name
Attach Biographical Sketch*:	1246-Mir Biosketch.pdf
Attach Current & Pending Support:	

Tracking Number: GRANT12187057
Funding Opportunity Number: PA-16-200 . Received Date: 2016-06-07T18:27:44.000-04:00

OMB No. 0925-0001 and 0925-0002 (Rev. 10/15 Approved Through 10/31/2018)

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: Mir Mohammad Ayoub

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

POSITION TITLE: **Associate professor** (College of Vet. Medicine, Western University of Health Sciences, CA)

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 Kashmir, India	BS	1992	Chemistry
University of Kashmir, India	MS	1995	Biochemistry
Saha Institute of Nuclear Physics, Kolkata, India	Post M.S diploma	1996	Biophysics
Saha Institute of Nuclear Physics, Kolkata, India	PhD	2002	Structural and Molecular Biology
University of New Mexico, Albuquerque, NM, USA	Post doc	2005	Molecular Virology

A. Personal Statement. I am a multidisciplinary virologist having 13 years of experience with the replication and therapeutic intervention of bio-medically important and bio-defense related RNA viruses. I have broad background and technical expertise in diverse areas of biochemistry, molecular biology, cell biology, infectious diseases and virology. I have significantly contributed to the basic biology of RNA virus replication, as discussed in detail in section C. Most importantly, on the basis of my basic work in hantavirology I have identified the viral nucloecapsid protein as a novel target for the therapeutic intervention of hantavirus disease. I developed the assays and identified the first chemical inhibitor, which is under development as first anti-hantaviral drug (patent filed). Apart from my significant contribution in other areas of basic virology (see section C), the current application stems and builds logically from our recently published work, as referenced below. I am a qualified, independent, well-equipped and motivated scientist with the necessary expertise to carry out the proposed work as an independent investigator. With a strong passion in molecular virology, I am enthusiastic to conduct the proposed experiments in my new lab at the college of veterinary medicine, Western University of Health Sciences, California. I have significant expertise in training undergraduate, graduate and postdoctoral fellows, as discussed in detail in facilities and resources page. I am dedicated to train a next generation of virologists with a background in veterinary sciences at the College of veterinary medicine.

1. **Mir, MA**, and A. T Panganiban*: A protein that replaces entire eIF4F complex, *EMBO Journal*, (2008), Dec 3;27(23):3129-39
2. **Mir, MA*** and A. T Panganiban: The triplet repeats of the Sin Nombre hantavirus 5' untranslated region are sufficient in cis for nucleocapsid-mediated translation initiation: *J. Virology*, (2010) Sep;84(17):8937-44.
3. **Mir, MA***, Sheema S, Abdul A, Haque A: Hantavirus nucleocapsid protein has distinct m7G cap and RNA binding sites, *J. Biol. Chem*. (2010) Apr 9;285(15):11357-68. Epub 2010 Feb 17
4. Haque, A and **Mir, MA*** : Interaction of hantavirus nucleocapsid protein with ribosomal protein S19 (RPS19). *J virology*, (2010). Dec;84(23):12450-3. Epub 2010 Sep 15
5. Cheng, E. Haque, A. Rimmer, MA. Hussein, I. Sheema, S. Little, A and **Mir, MA***: Characterization of the interaction between hantavirus nucleocapsid protein (N) and ribosomal protein S19 (RPS19): *J. Biol. Chem*, (2011). Apr 1;286(13):11814-24. Epub 2011 Feb 4.
6. Wang Z and **Mir MA***. Andes virus nucleocapsid protein interrupts PKR dimerization to counteract the host interference in viral protein synthesis**.** *J. Virol.* 2015 Nov 19. pii: JVI.02347-14. [Epub ahead of print

B. Positions and Honors

Positions and Employment:

1996-1997: Junior Research Fellow, Biophysics Division, Saha Institute of Nuclear Physics, India
1998-1999 Senior Research Fellow-I, Biophysics Division, Saha Institute of Nuclear Physics, India
2000- 2002 Senior Research Fellow-II, Biophysics Division, Saha Institute of Nuclear Physics, India
2003- 2005 Postdoctoral Fellow, University of New Mexico, Albuquerque, NM.
2006- 2008 Research Scientist, University of New Mexico, Albuquerque, NM
2008- 2009 Research Assistant Professor, University of New Mexico, Albuquerque, NM
2009-2014, Assistant Professor (tenure track), University of Kansas Medical Center, Kansas
2015-Present. Associate professor**,** College of Vet. Medicine, Western University of Health Sciences, CA

Professional Memberships:

American Society for virology (full member)
Biophysical Society (full member)
American Society for Biochemistry and Molecular Biology (Regular Member)

Honors and Awards:

1996 - Junior Research Fellowship from University Grants Commission and Center for Scientific and Industrial Research, India
1998 - Senior Research Fellowship from Department of Atomic Energy, Government of India
2000 - Qualified **GATE** (Graduate Aptitude Test), All India Rank of 211. This test is held in India to rank Indian Students on the basis of merit in all disciplines, including, Physical, Chemical, Mathematical, and Biological Sciences.
2007- MGM Staff Award, 2007. This award is given once a year by the Department of Molecular Genetics and Microbiology, University of New Mexico. MGM Staff Award have the objective of promoting and rewarding exceptional professionalism, dedication, loyalty, and manifest achievements that effect MGM department as a whole.
2010- International Patent (PCT/US09/004150) "A viral nucleocapsid protein as a multifunctional translation initiation factor and increased protein and polypeptide production using same"
2011- Travel award from American Society for Virology to attend the International Union of Microbiological Societies (IMUS 2011) meeting at Sapporo Japan.
2015- Patent (Docket number K1262.10071US01): A novel chemical Scaffold with broad-spectrum antiviral activity (application submitted)

C. Contribution to Science

The broad focus of research is to study the mechanism of RNA virus replication with an aim to identify novel targets for therapeutic intervention of viral diseases for which there is no antiviral drug or a vaccine available. Thus, the research in my lab is trans-disciplinary, both basic and translational in nature. I am working with hantaviruses from last thirteen years and have recently started working with Crimean Congo hemorrhagic fever virus (CCHFV), a tick born Nairovirus. These emerging RNA viruses cause significant human disease with high fatality rates in certain outbreaks. There is no FDA approved vaccine or antiviral drug against these emerging viruses of potential bio-terrorist threat. My five most significant contributions to the virology are as follows.

1. Cellular mRNA degradation and Bunyaviridae transcription initiation: More than three hundred negative strand RNA viruses from the *Orthomyxoviridae*, *Bunyaviridae* and *Arenaviridae* families use cap snatching mechanism for transcription initiation. These families include highly virulent and emerging category A viruses such as Lassa fever virus, Crimean Congo hemorrhagic fever virus (CCHF), Rift Valley fever virus and Hantaviruses. The diseases caused by these viruses are associated with hemorrhagic fever having significantly high mortalities in humans.

During cap-snatching mechanism of transcription initiation, the viral RNA dependent RNA polymerase (RdRp) cleaves the host cell mRNA 10-20 nucleotides downstream of the 5' cap. The resulting capped RNA fragment is used as primer by the viral RdRp to initiate viral mRNA synthesis. Interestingly, host cells contain an elegant decapping system to remove the 5' caps from host cell mRNAs after the completion of their translation. Decapping of host cell mRNAs is prerequisite for their degradation. Bulk host mRNA degradation takes place in cellular P-bodies. This raises questions that how are these viruses able to snatch caps from host cell mRNAs in the presence of active cytoplasmic decapping machinery. Our research is focused to determine how hantaviruses evade the host decapping machinery for efficient cap-snatching. We previously reported that hantavirus nucleocapsid protein specifically binds to the host mRNA caps and protects them from the attack of host decapping machinery. The rescued capped mRNA fragments up to 180 nucleotides in length are sequestered in P-bodies by hantavirus nucleocapsid protein. These sequestered capped mRNA fragments are processed by an unknown mechanism to generate short capped RNA primers for viral transcription initiation. We demonstrated that host cell mRNAs having a "G" residue located at 14th position downstream of the 5' cap are primarily targeted by viral RdRp for cap snatching. Our studies have revealed that hantavirus nucleocapsid protein binds to both the host mRNA 5' cap and viral RdRp. Our current working model is that hantavirus nucloecapsid protein recruits the RdRp to the mRNA 5' cap for the specific endonucleolytic cleavage of the target host mRNA at a G residue located 14 nucleotides downstream

of the 5' cap. This specific endonucleolytc cleavage guided by nucleocapsid protein generates a capped RNA primer of appropriate length and specificity. These findings have revealed novel targets for therapeutic intervention of viral diseases and have been published in following manuscripts. I served as primary or lead investigator in these studies.

1. **Mir. MA**, Hjelle, B. Ye, C and A. T Panganiban*: Cap snatching Revised: Viral storage of cellular 5'mRNA caps in P bodies: *Proc. Natl. Acad. Sci. USA*, 2008, 2008 Dec 9;105(49):19294-9.

2. Cheng E, **Mir MA***. Signatures of host mRNA 5' terminus for efficient hantavirus cap snatching. *J Virol*, (2012) Sep;86(18):10173-85. doi: 10.1128/JVI.05560-11. Epub 2012 Jul 11.

3. Cheng E and **Mir MA***. The interaction between hantavirus nucleocapsid protein and viral RdRp is required for viral mRNA synthesis *J Virol*. 2014 Aug 1;88(15):8706-8712. Epub 2014 May 21.

4. Mir, MA*, Sheema S, Abdul A, Haque A: Hantavirus nucleocapsid protein has distinct m7G cap and RNA binding sites, *J. Biol. Chem*. (2010) Apr 9;285(15):11357-68. Epub 2010 Feb 17

2. Translation control of viral mRNA: Despite the tight regulation of eukaryotic translation machinery, viruses have evolved selfish strategies to favor the translation of viral mRNAs in the host cell cytoplasm where cellular transcripts are competing for the same translation apparatus. For example, inhibition of the cap-dependent translation initiation by picornaviruses or degradation of cellular mRNAs by herpes simplex virus are the well characterized viral strategies that make host translation machinery abundantly available for the translation of viral transcripts. However, there are number of negative strand RNA viruses, including hantaviruses, which actively replicate in host cells without causing host translation shutoff or cytopathic effects to the host cell. This raises questions that how their mRNAs compete with the innumerable population of host cell mRNAs for the same translation machinery. Active replication of these viruses implies the active translation of their mRNAs by unknown mechanisms. We have recently found that hantaviruses use a novel translation initiation strategy that lures the host translation apparatus for the preferential translation of viral mRNA in the host cell. This translation initiation mechanism is operated by viral nucleocapsid protein (N-protein) that engages the 43S pre-initiation ribosome complex at the mRNA 5' cap, independent of eIF4F cap binding complex. N-protein specifically binds to both the viral mRNA 5' UTR and ribosomal protein S19 of the 40S ribosomal subunit. The ribosomes bound to N-protein are preferentially loaded on viral transcripts. The preferential ribosome loading on viral transcripts by N-protein mediated translation mechanism dramatically facilitates their translation in a competitive cytoplasmic environment where cellular transcripts are competing for the same translation machinery. Interestingly, our preliminary observations suggest that N-protein mediated translation mechanism also helps in the translations of multiple host factors required for virus replication. These studies have reviled novel targets for therapeutic intervention of viral disease, some of them have been explored for antiviral drug development. I served as primary or lead investigator in these studies.

1. **Mir, MA**, and A. T Panganiban: A protein that replaces entire eIF4F complex, *EMBO Journal*, (2008), Dec 3;27(23):3129-39

2. Haque, A and **Mir, MA*** : Interaction of hantavirus nucleocapsid protein with ribosomal protein S19 (RPS19). *J virology*, (2010). Dec;84(23):12450-3. Epub 2010 Sep 15

3. Safdar S Ganaie , Absarul Haque, Erdong Cheng, Tania S. Bonny, Nilsahd N. Salim and **Mir MA*.** RPS19 binding domain provides key insights into hantavirus N-mediated translation initiation mechanism. *Biochemical Journal*. 2014 Jul 25. [Epub ahead of print]

4. Cheng, E. Haque, A. Rimmer, MA. Hussein, I. Sheema, S. Little, A and **Mir, MA***: Characterization of the interaction between hantavirus nucleocapsid protein (N) and ribosomal protein S19 (RPS19): *J. Biol. Chem*, (2011). Apr 1;286(13):11814-24. Epub 2011 Feb 4.

1. ***Therapeutic intervention of Bunyaviral diseases***. We reported that hantavirus nucleocapsid protein (N-protein) binds to both the viral mRNA 5' UTR and ribosomal protein S19, a structural component of the 40S ribosomal subunit. N-protein associated ribosomes are selectively loaded on the 5' UTR of viral mRNA. This selective and efficient ribosome loading on viral transcripts rapidly favors their translation. We developed a fluorescence based high throughput screening assay to monitor the interaction between N-protein and viral mRNA 5' UTR. The assay was used to screen a chemical library of 100,000 chemical compounds to identify inhibitors that block the interaction between N-protein and viral mRNA 5' UTR. We have identified a novel small molecule inhibitor that selectively binds to the N-protein and inhibits N protein-UTR interaction with high potency. The inhibitor is well tolerated by cells and is currently being tested in Syrian hamster model as an anti-hantaviral therapeutic. A patent application has been filed for this inhibitor. Moreover, we found that

CCHFV nucleocapsid protein also binds viral mRNA 5' UTR and favors the translation of CCHFV mRNA. We are in process of developing a high throughput screening assays to target CCHFV nucloecapsid protein for therapeutic intervention. Hantaviruses also have two glycoprotein Gn and Gc. Gn has a cytoplasmic tail domain of 142 amino acids. The interaction between Gn tail domain and hantavirus nucleocapsid protein is required for selective packaging of viral nucleocapsids into virus particles. We have developed a novel assay to monitor the interaction between Gn tail domain and nucleocapsid protein. We have screened a chemical library of 1000 compounds and have identified a novel inhibitor that disrupts the interaction. The inhibitor is also under development as anti-viral therapeutic. The results from this research project are under review for publication. In short, the targets identified in our basic research program are targeted for anti-viral drug development in this research avenue. We have developed collaborations with medicinal chemists, animal model experts, computational biologists for the efficient promotion of this translational research avenue with a broad aim to target Bunyaviruses for therapeutic intervention. I served as lead investigator in these studies.

1. **Mir, MA*** and A. T Panganiban: The triplet repeats of the Sin Nombre hantavirus 5' untranslated region are sufficient in cis for nucleocapsid-mediated translation initiation: *J. Virology*, (2010) Sep;84(17):8937-44.
2. Salim N and **Mir MA***. A novel chemical scaffold with broad spectrum antiviral activity, 2015, (*PLos Pathogens*, Under review)
3. Roy S and **Mir MA***. A novel molecule disrupts the interaction between hantavirus Gn tail domain and nucloecapsid protein: A potential antiviral therapeutic, 2015 (*J. Virology*, in preparation)
4. **Patent (Docket number K1262.10071US01):** A novel chemical Scaffold with broad-spectrum antiviral activity (application submitted, Jan 2015)

4. ***Autophagic degradation of hantavirus glycoprotein*:** Hantavirus glycoprotein precursor (GPC) is post-translationally cleaved into two glycoproteins Gn and Gc, which are incorporated into viral envelope during virus assembly. We recently found that Gn is post-translationally degraded by the host autophagy machinery during the early stages of virus replication cycle. Interruption in autophagic degradation of Gn abrogates virus replication. Gn is an important structural component of the virion, it escapes autophagic degradation during the assembly and packaging phase of the virus replication cycle by an unknown mechanism. We have identified [redacted] protein [redacted] as a novel host factor likely regulating the expression of Gn through the hantavirus replication cycle. A good publication in this area is under preparation. I served as lead investigator in these studies.

1. Islam T. M. Hussein, Erdong Cheng, Michael J. Werle, Sheema Sheema and **Muhammad A. Mir** *: Autophagic clearance of Sin Nombre hantavirus glycoprotein Gn promotes virus replication in cells, J. *Virology*, (2012), Jul;86(14):7520-9. doi: 10.1128/JVI.07204-11. Epub 2012 May 2

2.Ganaie, S.S and **Mir MA ***. The role of viral genomic RNA and nucleocapsid protein in the autophagic clearance of hantavirus glycoprotein Gn. *Virus Res.* 2014 Jan 8. pii: S0168-1702(13)00480-2.

5. *Role of hantavirus nucleocapsid protein in hantavirus replication*. It is still a mystery that how the nucleocapsid protein of RNA viruses specifically recognizes the viral genomic RNA and packages it into new virions. We have found that trimeric hantavirus nucleocapsid protein forms a molecular complex that has potential to discriminate between viral and non viral RNAs inside the host cell. We demonstrated that trimeric nucleocapsid protein selectively recognizes the viral RNA panhandle structure formed by the partially complementary nucleotides at the 5' and 3' termini of viral genomic RNA. We propose this interaction selectively packages the viral genome into new virions during the virus assembly. In addition, we showed that hantavirus nucleocapsid protein has RNA helix unwinding activity that is likely required for transcription initiation in conjunction with viral RdRp. I served as primary investigator in these studies.

1. **Mir, MA** and A. T Panganiban. The hantavirus nucleocapsid protein recognizes specific features of the viral RNA panhandle and is altered in conformation upon binding. *J Virol*. (2005) Feb;79(3):1824¬35.

2. **Mir, MA** and A. T Panganiban. Characterization of RNA chaperon activity of Hantavirus Nucleocapsid protein activity. *J. Virol*, (2006) Jul;80 13:6276-85.

3. **Mir, MA** , B. Brown, B.L. Hjelle, W.A Duran and A. T Panganiban . Hantavirus N protein exhibits genus specific recognition of the v RNA panhandle, (2006), *J. Virol*. 13:6276-850

4. **Mir, MA** and A. T Panganiban. Hantavirus Nucleocapsid protein is an RNA Chaperon. *RNA*, (2006), 12:272-282.

D. Research Support

Ongoing Research Support: No Active grants at present

Completed Grant Support:

R01 AI095236 Mir (PI) 08/01/2011-07/31/2014
NIH/NIAID
Identification and Characterization of inhibitors for hantavirus replication
The major goals of this project are to develop an assay for the identification of inhibitors that can inhibit the interaction between the tail domain of glycoprotein Gn and nucleocapsid protein. These inhibitors should block the packaging of viral genome during virus replication in cells.

R21 AI097355 Mir (PI) 03/01/2012-02/28/2014
NIH/NIAID
Characterization of hantavirus N protein-mediated translation mechanism
The major goal of this project is to characterize the hantavirus nucleocapsid protein mediated translation initiation mechanism.

[redacted]

P20 RR016443 Mir (PI) 07/01/2010-06/30/2012
NIH/NCRR
Mechanics of hantavirus nucleocapsid protein mediated translation initiation of viral mRNA
Major goals of this project are to identify the exact component of 43s pre-initiation complex that interacts with N during ribosome loading on hantaviral mRNA.

R21 AI083672-01
NIH/NIAID Mir (PI) 07/01/2009-06/30/2011
Role of hantavirus nucleocapsid protein and cellular P bodies in viral mRNA "cap snatching"
The major goals of this project are to determine the role of cellular P bodies in Hantavirus replication, and to understand the mechanism of cap snatching in Hantaviruses

Pending grants:

[redacted]

OMB Number: 4040-0001
Expiration Date: 06/30/2016

RESEARCH & RELATED BUDGET - SECTION A & B, Budget Period 1

ORGANIZATIONAL DUNS*: [redacted]

Budget Type*: ● Project ○ Subaward/Consortium

Enter name of Organization: Western University of Health Sciences

Start Date*: 04-01-2017 **End Date*:** 03-31-2020 **Budget Period: 1**

A. Senior/Key Person

Prefix	First Name*	Middle Name	Last Name*	Suffix	Project Role*	Base Salary ($)	Calendar Months	Academic Months	Summer Months	Requested Salary ($)*	Fringe Benefits ($)*	Funds Requested ($)*
1 . Dr.	Mohammad		Mir	[redacted]	[redacted]		[redacted]			[redacted]	[redacted]	[redacted]

Total Funds Requested for all Senior Key Persons in the attached file

Additional Senior Key Persons: File Name:

Total Senior/Key Person [redacted]

B. Other Personnel

Number of Personnel*	Project Role*	Calendar Months	Academic Months	Summer Months	Requested Salary ($)*	Fringe Benefits*	Funds Requested ($)*
	Post Doctoral Associates						
1	Graduate Students	[redacted]			[redacted]	[redacted]	[redacted]
	Undergraduate Students						
	Secretarial/Clerical						
1	**Total Number Other Personnel**					**Total Other Personnel**	[redacted]
						Total Salary, Wages and Fringe Benefits (A+B)	[redacted]

RESEARCH & RELATED Budget {A-B} (Funds Requested)

Funding Opportunity Number: PA-16-200 . Received Date: 2016-06-07T18:27:44.000-04:00

RESEARCH & RELATED BUDGET - SECTION C, D, & E, Budget Period 1

ORGANIZATIONAL DUNS*: [redacted]

Budget Type*: ● Project ❍ Subaward/Consortium

Organization: Western University of Health Sciences

Start Date*: 04-01-2017 **End Date*:** 03-31-2020 **Budget Period: 1**

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

Equipment Item	**Funds Requested ($)***
Total funds requested for all equipment listed in the attached file	
Total Equipment	

Additional Equipment: File Name:

D. Travel	**Funds Requested ($)***
1. Domestic Travel Costs (Incl. Canada, Mexico, and U.S. Possessions)	[redacted]
2. Foreign Travel Costs	
Total Travel Cost	[redacted]

E. Participant/Trainee Support Costs		**Funds Requested ($)***
1. Tuition/Fees/Health Insurance		
2. Stipends		
3. Travel		
4. Subsistence		
5. Other: Internships		[redacted]
Number of Participants/Trainees	**Total Participant Trainee Support Costs**	[redacted]

RESEARCH & RELATED Budget {C-E} (Funds Requested)

Funding Opportunity Number: PA-16-200 . Received Date: 2016-06-07T18:27:44.000-04:00

RESEARCH & RELATED BUDGET - SECTIONS F-K, Budget Period 1

ORGANIZATIONAL DUNS*: [redacted]

Budget Type*: ● Project ❍ Subaward/Consortium

Organization: Western University of Health Sciences

Start Date*: 04-01-2017 **End Date*:** 03-31-2020 **Budget Period: 1**

F. Other Direct Costs	Funds Requested ($)*
1. Materials and Supplies	[redacted]
2. Publication Costs	
3. Consultant Services	
4. ADP/Computer Services	
5. Subawards/Consortium/Contractual Costs	
6. Equipment or Facility Rental/User Fees	
7. Alterations and Renovations	
8 . n/a	0.00
Total Other Direct Costs	[redacted]

G. Direct Costs	Funds Requested ($)*
Total Direct Costs (A thru F)	[redacted]

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)*
1 . MTDC	[redacted]	[redacted]	[redacted]
		Total Indirect Costs	[redacted]

Cognizant Federal Agency (Agency Name, POC Name, and POC Phone Number) DHHS, DCA Western Field Office, Karen Wong, 415-437-7820

I. Total Direct and Indirect Costs	Funds Requested ($)*
Total Direct and Indirect Institutional Costs (G + H)	[redacted]

J. Fee	Funds Requested ($)*

K. Budget Justification*	File Name: 1234-Budget justification-2-2.pdf (Only attach one file.)

RESEARCH & RELATED Budget {F-K} (Funds Requested)

Personnel Justification

Mohammad Mir, PI 3 Calendar Months; Will oversee the overall research directions of the project. He will work closely with the students and train them in all aspects of experimental design, data analysis and presentation, research ethics, data storage and manuscript writing. Dr. Mir will help in cloning and expression of N protein and other host factors in bacterial and mammalian systems, luciferase reporter assays, virus replication, gene knockdown assays, and all other experiments related to protein kinase R, proposed in specific aims 2.

Graduate students, TBN. 6 Calendar Months: The DVM students from the college of veterinary medicine and other graduate students from other colleges, especially the students from graduate college of Biomedical Sciences will be recruited in this project. The students with prior experience in Biochemistry or Molecular Biology will be prefered. The students under the close supervision of the PI will work on specific aim 1 during the 1st year, especially the cloning of host mRNA UTR sequences in reporter constructs and their role in N-protein mediated translation strategy. Students will also work to determine the role of host factors in virus replication. During the second year, the stdunets will work hand-in-hand with the PI to carry out *in silico* studies to identify the N-protein binding sites on the UTR sequences. Also the knockdown experiments aimed to identify the host factors playing a role in N-protein mediated PKR inhibition will be carried out in the second year. During the third year, the students will focus to determine the role of PKR in hantavirus organ tropism during the course of viral infection.

Undergraduate students, TBN. Few hours a week: The undergraduate students from California state polytechnic University (Cal Poly) at Pomona will be recruited to work in this project. Cal Poly is located at a distance of 3 miles from the CVM. Consistent with my undergraduate student recruitment plan, I will be giving seminars in Cal Poly to attract students for Biomedical research. The students interested to pursue a career in biomedical research will be provided internships to work throughout the academic year on proposed studies. Again the students will work under close supervision of the PI.

Supply Justification:

(i) Protein purification reagents, NiNTA packed Column, GST purification column

(ii) Restriction Enzymes, plasmid DNA purification kits, T7 transcription kits for the synthesis of RNA, RNeasy kits for the purification of RNA, Real time PCR reagents, Radioisotopes.

(iii) siRNA to knockdown vinculin, [redacted], glycoprotein [redacted] heat shock proteins [redacted] Plasmids for Lentivirus preparation used in gene knowkdown.

iv) Antibodies for western blot analysis of [redacted]

(v) Media, antibiotics and serum for mammalian cell cultures, media fro bacterial cultures, Reagents for purifying bacterially expressed His-tagged and GST-tagged fusion proteins

(vi) Reagents for Agarose gels, SDSPAGE, PCR reagents, protein and DNA markers, plastic ware including Petri dishes, flasks, tubes and pipette tips, films, PVDF membranes for western blots, reagents for making buffers

(vii) Reagents for luciferas reporter assay. Reagents for fluorescence binding assays and biolayer interferomery.
(viii) Cell lines and growth reagents for HUVEC, cardiac, pulmonary and glomerular microvascular endothelial cells.

Travel Justification:

PI needs to attend at least one national meeting each year on negative strand RNA viruses

RESEARCH & RELATED BUDGET - Cumulative Budget

	Totals ($)	
Section A, Senior/Key Person		[redacted]
Section B, Other Personnel		[redacted]
Total Number Other Personnel	1	
Total Salary, Wages and Fringe Benefits (A+B)		[redacted]
Section C, Equipment		
Section D, Travel		[redacted]
1. Domestic	[redacted]	
2. Foreign		
Section E, Participant/Trainee Support Costs		[redacted]
1. Tuition/Fees/Health Insurance		
2. Stipends		
3. Travel		
4. Subsistence		
5. Other	[redacted]	
6. Number of Participants/Trainees		
Section F, Other Direct Costs		[redacted]
1. Materials and Supplies	[redacted]	
2. Publication Costs		
3. Consultant Services		
4. ADP/Computer Services		
5. Subawards/Consortium/Contractual Costs		
6. Equipment or Facility Rental/User Fees		
7. Alterations and Renovations		
8. Other 1	0.00	
9. Other 2		
10. Other 3		
Section G, Direct Costs (A thru F)		[redacted]
Section H, Indirect Costs		[redacted]
Section I, Total Direct and Indirect Costs (G + H)		[redacted]
Section J, Fee		

Funding Opportunity Number: PA-16-200 . Received Date: 2016-06-07T18:27:44.000-04:00

PHS 398 Cover Page Supplement

OMB Number: 0925-0001

Expiration Date: 10/31/2018

1. Human Subjects Section

Clinical Trial? ❍ Yes ● No

*Agency-Defined Phase III Clinical Trial? ❍ Yes ❍ No

2. 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 proved scientific justification

3. *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)

Tracking Number: GRANT12187057

Funding Opportunity Number: PA-16-200 . Received Date: 2016-06-07T18:27:44.000-04:00

PHS 398 Cover Page Supplement

4. 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, please 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):

5. Inventions and Patents Section (RENEWAL)

*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 Research Plan

OMB Number: 0925-0001
Expiration Date: 10/31/2018

Introduction	
1. Introduction to Application (Resubmission and Revision)	1241-Introduction to application .pdf
Research Plan Section	
2. Specific Aims	1242-Specific aims.pdf
3. Research Strategy*	1243-Research Strategy.pdf
4. Progress Report Publication List	
Human Subjects Section	
5. Protection of Human Subjects	
6. Data Safety Monitoring Plan	
7. Inclusion of Women and Minorities	
8. Inclusion of Children	
Other Research Plan Section	
9. Vertebrate Animals	
10. Select Agent Research	
11. Multiple PD/PI Leadership Plan	
12. Consortium/Contractual Arrangements	
13. Letters of Support	1244-Letters of support combined.pdf
14. Resource Sharing Plan(s)	1245-Resource Sharing Plan.pdf
15. Authentication of Key Biological and/or Chemical Resources	
Appendix	
16. Appendix	

Introduction to application: I would like to thank all three reviewers of my initial application for their critical and constructive evaluation of my proposal. The concerns raised by reviewers are carefully addressed in this revised version. The changes are shown in italic letters.

REVIEWER 1: **Concern 1**: The reviewer 1 mentioned that studies proposed in specific aim 1 have not presented a clear prioritization of the experimental work to be done. **Response:** I would like to thank the reviewer for raising an interesting question. I have presented a clear prioritization plan for the work proposed in this aim. Briefly, the identified host factors have been grouped into three priority groups A, B and C. The prioritization is based on the fold change in their protein expressions levels and their role in the replication of other viruses. **Concern 2**: The conclusion that HTNV N protein does not interact with PKR is only based on results from CoIP, where it is well known that CoIP cannot detect many bona fide PPI. It would be important to confirm the CoIP results using for example the mammalian TH or confocal microscopy. **Response:** I agree with the reviewer. I have proposed the use of mammalian two-hybrid system and confocal microscopy to further confirm the CoIP results, as suggested by the reviewer. **Concern 3:** The host factors identified by the investigator have been reported to play a role in he replication of viruses that are entirely unrelated to HANTV. **Response**: To address the reviewer's concern, the siRNA knockdown of VCP (known to play a role in the replication of other viruses) revealed that VCP does play a role in hantavirus replication (Fig 3E-G). Similarly, we hope that other host factors also play a role in HTNV replication. **Concern 4**: Why not focus on two or three of the identified candidate protein molecules to validate the concept being explored prior extending the research to the large collection of candidates. **Response**: We have decided to test the 5' and 3' UTR sequences of ten transcripts from priority groups A and B. This slightly broader study will help to identify the best cis-acting RNA sequence preferred by N-protein to boost the mRNA translation. Such a sequence will be used in future to generate a translation system to facilitate the translation of desired mRNA of interest with the assistance of N-protein. Moreover, the data obtained from the analysis of panel of mRNA molecule will be used in *in-slico* studies to identify a potential RNA structural motif that binds N-protein. Such a structural motif will be helpful in future for the design of an inhibitor to shutdown the N-protein mediated translation strategy. Thus we felt that analysis of a panel of mRNA molecules might be highly helpful.

REVIEWER 2: **Concern 1**: There is a possibility that host mRNA that bear the UAGUAGUAG sequence in their UTRs are not important for viral replication, and the presence of this sequence is random. If this were the case, the impact of the first aim would be diminished. **Response:** The N-protein mediated translation up-regulation of host cell mRNA bearing UAGUAGUAG sequence or other structural determinant may or may not be important for virus replication, although the role of translation up-regulation of VCP in hantavirus replication suggest that may not necessarily be the case. In addition, even if translation up-regulation of certain host factors does not play a role in virus replication, the cis acting sequences in their mRNAs required by N-protein to promote their translation will be useful to generate novel translation system to boost the translation of desired proteins of interest such as therapeutic antibodies. **Concern 2**: Obviously, knockdown of any gene that is important for normal cellular function could adversely affect virus production indirectly, so drawing conclusions may not be as straightforward as indicated here. **Response.** We agree with the reviewer that drawing conclusions from knockdown experiments is not that straightforward. However, such experiments provide insights about he pathways that might be indirectly involved in virus replication. In addition, knockdown of housekeeping genes important for cellular function usually show cytotoxic effects, which will be taken into consideration while interpreting the data. **Concern 3**: It is unclear yet how the environment at PIs new institution will support this proposed work. **Response:** The CVM has significantly upgraded its research facilities. I would like to mention that research facilities at the Western University are excellent and the overall environment is highly supportive. **Concern 4:** The description of availability of students is somewhat light and the letter of support from the VPR at Western University is similarly vague. If undergraduate students are coming to Western from, e.g. the Claremont colleges for research experiences, this collaboration should be more clearly described. **Response**: I have already hired two DVM students this year in my research program. The hiring plan for students in this research project has been incorporated. The two letters of support from California Poly technique University, a major undergraduate institution in California, have been provided. The VPR of Western University has provided a new letter of support. I hope it meets the expectations.

REVIEWER 3: **Concern 1**: The number of transcripts to be screened in specific aim 1 was not well defined. **Response:** I have provided the prioritization plan in this revised version. **Concern 2**: It is not clear from the application how many DVM and undergraduate students will be involved in this research and how the students will be recruited. Letters of support from undergraduate universities would strengthen the application. **Response**: We plan to recruit two undergraduate and two graduate students. This number may go up depending upon the free time each student has to work on the project. A recruitment plan has been incorporated and the letters of support have also been included as requested by the reviewer.

Specific aims: The majority of eukaryotic mRNA translation is m7G cap dependent and is initiated by the formation of a ternary complex between methionine-loaded initiator tRNA, eIF2 and GTP (Fig 1A). The ternary complex then joins the 40S ribosomal subunit along with other initiation factors and forms the 43S-preinitiation ribosome complex, which is loaded on eIF4F cap binding complex assembled on the mRNA 5' cap. The eIF4F complex is composed of three initiation factors eIF4E, eIF4A (8, 9) and eIF4G (12). The ribosome loading on an mRNA molecule is the rate-limiting step in eukaryotic translation. As an antiviral response, host cells interrupt the formation of ternary complex by the phosphorylation of α subunit of eIF2, carried out by protein kinase R (PKR). As a result the host translation machinery is transiently shutdown to create roadblocks for the synthesis of viral proteins. Interestingly, we found that hantavirus N-protein prevents the host induced translation shutdown by inhibiting PKR in virus infected cells. Despite the PKR inhibition, the viral mRNAs still have to compete with the host cell transcripts for the same translation apparatus. To avoid such competition, we reported that hantavirus N-protein lures the host translation machinery for the preferential translation of viral mRNA. We showed that N-protein binds to the ribosomal protein S19 (RPS19), a structural component of the 40S ribosomal subunit (14). N-protein also binds to a highly conserved triplet repeat sequence (UAGUAGUAG) of the viral mRNA 5' untranslated region (5'UTR) (18, 20). N-protein associated ribosomes are preferentially loaded on viral mRNA 5' UTR to boost the translation of downstream open reading frame (ORF). Thus, N-protein binding with RPS19 likely reserves a population of host cell ribosomes that remain dedicated for the translation of viral mRNA in virus infected cells. Based on these observations we asked whether host cell mRNAs whose 5' UTRs share a sequential or structural homology with the viral mRNA 5' UTR are also preferentially translated by the N-protein mediated translation strategy. Our preliminary data shows that N-protein favors the translation of a subset of cellular mRNAs, likely encoding host factors required for efficient virus replication. *The central focus of this grant application is to delineate the molecular mechanism for PKR inhibition and selective translation of some host cell mRNAs by N-protein mediated translation strategy*. Based on our preliminary data we hypothesize that certain host cell mRNAs harbor high affinity N-protein binding sites upstream of the start codon. These binding sites selectively engage N-protein associated ribosomes similar to viral mRNA 5'UTR to boost the translation of downstream ORF. We also hypothesize that N-protein either directly binds to PKR or uses a host cell factor to inhibit PKR dimerization, a crucial step required for its activation. We propose following aims to test these hypotheses.

Specific aim 1: To delineate the mechanism for the preferential translation of host cell mRNAs by N-protein mediated translation strategy: We examined the effect of N-protein upon the regulation of host gene expression at the translation level, using two dimensional in gel-electrophoresis (2D-DIGE). We identified numerous host cell factors that are regulated at translational level by N-protein mediated translation mechanism. Some of these factors have been previously reported to play a role in virus replication. To further confirm this observation, we will express translation deficient N-protein mutant in HUVEC cells and examine its impact upon the translation of identified host factors using western blot analysis. We will identify and characterize the mechanism for the preferential translation of identified host factors by N-protein mediated translation strategy. We will demonstrate how their translation regulation plays a role in virus replication. These studies will provide insights about the molecular mechanism of hantavirus pathogenesis and will reveal new targets for therapeutic intervention of hantavirus disease.

Specific aim 2: How N-protein inhibits PKR dimerization to counteract the host interference in viral protein synthesis?

Host cells primarily activate PKR-eIF2α pathway as an antiviral response to shutdown the translation machinery and create barriers for the synthesis of viral proteins. However, it is well known that hantavirus infected cells do not undergo translation shutdown. PKR undergoes dimerization, which is required for its autophosphorylation to gain activity. Our preliminary data shows that N-protein inhibits PKR dimerization and subsequent autophosphorylation. We will use multifaceted experimental avenues to demonstarte how N-protein either with the assistance of an endogenous host cell factor or by direct interactin with PKR inhibits its dimerization to ensure the availability of active host translation machinery for the synthesis of viral proteins during the course of infection.

(a) Significance: Hantavirus cardiopulmonary syndrome (HCPS) and hemorrhagic fever with renal syndrome (HFRS) have mortality rates of 15 and 50 percent, respectively. Annually, 150,000 to 200,000 cases of hantavirus infection are reported worldwide, and there is no treatment for hantavirus associated diseases at present. Lack of FDA approved vaccine and antiviral therapeutic agents make hantavirus disease a global health problem. Proposed studies in specific aim 1 will demonstrate how a viral translation initiation strategy regulates the expression of host cell factors at translational level to promote virus replication and dissemination to neighboring cells. These studies will provide critical insights about the pathogenesis of HFRS and HCPS, and will reveal new targets for therapeutic intervention of hantavirus infection. The proposed studies in specific aim 2 will reveal new host factors required by a newly discovered PKR inhibitor (N-protein) to ensure that host translation machinery remains abundantly available without interruption for the synthesis of viral proteins. The specific aim 2, focused to reveal new conceptual insights about the role of PKR in hantavirus tissue tropism and potential to cross species barriers with the assistance of endogenous host factor, is of potential significance to general virology and the biology of PKR.

The proposed studies will expose the students of Western University to modern technologies in Microbiology and infectious disease research. In addition, the proposed research studies will greatly strengthen the over all research environment at the Western University of Health Sciences, especially in the College of veterinary medicine.

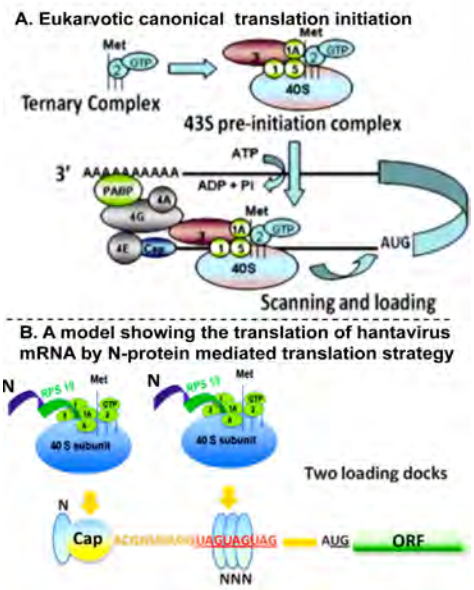

Fig 1: A. Canonical eukaryotic translation initiation: A ternary complex is formed between eIF2, GTP and methionine loaded initiator tRNA. The ternary complex binds to the 40S ribosomal subunit along with other initiation factors. The 43S pre-initiation complex is loaded on eIF4F complex composed of eIF4E, eIF4G and eIF4A. Some initiation factors have been omitted for simplicity. **B. A model showing ribosome loading on 5' UTR of viral mRNA.** Two ribosome loading dock model is proposed. Monomeric and trimeric N-protein molecules bind to the mRNA 5' cap and triplet repeat sequence (red) at the viral mRNA 5' UTR, respectively. Another molecule of N-protein binds to RPS19 of the 40S ribosomal subunit. We propose that N-protein associated ribosomes are loaded on both the 5' cap and triplet repeat sequence of the viral UTR. N-protein associated ribosomes can also be loaded internally on the triplet repeat sequence, as N-protein mediated translation strategy can also facilitate translation uncapped mRNA, although the efficiency is less compared to capped mRNAs. **The results pertinent to this model have been published (1-7).**

(b) Innovation: It is our assessment that the project is quite innovative with regards to the concepts, the disease and the proposed experimental approach. Eukaryotic cells have evolved a complex and tightly regulated translation initiation mechanism involving a constellation of initiation factors to avoid breaches in the translation process by invading pathogens. We have found a novel translation initiation mechanism, operated by a single viral protein that evades the function of eIF4F complex, an amalgam of three-initiation factors eIF4E, eIF4G and eIF4A. Further studies to delineate the role of this viral mechanism in the regulation of host gene expression at the translational level will bring significant innovation in the research on translation control and viral pathogenesis. The observations that N-protein regulates the host gene expression post-transcriptionally, and selectively facilitates the translation of host cell factors required for virus replication are very interesting and novel. Similarly, the hypothesis that N-protein either by direct interaction or with the assistance of a host cell factor inhibits PKR to counteract host interference in viral protein synthesis is conceptually innovative. The proposed studies will reveal novel host factors of potential therapeutic importance for the *Bunyaviridae* family of viruses.

(C) Approach: *preliminary studies*: Since most of the preliminary data related to this application is published (1-7), I will briefly summarize it below. During routine transfection experiments, we cotransfected HeLa cells with N-protein expression plasmid along with either GFP or luciferase reporter plasmid and surprisingly observed a concomitant five fold increase in both GFP and *Luc* expression. Real time PCR analysis showed that N-protein expression did not detectably affect the endogenous indicator mRNA levels, suggesting that N-protein augmented expression at the translational level (5). Further in-depth studies revealed that N-protein facilitated the translation of both capped and uncapped mRNAs, although the effect of N-protein on mRNA translation was more efficient and manifested at a lower N-protein concentration when indicator mRNA contained a 5' m7G cap (5). While defining the mechanism of N-protein mediated translation strategy, we found that N-protein binds to the mRNA 5' cap

and requires four nucleotides adjacent to the 5' cap for high affinity binding (5). We demonstrated that N-protein has distinct m7G cap and RNA binding domains (7). The cap binding activity of N-protein is consistent with its preferential translation of capped mRNAs. To determine how N-protein preferentially facilitated the translation of capped mRNA, we demonstrated that N-protein interacts with the 40S ribosomal subunit and efficiently loads it onto the 5' mRNA cap during translation initiation (5). Since the 40S subunit is a blend of 18S rRNA and multiple ribosomal proteins, it was obvious to identify the exact component of this huge complex that interacts with N-protein. Using multifaceted experimental avenues, including far-western blot, LC/MS/MS mass spectrometry and immunoprecipitation, we showed that N-protein interacts with ribosomal protein S19 (RPS19), a structural component of the 40S subunit (14). Using fluorescence spectroscopy, we reported the detailed characterization of N protein-RPS19 interaction (31). The RPS19 does not contain a cystine residue in its sequence. We generated an RPS19 mutant (RPSm1, Fig 2) containing a single cystine residue at the N terminus next to the initiating methionine. The cystine side chain of mutant RPSm1 was covalently modified with 1,5 IAEDANS, a thiol reactive fluorescent probe using a standard protocol (16). The point mutation and fluorescence labeling did not affect the structure and function of RPS19 (31). We observed a consistent decrease in the fluorescence quantum yield of IAEDANS labeled RPSm1 due to the interaction with purified N-protein (Fig 2). Analysis of fluorescence binding data revealed a dissociation constant (K_d) of 95 nM for N protein-RPS19 interaction. Binding stoichiometry analysis (Fig 2 D1 & D2) revealed that N-protein and RPS19 interact with a molar ratio of 1:1. Using numerous experimental avenues such as immunoprecipitation, His and GST tag pull-down assays, circular dichroism (CD) spectroscopy and biolayer interferometry, we mapped and characterized the RPS19 binding domain at the N-

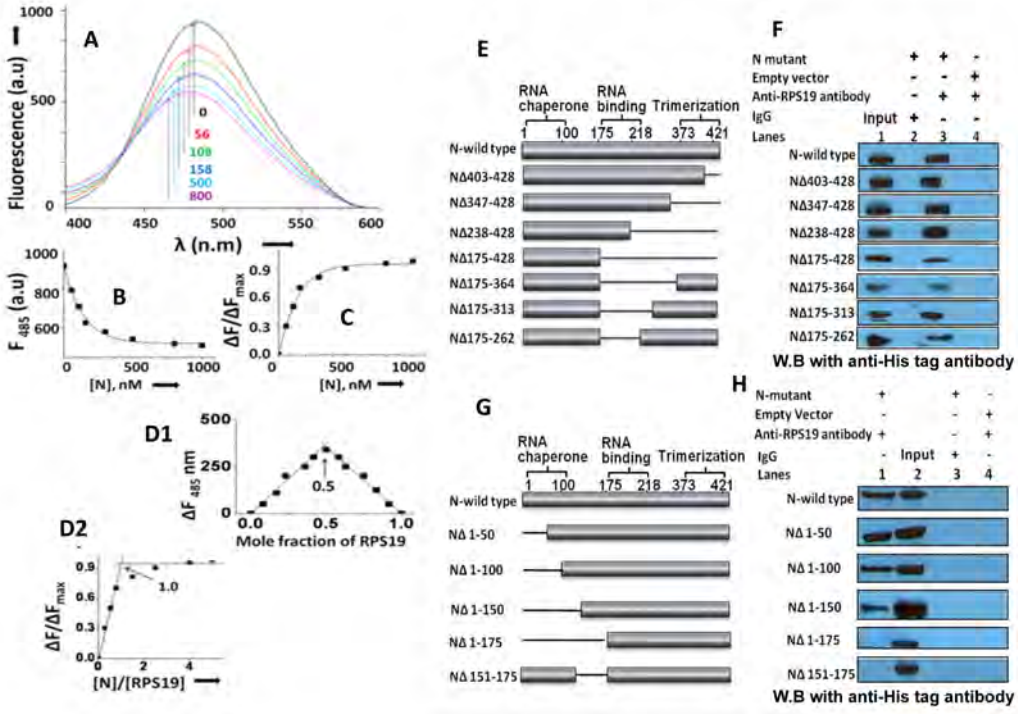

Fig 2: (A). A fixed concentration of IAEDANS labeled RPSm1 (200 nM) was excited at 336 nm, and fluorescence spectrum from 400-600 nm was recorded (black line). The experiment was carried out at 25°C. The fluorescence signal from free binding buffer in the absence of RPSm1 was subtracted wherever required. The fluorescence spectra of IAEDANS labeled RPSm1 at increasing concentrations of purified N protein are shown in different colors. The input concentration of N protein (nM) corresponding to each spectrum is shown by arrow. **B.** The fluorescence intensity of IAEDANS labeled RPSm1 at 485 nm was recorded at each input concentrations of N protein. **(C).** The data from panel D was used to generate a plot of $\Delta F/ \Delta F_{max}$ versus N protein concentration for the calculation of dissociation constant (K_d) as previously reported (11). **D1** . The data from panel D was re-plotted for the calculation of binding stoichiometry as previously reported (13). **D2**. Binding stoichiometry was confirmed using continuous variation plot, as previously reported (11, 13). **E & G**. Diagrammatic representation of wild type and mutant N-protein used in this study. Thin line represents the amino acid sequence that was deleted from the wild type N protein. **F and H**. HeLa cells in 60 mm dishes were transfected with either empty vector or plasmid expressing the N mutant of interest. Cells were harvested thirty-six hours post transfection and lysed in 400 µl of RIPA buffer. 300 µl of the resulting cell lysates were incubated overnight at 4 °C with 1 µg of either anti-RPS19 antibody or IgG (negative control), followed by further incubation with 50 µl of washed sepharose G beads for six hours at 4 °C. Beads were washed three times with 1X PBS and incubated with 60 µl of SDS loading dye on boiling water bath. After brief centrifugation 30 µl of the supernatant were loaded on 12% SDS PAGE and examined by western blot (W.B) analysis using anti-His tag antibody. Input represents the 8-10% of the total cell lysate, loaded as positive control. In addition, all mutants were stably expressed in cells, except the mutant (NΔ238-428) was slightly degraded. The degradation products also interacted with RPS19, which are not shown. Some of the mutants shown in panels A and D were also used in our previous publication (15). **Note: The data shown in Fig 2 is published in (1, 2)**

terminus of N-protein (2). Its deletion did not influence the secondary structure, but affected the conformation of trimeric N-protein molecules. The N-protein mutant lacking the RPS19-binding region was able to bind the mRNA 5' cap. In addition, the mutant formed stable trimers similar to wild-type N-protein. Use of this deletion mutant in multiple experiments provided insights into the mechanism of ribosome loading during N-protein mediated translation strategy. The preliminary study suggested that N-protein molecules individually associated with the mRNA 5' cap and RPS19 of the 40S ribosomal subunit undergo N protein-N protein interaction to facilitate the engagement of N-protein associated ribosomes at the mRNA 5' cap, without the requirement of eIF4F cap binding complex (Fig 1) (5). During translation initiation, cellular mRNAs are circularized by the interaction of poly A tail with poly A binding protein (PABP), (see Fig 1A). We showed that N-protein does not interact with any component of eIF4F complex or PABP, suggesting that N-protein mediated translation strategy likely does not circularize the mRNA (5). This idea is consistent with the previous observations that hantavirus S and L segment mRNAs don't have a poly A tail.

Although N-protein mediated translation strategy facilitated the translation of capped mRNAs, it was obvious to determine whether it had some preference for the translation of viral transcripts. Hantaviral viral mRNAs are 5' capped, lack 3' poly A tail, and contain highly conserved triplet repeat sequence (UAGUAGIUAG) in the short 5' untranslated region (5'UTR) of 36-52 nucleotides in length. The GFP or luciferase reporter mRNAs containing either 5' viral mRNA UTR or a non-viral UTR of same length were synthesized *in vitro* by T7 transcription reaction. The resulting reporter mRNAs in equimolar ratios were translated in rabbit reticulocyte lysates in the presence or absence of bacterially expressed and purified N-protein. These *in vitro* competitive translation assays revealed that N-protein preferentially favored the translation of any reporter mRNA containing the 5' viral mRNA UTR derived from either S or M or L segment genome (17). Further studies showed that a monomeric and a trimeric N molecule bind with specificity at the viral mRNA 5' cap and triplet repeat sequence (UAGUAGUAG), respectively. The binding of trimeric N molecule at the triplet repeat sequence significantly favored ribosome loading onto the 5' UTR of viral mRNA and dramatically enhanced the translation of downstream ORF (17). Mutations in the UAGUAGUAG sequence abrogated both the N-protein binding to the UTR and preferential translation of the downstream ORF. We propose that hantaviral mRNA 5'UTR has two ribosome loading docks, one at the 5' cap and another at the triplet repeat sequence of the 5'UTR (Fig 1B). The ribosome loading at the triplet repeat sequence occurs through IRES (internal ribosome entry site) type of mechanism, based on the fact that N-protein can favor the translation of uncapped mRNA **(5)**. The ribosome loading at both the 5' cap and triplet repeat sequence does not require eIF4F cap binding complex **(5).**

Based on these observations, we hypothesize that host cell transcripts having viral sequence (UAGUAGUAG) in the 5' UTR or having a structural homology with the viral mRNA 5' UTR will also be preferentially translated by the N-protein mediated translation mechanism. To test this hypothesis, we packaged lentiviruses expressing either N-protein or GFP as negative control. HUVEC cells were infected with the resulting lentiviruses and cells were lysed 48 hours post-infection. Cell lysates were examined to determine the impact of N-protein expression on the translation of host cell mRNAs, using 2D-DIGE and MALDI-TOF/TOF approach (Fig 3). A total of 58 well resolved protein spots whose translation was altered by ≥ 1.4 fold by N-protein in comparison to GFP control were identified and selected for identification by tandem mass spectrometry (MS/MS). Around half of the spots were translationally up-regulated in the range of 1.4-2.6 fold due to N-protein expression. The MS/MS analysis with high confidence score revealed the identification of up-regulated proteins as: [redacted]

To confirm that alterations in the expression levels of these proteins was not due the alterations in their mRNA levels in N-protein expressing HUVEC cells, we purified total RNA from the cell lysates and quantitatively estimated the mRNA levels by real time PCR. As shown in Fig 3B, we did not observe a noticeable change in their mRNA levels due to N-protein expression, suggesting that alteration in their protein expression levels was post-transcriptional. To further confirm these observations, we infected

HUVEC cells with sin nombre hantavirus and examined the expression of these genes at transcriptional level by real time PCR (Fig 3C). We also confirmed the expression levels of few proteins using western blot analysis. As shown in Fig 3C, we again did not observe a noticeable change in their mRNA expression levels in virus infected cells as compared to mock infection. Other investigators who monitored alterations in host gene expression at mRNA level using microarrays also did not observe any change in the levels of above-mentioned mRNA molecules due to hantavirus infection in cells (18). However, the protein expression of four tested gens was significantly altered consistent with similar observations from 2D-DIGE (Fig 2D). For western blot analysis, we selected few genes whose role in virus replication has been previously reported. For example, VCP has been recently reported to help poliovirus replication by playing a role in the protein secretion pathway in poliovirus infected cells (19). The [redacted] along with Gag protein has been reported to help HIV to counteract the anti-viral stress response by blocking the formation of stress granules, the translationally silent RNA triage sites, during the course of infection. Depletion of [redacted] impaired virus production and infectivity. This new [redacted] mechanism has laid the groundwork for new therapeutic strategies to bolster host immune defenses against HIV and other pathogens (20). [redacted] has been reported to regulate cell surface cadherin expression by binding to beta catenin, and Andes hantavirus has been reported to down-regulate vascular endothelial cadherin to disrupt the endothelial cell barrier, a distinct pathology observed in hantavirus infected patients (21, 22). The dramatic increase in endogenous [redacted] levels by N-mediated translation mechanism (Fig 3D) is likely involved in increased vascular

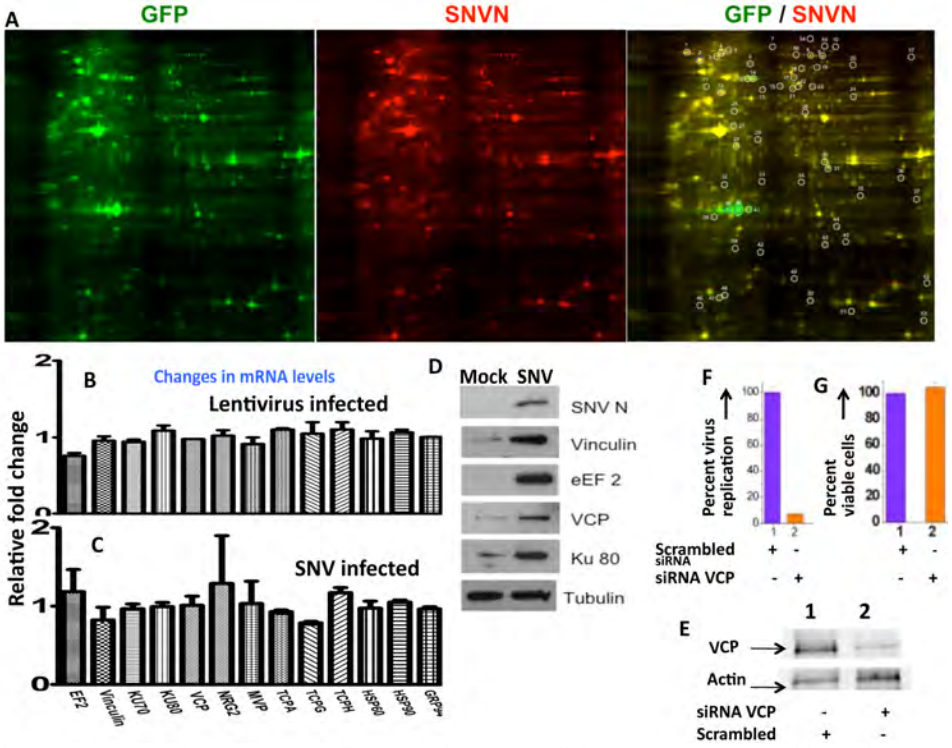

Fig 3: Fig 1: **Panel A**. HUVEC cells grown in 10 mm dishes were infected with lentiviruses expressing either N-protein or GFP. Cells were lysed 48 hours post-infection and 1/4th of the total lysate was saved for real time PCR analysis. Equal volume of cell lysates from N-protein or GFP expressing cells containing equal amount of total protein were labeled with cy3 (red) and cy2 (green), respectively. As an internal control equal volume of cell lysates containing equal amount of total protein from N-protein and GFP expressing cells were mixed together and labeled with cy5. The three protein samples were mixed together and separated on a single gel by 2D electrophoresis. The gel was scanned and computer aided image analysis was performed to examine the signals from labeled protein spots. Normalized intensities from cy2 and cy3 channels were compared to quantitatively estimate the effect of N-protein on the expression of each protein spot. A total of 58 well resolved protein spots (encircled) were examined and 25 of them were precisely excised from the gel and examined by tandem mass spectrometry (MS/MS) analysis to determine their identity. The superimposed image from cy2 and cy3 channels is shown as GFP/SNV. **Panel B**. Total RNA was purified from the lysates saved in panel A, using RNeasy kit. The RNA was reverse transcribed and expression levels of mRNAs of interest were determined by real time PCR, using tubulin as internal control. Each bar represents the fold change in the levels of mRNA of interest in N expressing cells related to cells expressing GFP. **Panel C**. HUVEC cells were infected with SNV at an MOI of 1.0 or mock infected, followed by lysis 48 hours pos-infection and 3/4th of the cell lysates were saved for western blot analysis. Total RNA was purified from remaining 1/4th of the lysate and the expression levels of same mRNAs shown in panel B were determined by real time PCR, as mentioned in panel B. Each PCR reaction in panels B and C was performed in triplicates for the calculation of error bars. **Panel D**. The saved 3/4th of the lysate from panel C was used in western blot analysis to examine the expression of indicated proteins using appropriate antibodies. **Panel E**. A western blot showing the expression of VCP and β-actin in HUVEC cells transfected with either scrambled siRNA (lane 1) or siRNA targeting VCP (Lane 2). Cells were lysed 36 hours post siRNA treatment. **Panel F**. HUVEC cells transfected with either scrambled siRNA or siRNA against VCP for 36 hours were infected with Andes virus and virus replication was monitored by real time PCR analysis of S-segment RNA. Knockdown of VCP significantly inhibited virus replication (compare yellow bar with purple bar). **Panel G**. HUVEC cells transfected with either scrambled siRNA (purple bar) or siRNA against VCP (yellow bar) for 36 hours were examined for viability using cell titer glow reagent (Promega).

leakage during hantavirus infection. The depletion of [redacted] has been reported to delay HIV replication in cells (23). These observations lead to a hypothesis that N-mediated translation mechanism may promote the translation of host cell factors required for hantavirus replication. To briefly test this hypothesis, we used siRNA to knockdown VCP expression, one of the identified host factors (Fig 3E). Its down-regulation did not impact the cell survival (Fig 3G). However, the knockdown of VCP significantly inhibited hantavirus replication in cells (Fig 3F). This observation supports the hypothesis that N-protein may favor the translation of host cell factors required for hantavirus replication.

Specific aim 1: To delineate the mechanism for the preferential translation of host cell mRNAs by N-protein mediated translation strategy:

Rationale and hypothesis: Our previously published studies suggest that N-protein mediated translation mechanism favors the translation of any mRNA containing the viral mRNA 5' UTR (6). Further studies revealed that high affinity binding of N-protein to a highly conserved triplet repeat sequence UAGUAGUAG of the viral mRNA 5' UTR (K_d=95 nM) is required for such preferential translation (6). Based on these observations, we hypothesize that 5' UTR of host cell mRNAs having sequence or structural homology with the viral mRNA 5'UTR are preferentially translated by N-protein mediated translation mechanism. Alternatively, host mRNAs containing sequentially different high affinity binding sites for N-protein at either 5' or 3' UTR, serving as internal ribosome loading sites (IRES) for N-protein associated ribosomes (see Fig 4), will be preferentially translated by N-protein mediated translation strategy. This alternative hypothesis is supported by the fact that N-protein favors the translation of uncapped mRNAs in rabbit reticulocyte lysates (5). The ribosome loading through structured 3' IRES elements is well known in plant viruses (24). The observation that numerous host cell mRNAs generated elevated protein levels in HUVEC cells expressing N-protein without noticeable alterations in their endogenous steady state levels support the hypothesis that these transcripts may contain *cis-acting* high affinity binding sites for N-protein at either 5' or 3' UTR to facilitate the loading of N-protein associated ribosomes *(Fig 4).* Some of these transcripts encode proteins that have been previously reported to play a role in virus replication. It is likely that hantaviruses have evolved N-protein mediated translation strategy not only to boost the translation of viral transcripts but also to promote the translation of host factors that support virus replication. *The proposed studies have broad range significance. For example, the Identification of cis-acting sequences in host cell transcripts, serving as IRES for N-protein mediated translation strategy, can be used in future to design novel translation systems to boost the translation of desired proteins, such as, therapeutic antibodies or tumor suppressor proteins. In addition, the preferential translation of host cell factors playing a role in virus replication will reveal new cellular pathways and novel targets for therapeutic intervention of hantavirus disease.*

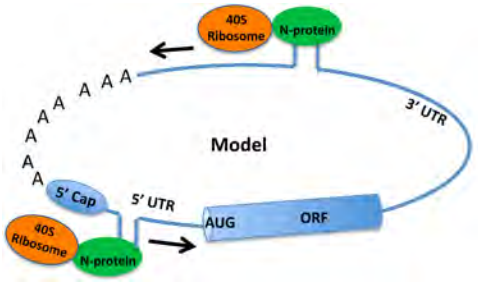

Fig 4: The high affinity binding sites for N-protein are located at either 5' or 3' UTR, and N-protein associated 40S ribosomal subunits are loaded on these sites, which serve as IRES for N-protein mediate translation strategy. The correct ribosome loading boosts the translation of the mRNA.

Experimental design: We will use a translation deficient N-protein mutant to further reconfirm the observation that over-expression of host cell factors in HUVEC cells (Fig 3) is due to N-protein mediated translation mechanism. N-protein contains three crucial domains required to bind RNA, mRNA 5' cap and the 40S ribosomal subunit via the ribosomal protein S19 (RPS19). The RNA binding domain has been mapped to the region from amino acids 175-218 (25). We recently mapped the RPS19 binding domain to the region from amino acids150-175 (2). The cap-binding domain has not been mapped yet. However, we have an N-protein mutant that lacks the entire region from 100-230 amino acids, which includes both the RPS19 and RNA binding domains. This mutant is deficient in translation and likely lacks the cap-binding domain, as N-protein mutants deficient in translation with intact cap binding pocket are dominant negative and globally shutdown the translation machinery of the host cell. However, our existing translation deficient N-protein mutant does not shutdown the global translation of the host cell. We will package lentiviruses expressing either wild type N-protein or N-protein mutant deficient in translation or GFP as negative control. HUVEC cells cultured in six mm dishes will be infected with the resulting lentiviruses. Cells will be lysed 48 hours post-infection and half of the cell lysates will be examined by

western blot analysis to monitor the expression levels of host factors that show over-expression in previously performed 2D-DIGE experiment (Fig 3). Total RNA will be purified from remaining half of the cell lysates and mRNA levels of the host factors will be examined by the real time PCR, as reported in Fig 3 (panels B and C). We expect that wild type N-protein unlike translation deficient mutant and GFP will facilitate the translation of host factors without affecting their endogenous mRNA levels.

We prioritized the identified host factors into three groups for further study. Group A: [redacted] includes the host factors whose translation up-regulation by N-protein mediated translation strategy was significant and was confirmed by both 2D-DIGE and western blot analysis (Fig 3). In addition, these host factors have been reported to play a role in virus replication. Group B: ([redacted]) includes the host factors whose translation upregulation by N-protein mediated translation strategy was also significant and was confirmed only by 2D-DIGE and not by western blot analysis. These host factors have also been previously reported to play a role in virus replication(26-30). *Remaining all host factors showed a moderate upregulation and their role in virus replication has not been well documented. These host factors were placed in Group C.*

We will next test the hypothesis that cellular transcripts whose translation is up-regulated by N-protein mediated translation mechanism have either a sequence or structural homology with the viral mRNA 5' UTR or simply contain a high affinity binding site for N-protein in their 5' UTR. We will generate GFP or luciferase reporter constructs expressing the reporter mRNA containing the 5' UTR sequences from the mRNAs in groups A and B. HeLa or HEK293T cells will be co-transfected with plasmids expressing either wild type N-protein or translation deficient N-protein mutant along with another plasmid expressing GFP or luciferase reporter mRNA. We expect that fusion of 5' UTR from the mRNA encoding the host factor will boost the reporter expression, provided N-protein mediated translation strategy uses the cellular 5' UTR similar to the 5' UTR of viral mRNA. To further confirm that N-protein with the assistance of 5' UTR from the mRNA encoding the host factor augments the reporter expression, we will perturb the 5' UTR by either deletion mutation or randomization of the sequence and repeat the above experiment. We expect that disruption of structural integrity or perturbation in the nucleotide sequence of the 5' UTR will compromise the preferential translation of reporter mRNA by N-protein mediated translation mechanism. If this preliminary study demonstrates that 5' UTR of the host mRNA contains the determinants required for N-protein mediated translation mechanism, we will next examine the affinity of 5' UTR sequences for binding to N-protein. We will use T7 RNA polymerase to synthesize the wild type 5' UTR, randomized 5' UTR and ORF of the transcript encoding the host factor of interest. We have extensively used T7 RNA polymerase for *in vitro* RNA synthesis (4-7, 31). Briefly, the total RNA from the cells will be purified by RNeasy kit and reverse transcribed using random primers. The cDNA will be used to amplify the ORF or the 5' UTR sequence of the mRNA of interest using appropriate primers. The PCR product will be cloned in a vector containing the T7 promoter. The resulting vector will be used in an *in vitro* T7 transcription reaction for the synthesis of RNA of interest. The RNA will be radiolabeled during synthesis and the binding affinity of the resulting RNA with both the wild type and mutant N-protein will be carried out by filter binding assay, as previously reported (4-7, 31). Alternatively, we can use the fluorescence binding assay or gel shift assay, as previously reported (1). Another alternative approach will be to use biolayer interferometry to examine the binding affinity of RNA with N, as previously reported (2). Similar binding reaction of N-protein with viral mRNA 5' UTR will be carried out side wise as a positive control. We expect that similar to viral mRNA 5' UTR, the 5' UTR of the mRNA encoding the host factor will bind N-protein with high affinity (K_d~95 nM).

If we find that 5' UTR neither binds to N-protein with high affinity nor does it play a role in N-protein mediated translation of host cell factors, we will next determine whether the 3' UTR from the mRNAs encoding the host factor in groups A and B play a role in N-protein mediated translation. The 3' UTR of the mRNA encoding the host factor of interest will be cloned at the 3' terminus of GFP or luciferase ORF. The resulting reporter constructs will be transfected into HEK293T or HeLa cells along with the plasmids expressing either wild type N-protein or N-protein mutant deficient in translation. The reporter expression will be monitored to determine whether N-protein with the assistance of 3' UTR favors the translation of reporter mRNA. If we find that 3' UTR plays a role in N-protein mediated translation, we will next determine the affinity of 3' UTR with the N-protein using either filter binding or fluorescence binding or gel shift or biolayer interferometry assays, as mentioned above. These studies will identify the cis-acting determinants of endogenous transcripts exploited by a viral protein to boost the translation of host factors that possibly support virus replication in cells.

Although this initial study is focused on ten transcripts from groups A and B, it is not time consuming based on our extensive experience in the approach employed. This initial study has several advantages. One: it will provide mechanistic insights upon the mechanism employed by N-protein to boost the translation of endogenous host cell factors. Two: the analysis of ten transcripts will identify the best cis-acting sequence preferred by N-protein for translation up-regulation. Such a sequence can be used in future in the development of translation assays to boost the translation of desired proteins in cells. For example the identified cis-acting sequence can be fused with the transcript encoding a protein of interest such as a therapeutic antibody. The co-expression of engineered mRNA along with N-protein can boost the translation of protein of interest. Third: The cis-acting sequences from ten transcripts will be used in in-slico studies for the identification of a common RNA structural motif binding to N-protein, as discussed further in this aim. Such a structural motif can provide insights for the future design of an inhibitor to shut down the N-protein mediated translation mechanism,

We will next determine whether the preferential translation of cellular transcripts encoding crucial host factors play a role in virus replication. The mRNA encoding the host factors from groups A and B will be knocked down in HUVEC cells using siRNA. Alternatively, the knockdown can be performed using an shRNA targeted to the 3' UTR of the mRNA of interest. The shRNA will be delivered to HUVEC cells by a lentivirus delivery system. We routinely use both of these approaches for gene knockdown in cells. HUVEC cells deficient in the host factor of interest will be infected with SNV at an MOI of 1.0 and virus replication will be monitored over time by quantitative estimation of viral S-segment RNA, as previously reported **(1-7)**. We expect significant inhibition of virus replication due to the lack of an endogenous host factor whose elevated levels are required for efficient virus replication in the cell. We will perform complementation assays to rule out the off-target effects of the shRNA knockdown. The cells expressing an shRNA targeted to the 3'UTR of the endogenous mRNA of interest will be co-transfected with a plasmid expressing the same mRNA lacking the 3' UTR. Thus, the mRNA expressed from the transfected plasmid will be resistant to the shRNA knockdown. In addition, the mRNA expressed from the transfected plasmid will be preferentially translated by N-protein, provided the high affinity binding site for N-protein serving as IRES for N-protein mediated translation strategy is located at the 5' terminus of the mRNA. Thus, continuous over-expression of the host factor will revert the effects of shRNA knockdown in virus infected cells and support virus replication. *Although host factors from groups A and B have been reported to play a role in virus replication, it is possible that some of these host factors do not play a role in hantavirus replication. Under such highly unforeseen circumstances, we will test the role of host factors from group C in hantavirus replication. This experimental avenue will reveal cellular pathways playing a role in hantavirus replication, and will provide insights about the new targets for therapeutic intervention of the viral disease. It is possible that some of the transcripts harboring the cis-acting sequences in the 5' and 3' UTR for N-protein binding are preferentially translated but their translation up-regulation does not play a role in virus replication. The cis-acting sequences from such transcripts can still be useful in the development of translation strategies to boost the expression of desired proteins, and also will help in in-slico studies for the identification of common RNA structural motif binding to N-protein.*

Once we determine whether 5' or 3' UTR contains the structural determinants for preferential translation by N-protein, we will next identify and characterize the binding site on the UTR. Since N-protein has been reported to bind to the panhandle or hairpin like RNA structures, it is possible that mRNAs receiving translational favor by N-protein mediated translation strategy contain a similar structural motif having high affinity for N-protein binding. It is equally possible that UTR may harbor the viral triplet repeat sequence. We will first focus on one UTR sequence having highest affinity for N-protein binding. Deletion mutations will be carried out in the UTR sequence and the impact upon N-mediated translation will be examined by luciferase or GFP reporter assay as discussed above. Alternatively, the mutant UTR sequences will be synthesized by T7 RNA polymerase and their interaction with the purified N-protein will be examined *in vitro* using filter binding, fluorescence spectroscopy or biolayer interferometry assays, as previously mentioned. Once the minimal possible region on the UTR required for N-protein binding is identified, the UTR sequence will be folded using *in-slico* assays to determine whether the identified region forms a secondary structure with other regions of the UTR sequence. If a potential secondary structure is predicted with high confidence by *in-slico* analysis, the secondary structure will be perturbed by mutational approach and the impact upon N-protein binding and N-protein mediated translation activity will be examined. This experimental approach will help to identify

a possible secondary structure that constitutes the biding site for N-protein. Once the binding site is identified and characterized on one UTR, the information will be used to scan other UTR sequences to determine potentially similar binding sites for N-protein. Dr. Luo from the college of Pharmacy, Western University of Health Sciences has extensive experience in Bioinformatics, she will help in *in-slico* studies to determine whether other UTR sequences contain a similar structural motif for N-protein binding (see Dr. Luo's letter of support). Again mutational approaches will be used to confirm the N-protein binding sites on other UTR sequences and their role in N-protein mediated translation strategy. These studies will provide crucial insights about a common RNA secondary structural motif in the UTR that might bind N-protein to guide the precise ribosome loading. Such a structural motif will set the stage for the development of a potential inhibitor in the future to shutdown the preferential translation of host genes by N-protein mediated translation strategy. Moreover, identification of such a structural motif can be used to generate novel protein expression systems to boost the translation of important proteins such as monoclonal antibodies and proteins of therapeutic importance using N-protein translation strategy.

Potential pitfalls and alternative approaches: We do not expect any major pitfalls in the proposed studies, as the procedures used in this study are pretty standard and routinely used in our lab. The N-protein mutant deficient in translation to be used in this study already exists in our lab.

Specific aim 2: How N-protein inhibits PKR dimerization to counteract the host interference in viral protein synthesis?

Rationale and hypothesis: Host cells shutdown translation machinery as first line of defense in response to viral infection to create obstacle for the manufacture of viral protein. However, it is well known that hantavirus infected cells fail to shutdown their translation machinery and thus continuously support the synthesis of viral proteins during the course of viral infection. Host cells primarily activate PKR-eIF2α pathway as antiviral response to shutdown the translational machinery. The protein kinase R (PKR) binds to double stranded RNA (dsRNA), undergoes dimerization and autophosphorylation. The autophosphorylation activates PKR, which then phosphorylates the downstream target eukaryotic translation initiation factor 2α (eIF2α) that inhibits translation initiation. Our preliminary data demonstrates that N-protein from sin nombre virus (SNV) and Andes hantaviruses (ANDV) inhibits PKR dimerization (10), a crucial step required for its activation. As a result the eIF2α remains constantly active in virus-infected cells. The active eIF2α maintains both canonical and N-protein mediated translation systems in functional state to ensure continuous synthesis of both host and viral protein. We will determine the mechanism by which N-protein inhibits PKR dimerization.

Also, we will determine whether N-protein from old and new world hantavirus species inhibit PKR with similar potencies. During the oliguric phase of hantavirus disease the viral infection manifests to different organs. The new world hantaviruses, such as SNV and ANDV, causing hantavirus cardiopulmonary syndrome, predominantly target lungs and heart. The old world hantavirus causing hemorrhagic fever with renal syndrome selectively target kidney. The molecular mechanism of such organ tropism

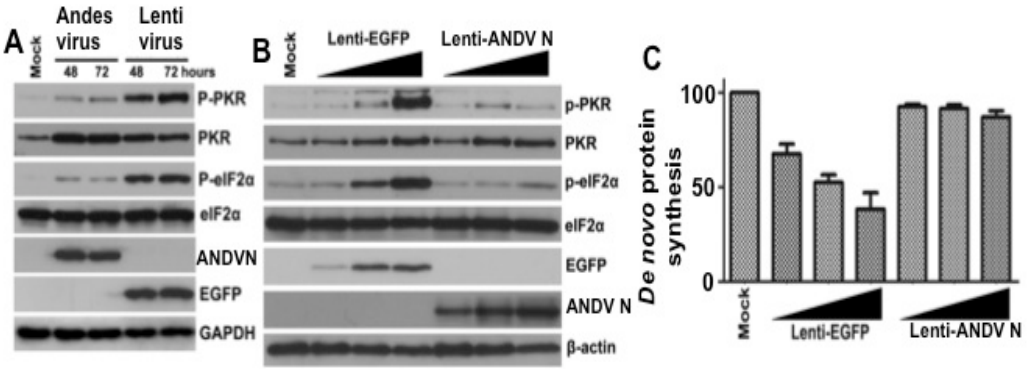

Fig 5. Panel A: HUVEC cells grown in six well plates were infected with either Andes virus or lentivirus expressing EGFP at an MOI of 1.0. Cells were lysed 48 or 72 hours post-infection and cell lysates were examined for the expressed of phosphorylated PKR (P-PKR) and phosphoylated eIF2α (P-eIF2α), total PKR and eIF2α. Cell lysates were also examined for the expression of ANDV N, EGFP and GAPDH as internal control. **Panel B**. HUVEC cells in six well plates were infected with lentivirus at increasing MOI, expressing either EGFP or ANDVN. Cells were lysed 48 hours post infection and examined for the expression of PKR, P-PKR, P-eIF2α, eIF2α, EGFP, ANDV N and β-actin as internal control. **Panel C**. HUVEC cells in six well plates were infected with lentivirus expressing either EGFP or ANDV N as mentioned in panel B. 48 hours post infection, cells were chased for de novo protein synthesis by metabolic labeling using S^{35} Methionine. The band intensity from three randomly chosen areas in the autoradiogram were used to generate the bar graph shown in panel C. ***This data was recently publication*** (10)

is unknown. Since PKR is the major host restriction factor that inhibits virus replication in the host. We would like to determine whether N-protein from old and new world hantaviruses show selectivity in PKR inhibition in the kidney and heart, respectively, which in turn might support organ tropism of hantavirus disease. *Based on our preliminary data, the N-protein either directly binds to PKR or uses an endogenous host factor to inhibit PKR dimerization.* Moreover, the N-protein from old and new world hantaviruses shows significant variation in the primary amino acid sequence. It is possible that N-protein from old and new world hantaviruses use different host factors to inhibit PKR. Although PKR expression in kidney and heart may not be significantly different, but the expression of host factors required to inhibit PKR may be different in heart and kidney. Such differential expression of the host factor could offer selectivity in PKR inhibition, and hence lead to organ tropism. These studies will provide mechanistic insights in the pathogenesis of hantavirus diseases.

Preliminary data: We infected HUVEC cells with either ANDV or lentvirus as positive control and examined the expression of both PKR and eIF2α. It is evident from Fig 5A that unlike eIF2α, total PKR levels in virus infected cells are high compared to mock. Although PKR expression was induced due to Andes virus infection, we did not observe a noticeable change in the phosphorylated forms of both PKR and eIF2α. In comparison, the lentivirus induced PKR expression resulted in the phosphorylation of both PKR and downstream target eIF2α (Fig 5A). We next asked whether inhibition of PKR and eIF2α, observed in ANDV infected cells was due to the expression of ANDV nucleocapsid protein (ANDV N). As lentivirus infections are know to induce innate immune response, promote the expression and activation of PKR in cells (32, 33), HUVEC cells were infected with lentivirus at increasing MOI, expressing either ANDV N or EGFP as negative control. It is evident from Fig 5B that both lentiviruses induced the PKR expression in a dose dependent manner, without a noticeable change in the total expression levels of eIF2α. However, unlike EGFP, the ANDV N expression inhibited the phosphorylation of both PKR and eIF2α. Cells infected with lentivirus expressing either EGFP or ANDV N were chased for denovo protein synthesis 48 hours post-infection, using S^{35} labeling. This analysis revealed that activation of PKR-eIF2α pathway by EGFP expressing lentivirus caused global translation shutoff and ANDV N expression prevented such translation shutoff in cells (Fig 5C). The initiation factor eIF2α is phosphorylated by four kinases PKR, HRI, PERK and GCN2, which sense different aspects of stress response in the cell. Using multifaceted experimental avenues, we found that ANDV N does not inhibit the phosphorylation of eIF2α induced by the activation of HRI, PERK and GCN2 (10). Since PKR requires dsRNA for activation, one would expect that sequestration of dsRNA by ANDV N may lead to PKR inhibition. However, ANDV N mutant lacking the RNA binding domain inhibited PKR activation similar to wild type N protein, suggesting that RNA binding activity of N-protein does not play a role in PKR inhibition (10). Moreover, both wild type and mutant ANDV N inhibited PKR

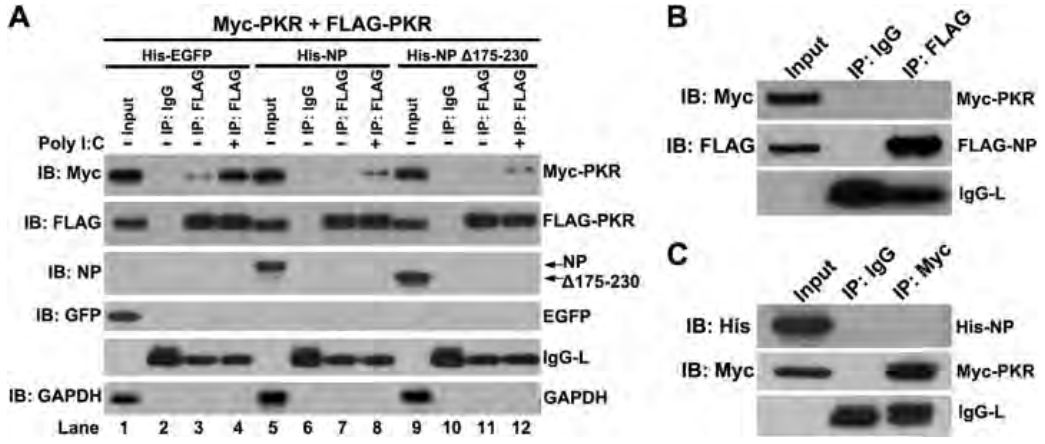

Fig 6: Andes virus N-protein inhibits PKR dimerization. (**A**) HEK293T cells were cotransfected with plasmids expressing either EGFP or wild-type or mutant His-tagged NP along with two additional plasmids: one expressing Myc-tagged PKR and another expressing FLAG-tagged PKR. Cells were lysed at 48 h post-transfection, and one-third of the cell lysate was treated with poly(I·C) (1 μg/ml) for 30 min. Cell lysates were immunoprecipitated with either IgG or anti-FLAG ant body, and immunoprecipitated material was examined by Western blot analysis (IB) using anti-Myc antibody to detect Myc-tagged PKR, anti-FLAG ant body to detect FLAG-tagged PKR, anti-NP antibody to detect His-tagged NP, anti-GFP to detect GFP, or anti-GAPDH antibody to detect GAPDH. (**B**) HEK293T cells were cotransfected with plasmids expressing Myc-tagged PKR and FLAG-tagged NP. At 48 h posttransfection, cells were lysed with 0.5% NP-40 lysis buffer. Cell lysates were immunoprecipitated with either anti-FLAG antibody or IgG. The immunoprecipitated material was examined by Western blot analysis using either anti-Myc antibody (top) or anti-FLAG antibody (middle). The bottom panel shows the IgG light chain (IgG-L). (**C**) The experiment was performed in the same way as that for panel B, except that immunoprecipitation was carried out using anti-Myc antibody and Western blot analysis was carried out using anti-His antibody. ***This data was recently publication*** (10). *Note*: Both NP and N used in the text and figure legend mean the nucleocapsid protein.

activation in cells triggered by poly I:C treatment. In addition, HEK293T cells were co-transfected with plasmids expressing both FLAG and Myc tagged PKR along with another plasmid expressing N-protein. Cell lysates were immunoprecipitated with ant-FLAG antibody and examined by western blot using nati-Myc antibody. It is evident from Fig 6A, that ANDV N expression inhibited the interaction between FLAG and Myc tagged PKR molecules. Further studies (Fig 6B and C) showed that binding of ANDV N to the PKR is not required to inhibit intermolecular interaction between PKR molecules, *although this observation needs further verification*. Our preliminary data clearly shows that N-protein from both ANDV and SNV inhibit PKR activation by perturbing its dimerization, a crucial step in the activation process of PKR (See (10) for details). The molecular mechanism to inhibit PKR dimerization remains unclear.

Research Design: *Although Fig 6B and C demonstrates that hantavirus N-protein does not bind to PKR (Fig 6), suggesting that direct interaction between PKR and N-protein likely does not play a role in the inhibition of PKR dimerization. We will use mammalian two hybrid system and confocal microscopy to further confirm the lack of interaction between N-protein and PKR. We have previously used the dual luciferase mammalian two-hybrid system (Promega) to re-conform the protein-protein interaction in mammalian cells. Briefly, N-protein and PKR will be cloned in pBIND and pACT vectors and expressed as fusion proteins with GAL4 DNA binding domain and herpes simplex virus VP16 activation domain, respectively. The pBIND plasmid also expresses renilla luciferase under SV40 promoter. Similarly, the vaccinia virus K3L protein and influenza virus PB2 protein will be cloned in pBIND vector and used as positive and negative controls, respectively. The K3L protein is a pseudosubstrate for PKR and is know to directly bind to PKR* (34-36)*. The PB2 protein from influenza virus on the other hand has not been reported to show any direct interaction with PKR. The pBIND and pACT plasmids expressing the fusion proteins will be co-transfected to cells along with pG5luc reporter plasmid expressing fire fly luciferase. The pG5Luc has five tandem GAL4 binding sites. Cells will be lysed 72 hours post transfection and dual luciferase activity will be monitored. We expect a significant increase in the relative firefly luciferase activity if N-protein and PKR interact with each other. A comparison of relative fire fly luciferase signal with the positive control (K3L) and negative control (PB2) will confirm the potential interaction between N-protein and PKR. Alternatively, N-protein and PKR will be expressed as GFP and FLAG tag fusion protein in cells. Using anti FLAG antibody, the confocal imaging will be carried out to determine the potential interaction between N-protein and PKR, as previously reported* (15, 37)*. If this initial study reveals that N-protein binds to PKR, we will next use an existing panel of twenty N protein mutants to identify an N-protein mutant deficient in binding to PKR. Such N-protein mutant will be assayed to examine its impact upon PKR dimerization and inhibition of PKR-eIF2α pathway, as previous reported* (10)*.*

However, if it turns out that N protein does not bind to PKR we will next test the hypothesis that N-protein with the assistance of host cell factors interferes in PKR dimerization to exert its inhibitory effects. The well characterized endogenous PKR inhibitors include $p58^{IPK}$, TRBP, glycoprotein p67, nucleophosmin (NPM), heat shock proteins HSP90 and HSp70. These endogenous PKR inhibitors either directly or indirectly bind to PKR and inhibit its kinase activity. For example $P58^{IPK}$ directly binds to PKR and inhibits its autophosphorylation by perturbing its dimerization (38). The influenza virus recruits $p58^{IPK}$ to repress PKR induced eIF2α phosphorylation (39). It is possible that similar to influenza virus, hantaviruses may recruit an endogenous PKR inhibitor to repress the PKR mediated eIF2α phosphorylation. It must be noted that N-protein mediated translation strategy facilitates the translation of HSP90, a known PKR inhibitor (Fig 3). To test our hypothesis, we will individually knockdown $p58^{IPK}$, TRBP, glycoprotein p67, nucleophosmin, heat shock proteins HSP90 and HSp70 in human cardiac and pulmonary microvascular endothelial cells using siRNA. The cardiac and pulmonary endothelial cells will be used because new world hantaviruses infect endothelial cells and selectively target lungs and heart in infected patients. Alternatively, the gene knockdown can be performed using shRNA targeted by a lentivirus delivery system. We routinely use these approaches for gene knockdown in our laboratory (10, 15, 37). The knockdown cells will be infected with lentivirus at increasing MOI, expressing the N protein from SNV or ANDV or EGFP as negative control, as mentioned in Fig 5B. Cells will be lysed 48 hours post infection and resulting cell lysates will be examined for both the induction and phosphorylation of PKR and eIF2α, using western blot analysis. If N-protein requires assistance from any endogenous PRK inhibitor, its knockdown will inhibit N-protein mediated PKR inhibition, observed by the rapid phosphorylation of both PKR and eIF2α. We will use a complementation assay to further confirm the

requirement of identified endogenous PKR inhibitor in N-protein mediated PKR inhibition and rule out the possibility of off-target effect of siRNA treatment. The identified endogenous PKR inhibitor will be down-regulated by shRNA targeted against the 3' untranslated region (UTR) of the encoding mRNA. During complementation, the endogenous inhibitor will be expressed from a plasmid harboring the open reading frame (ORF), devoid of 3' UTR. The expression of endogenous PKR inhibitor from a transfected plasmid should overcome the effects of shRNA knockdown to ensure that observed phosphorylation of PKR and eIF2α was due to the knockdown of the endogenous PKR inhibitor and not due to the off-target effect of siRNA treatment.

The role of endogenous PKR inhibitor in conjunction with N-protein to counteract the PKR-mediated translation shutoff in virus infected cells will be further evaluated. The endogenous PKR inhibitor will be down-regulated in cells using either siRNA or shRNA, followed by hantavirus infection. We expect that transient loss of endogenous PKR inhibitor will cause PKR-mediated translation shutdown in virus-infected cells, which will inhibit virus replication due to obstructions in the synthesis of viral proteins. The transient translation shutoff in virus infected cells will be directly monitored by quantitative estimation of de novo protein synthesis using S^{35} Methionine labeling, as mentioned in Fig 5C. Since PKR is a versatile antiviral barrier in different animal families, numerous viruses have evolved antagonists to evade the PKR antiviral response to successfully establish infection in their hosts. The potential of an antiviral antagonist to inhibit PKR from diverse host species determines the ability of a virus to cross species barriers and establish infection in new hosts. Once the host factor required by hantavirus N-protein to inhibit PKR dimerization is identified, it will set the stage to determine whether such host factors are evolutionary conserved among diverse animal species. Such future studies will be of potential significance to evaluate the ability of hantaviruses to cross species barriers. If this gene knockdown approach reveals that none of the above mentioned endogenous PKR inhibitors play a role in the N-protein mediated inhibition of PKR activation, the alternative approach will be to use a small siRNA library targeted against all individual components regulating the PKR-eiF2α pathway. This alternative approach will help to identify the potential new host factors required in N-protein mediated inhibition of PKR dimerization. We have previously used this approach in another research project, which helped to identify a novel host factor mediating the degradation of hantavirus glycoprotein during the early stages of virus replication. Thus, we are aware of potential off targets effects of siRNA library and the use of complementation assays as described above to further confirm the results.

As mentioned briefly in the rationale above that old and new world hantaviruses show a unique organ tropism, which likely plays a role in the prognosis of hantavirus disease. The hemorrhagic fever with renal syndrome (HFRS), an acute illness in which kidney is selectively targeted by old world hantaviruses, has a mortality rate of 15%. In comparison, the hantavirus cardiopulmonary syndrome (HCPS), in which lungs and heart become the target of new world hantavirus infection, has mortality rate of 50%. The infection to highly differentiated and specialized microvascular endothelial cells in these organs breaks the endothelial barrier, causing acute kidney injury in HFRS patients or sever pulmonary and cardiac distress in HCPS patients. Since the N-protein from old and new world hantaviruses has significant differences in the primary amino acid sequence, they likely use different host cell factors to inhibit PKR. It is highly probable that host factor required by N-protein from old world hantaviruses is highly expressed in kidney as compared to lungs and heart. As a result, old world hantaviruses easily overcome the PKR antiviral barrier in the kidney and successfully establish infection in there. They fail to do so in lungs and heart due to poor expression of such a host factor. The vice versa is like true for new world hantaviruses. To test this hypothesis we will transfect human cardiac, pulmonary and glomerular microvascular endothelial cells with plasmids expressing either old world hantavirus N-protein from Puumala and Seoul viruses or new world hantavirus N-protein from SNV and ANDV. If the transfection efficiency was poor, the alternative approach will be to use the lentivirus delivery system as reported in Fig 5. Cells will be lysed 48 hours post-transfection and half of the resulting cell lysates will be treated with poly I:C (1ug/ml) for 30 minutes to trigger the activation of PKR-eIF2α pathway, as mentioned in Fig 6. Cell lysates will be examined by western blot analysis to monitor the phosphorylation of PKR and eIF2α, as mentioned in Fig 6. We expect that N-protein from old world hantaviruses will selectively inhibit the phosphorylation of both PKR and eIF2α in glomerular microvascular endothelial. The N-protein from new world hantavirus will be expected to specifically inhibit the phosphorylation of PKR and eIF2α in cardiac and pulmonary microvascular endothelial cells. To further confirm these observations, the cells

will be infected with old and new world hantaviruses and cells will be lysed at increasing time intervals post infection. Half of the resulting cell lysates will be examined for virus replication by quantitative estimation of viral S-segment RNA by real time PCR as previously reported **(1-7)**. Remaining half of the cell lysates will be examined for the phosphorylation of both PKR and eIF2α by western blot analysis as mentioned in Fig 6. We expect that old world hantaviruses will inhibit PKR activation and efficiently replicate in glomerular microvascular endothelial cells as compared to cardiac and pulmonary microvascular endothelial cells. However, new world hantaviruses will be expected to inhibit PKR and replicate efficiently in cardiac and pulmonary microvascular endothelial cells as compared to glomerular microvascular endothelial cells. To further confirm these results the identified host factor required by new world hantavirus N-protein will be knockdown using siRNA or shRNA and its impact upon virus replication and inhibition of PKR-eiF2 pathway will be examined. If old and new world hantavirus N-protein uses different host factors to counteract PKR antiviral response, we expect that knockdown of the identified host factor will selectively impact replication of new world hantaviruses in cardiac and pulmonary endothelial cells, and will have no impact upon the replication of old world hantaviruses in glomerular microvascular endothelial cells. These experiments will set the stage for the identification of host factor for the old world hantaviruses.

It is equally possible that both old a new world hantaviruses infect cardiac, pulmonary and glomerular microvascular endothelial cells with same efficiencies, their N-protein molecules use same host factor to equally inhibit PKR-eIF2 pathway in these cell lines. These observations will demonstrate that inhibition of PKR by N-protein does play a role in organ tropism of hantavirus infection. Such observations will set the stage to look for other possible mechanism for hantavirus organ tropism.

Potential pitfalls and alternative approaches: The knockdown of endogenous PKR inhibitors using siRNA has been previously carried out and should not be problematic. Human cardiac, pulmonary and glomerular microvascular endothelial cells are commercially available. Their cell culture conditions have been well standardized and we do not expect any problem in working with these cell lines.

Time line: In the first six months of year 1, we will carryout luciferase assays and *in vitro* RNA-protein binding studies to determine the role of 5 and 3' UTR sequences of host transcripts in N-protein mediated translation (aim 1). In the last six months of year 1, we will demonstrate the role of host factors in virus replication using the gene knockdown approach (aim 1). In the first six months of year 2, we will carryout deletion mutation and *in slico* studies to identify a common RNA secondary structural motif in the host UTR elements required for N-protein binding (aim 1). The last six months of year 2 will be focused on mammalian two-hybrid system to determine the potential interaction between N-protein and PKR (aim 2). In the last year of funding (year 3), we will demonstrate whether N protein with the assistance of host factor or direct interaction with PKR inhibits PKR-eIF2 pathway. We will also determine the possible role of PKR inhibition in the organ tropism of hantavirus diseases (aim 2).

Impact of the proposed research plan to strengthen the research environment at the Western University of Health Sciences: The proposed studies in this application will expose the students to modern biomedical technologies, such as gene cloning and sequencing, gene mutations, expression and purification of proteins in bacterial and mammalian systems, viral, bacterial and mammalian cell culture, gene knockdown using siRNA and lentiviral approaches, *in silico* studies on RNA structures, training in BSL3 lab environment to handle highly infectious category A pathogens. The students will be trained in experimental design, data analysis and presentation, manuscript preparation and literature surveys in cutting edge areas of biomedical science. I believe this exposure will greatly stimulate the students to consider a career in biomedical research. I believe that my past experience in training researchers, availability of huge reagent resource from our past work and my significant experience in proposed experiments will greatly add to the success of this project. All these credentials will strengthen the over all research environment at Western University of Health Sciences.

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Resource Sharing Plan

As for our plan to share materials and our management of intellectual property, we will adhere to the NIH Grant Policy on Sharing of Unique Research Resources including the Principles and Guidelines for Recipients of NIH Research Grants and Contracts on Obtaining and Disseminating Biomedical Research Resources issued December 23, 1999. All the reagents generated by this project will be distributed widely or deposited into a repository/stock center making them available to the broader research community, either before or immediately after publication, in accordance with University policies. If we assume responsibility for distributing the newly generated reagents, we will fill requests in a timely fashion. In addition, we will provide relevant protocols and published genetic and phenotypic data upon request. Material transfers will be made with no more restrictive terms than in the Simple Letter Agreement (SLA) or the Uniform Biological Materials Transfer Agreement (UBMTA) and without reach through requirements. Should any intellectual property arise which requires a patent, we will ensure that the technology (materials and data) remains widely available to the research community in accordance with University policies and the NIH Principles and Guidelines document.