

**Influenza virus strategies to regulate S1P-metabolizing enzymes and evade
host innate immunity for robust viral replication**

A Dissertation
presented to
the Faculty of the Graduate School
at the University of Missouri-Columbia

In partial fulfillment
of the requirements for the degree
Doctor of Philosophy
in Microbiology and Immunology

By
Jennifer Jeanette Wolf
Dr. Bumsuk Hahm, Dissertation Mentor
July 2021

The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

Influenza virus strategies to regulate S1P-metabolizing enzymes and evade host innate immunity for robust viral replication

presented by Jennifer Jeanette Wolf, a candidate for the degree of Doctor of Philosophy in Microbiology and Immunology, and hereby certify that, in their opinion, it is worthy of acceptance.

Bumsuk Hahm, PhD

David Pintel, PhD

Marc Johnson, PhD

Margaret Lange-Osborn, PhD

Xiao Heng, PhD

Dedication

To my mom, Dr. Barbara Sumner, my dad, Dr. Lloyd Sumner, my brother, Keith Sumner,
and my husband, Joseph Mangieri.

Acknowledgements

I would like to thank my advisor Dr. Bumsuk Hahm for all of his support, patience, consistency, care, and guidance over the years since joining his lab. Dr. Hahm is a brilliant scientist that has taught me to do the best work possible. I have always appreciated the time, effort, and resources he has put into my education, scientific development, and well-being. Whenever I had trouble with an experiment or understanding a concept, Dr. Hahm would always take the time to help me in whatever way he could. He does whatever he can to help his students succeed. I feel that I really learned to become a scientist and think like one thanks to Dr. Hahm. I am very grateful that Dr. Hahm took a chance on me and allowed me to do research in his lab.

I would like to thank my graduate committee members Dr. David Pintel, Dr. Xiao Heng, Dr. Marc Johnson, and Dr. Margaret Lange-Osborn for their constructive criticism, encouragement, and valuable suggestions on my research projects. I am extremely grateful to them for readily sharing reagents, equipment, and ideas throughout the years.

Dr. Hahm's lab has been a very supportive and encouraging environment, and I am very grateful for the time I have spent in it. Every lab member I have worked with has always been willing to help each other out, whether it is with finishing an experiment up if they had a meeting, proofreading each other's manuscripts, showing each other techniques, or bouncing ideas off of one another. I was trained by and worked closely with former postdoc Dr. Chuan Xia when I first joined the lab. We worked a lot together in my first year in the lab, with the ultimate goal of getting as much done as possible before he left for his professor position, and much of the data resulted in publications. I am especially grateful for Dr. Caleb Studstill. For some time, it was just Caleb and me in the lab, our desks back-to-back. Caleb was always willing to help me with anything or talk about anything, no matter the time or place. He has been and continues to be a great friend, and I am very thankful for his friendship. No matter the time or place, Caleb was and is always

willing to offer his support, whether it be helping me with an experiment, attending an event I was running, making a coffee run, or just listen to me rant. We have had many fun times together, and I have really valued our friendship. I am also thankful for Gina Wamsely, the newest addition in the lab. She is very friendly and always does her best, and I am grateful that she has made efforts to check up on me and keep in touch while I write my dissertation. I have also had the privilege of meeting former graduate student Dr. Madhuvanathi Vijayan, and I am grateful for her advice with certain procedures. During my time in Dr. Hahm's lab, I have worked with several undergraduates. Among them, Hanh Ngo and Paige Williams were wonderful students. I appreciated their "can do" attitude, the hard work they both put in to ensure they understood what they were doing, and that their experiments were done correctly.

Prior to joining Dr. Hahm's lab, I had the privilege of being in the lab of Dr. Stefan Sarafianos for two years before the lab moved to Emory in Atlanta GA. I learned a lot in Stefan's lab, and I got to do and try many things that I wouldn't have been able to otherwise. I am grateful for my time in there. I would like to thank Stefan for teaching me to effectively communicate science as well as the importance of collaboration with others. I also had the pleasure of working with several fantastic people, including Dr. Karen Kirby, Dr. Philip Tedbury, Dr. Kamal Singh, Dr. Andrew Huber, Dr. Mary Casey, Seongmi Kim, Dr. Maria Boftsi, Dr. Anna Gres, Dr. Dandan Liu, Dr. Obiaara Ukah, Dr. Maritza Puray-Chavez, Dr. Assi Vincent Yappo, Jacqueline Flores, Juan Ji, and Grace Yang. Thank you for your patience with me and for your friendship and teaching. I learned so much, and it was truly a pleasure to work with everyone. Thank you to my former undergraduate student Cortney Hutson for always doing your best and working with my hectic schedule.

During my time in graduate school, I was able to be a part of the graduate student organization Alternative Career Exploration in the Sciences (ACES). I met and worked with many exceptional people in this organization. Big thank you to Nicholas Olivarez for

all of your help and support over the years, I treasure your friendship. Thank you to Omiya Hassan, Dr. Rayne Lim, Jacob Bouchier, Ali Hosseini Taleghani, Dennis Perez-Lopez, Dana Brown, Ashley Smith, Dr. Cate Omosule, LaCassidy Broadnax, Dr. Rachel Owen, Dr. Michelle Williams, Dr. Emilia Asante, and others for your help, support, and time to make events happen for our members. While the events were usually hectic, our planning meetings at the Heidelberg were a blast!

In addition to Caleb, my graduate class was filled with great friends. Dr. Lexy Cattin-Roy is a great friend of mine. I have really valued Lexy's friendship, support, and encouragement over the years. We have had many great times together. Caleb, Lexy, Dr. Clifton Ricaña, Matt Burch, Dr. Kyle Hoffman, and Nicholas Griswold are all wonderful people, and we have had a lot of fun and crazy times together, from taking those first two years of classes, surviving TA'ing and comprehensive exams, to just hanging out. Along with all of the people I have mentioned so far, I have had lots of fun times with many others. Thank you, Dr. Leah Cardwell, for your friendship, encouragement, advice, and guidance over all of these years. I have also had many great times and science conversations with Dr. A.E. Wolfe, Dr. Kinsey Hilliard, Alexander Lucido-Johnston, Dr. Mindy Miller, Dr. Alexis Dadelahi, Michael Quaney, Dr. Lauren Quaney, Dr. Kevin Kaifer, Fiorella San Martin, Dr. Yue Guan, Dr. Curtis Pritzl, Alexander Earhart, Jorge Durand-Heredia, Christa Jackson, Dr. Rachel Olson, Dr. Greg Lambert, Gustavo Santiago-Collazo, Dr. Geralisa Van Gronigen Case, Sean Kraus, Skylar Kramer, and more.

I would like to thank members of the MPT, MMI, VPB, and Surgery departments for their assistance. I would also like to thank Dr. Mick Calcutt and Dr. George Stewart for their guidance and support over the years. Thank you Dr. Manijeh Heidari for looking out for me and checking in on me, even after I finished TA'ing. Additionally, I am very thankful for Jana Clark, Shelly Crawford, Kristen Clark, Karen Ehlert, Ami Patel, and Kathy LaMere, who have helped me in many ways.

During my time at University of Missouri, I had the privilege of working with many different core facilities for my research. I would like to thank the Electron Microscopy core for their help, support and training. I always appreciated Dr. Tommi White and Deanna Grant's willingness to help me and teach me. I would also like to thank the Molecular Cytology core, particularly Dr. Alexander Jurkevich and Dr. Frank Baker, for their help and training. Additionally, I had the unique privilege of working with Dr. Lloyd Sumner and Dr. Barbara Sumner in the Metabolomics core, and I am grateful for their help, support, and training. I would also like to thank the Charles W. Gehrke Proteomics Center, particularly Dr. Brian Mooney, for their help with experiments. The Cell and Immunobiology core has also been essential for my studies, and I am grateful for their help, especially Dr. Kathy Schreiber.

During the first four years of my graduate education, I was very fortunate to have a Life Sciences Fellowship. It was one of the motivating factors that brought me to University of Missouri. I learned so much from the Life Science Fellows program, whether it be networking, presentation, or knowledge from the extra classes I would not have taken otherwise. I also met many exceptional people through the program and had many opportunities that I would not have had otherwise. I am very grateful for the assistance of Mrs. Debbie Allen and Dr. Mark Hannink during my time at MU. They have given me much advice, and I have learned a lot that I would not have had I not been a life science fellow.

Biorender.com was used to make Figures 4, 5, 6, 23, and 33.

Since the onset of COVID-19, many in-person events have not been able to happen. However, it is precisely because of this that I was able to form and strengthen friendships with people many miles away. I would like to thank Ben Homer Dodson Jr., Evan Volpe, Ava Volpe, Zach Conine, Kathy Palmer, Michael Shay, and Trevor Leffert for being cool people and for all of their support. I have really enjoyed our weekly online trivia nights, LearnedLeague tournaments, and game sessions. Here's hoping for many more!

I would also like to thank my biotechnology teacher at the Southern Oklahoma Technology Center, Dr. Fiona McAlister, for teaching me the techniques, skills, and concepts that launched me into lab work and ultimately made me want to go into this field. It is very likely that I would not have gone on this path if it were not for my experience in her biotechnology class, and I am very thankful for the time and effort she invested in my education. Summers at the Noble Foundation in Ardmore, OK during my undergraduate education were also essential to my scientific development. Dr. Chunxiang Fu taught me many concepts and techniques that I still use, and I am grateful for his mentorship and guidance. I would also like to thank Dr. Zengyu Wang for hiring me and ultimately giving me great career advice. Laura Nelson, Dr. Hiroshi Hisano, Dr. Frank Hardin, Steven Tudor, Tim Hermanas, Dr. Yaxim Ge, Xirong Xiao, and Kathy Zhou were all great to work with, and I am very grateful to have worked in the same lab as them. I would also like to thank my teachers at Ardmore High School for instilling concepts in me that I use to this day. My writing used to be very flowery, and Mrs. Debbie Herndon taught me to simplify it. Ms. Laura Brooks was my speech and debate coach, and she helped me gain the courage to speak and perform in front of others. I would also like to give a special acknowledgement to Mrs. Sherry Wheatley Ramsey and Dakota Wheatley for their support.

My mom and dad, Dr. Barbara Sumner and Dr. Lloyd Sumner, as well as my brother, Keith Sumner, have been a constant source of support for me throughout my life, and I cannot thank them enough for all that they have done for me. My mom raised me on her own after the passing of my biological father, Dr. Kevin L. Wolf, until she married my dad. She has made many sacrifices for my well-being, and she also put in a lot of her time and effort over the years to ensure I could be successful in whatever I wanted to pursue. My dad has done his best since marrying my mom to treat me as one of his own, and I am really grateful for that. He has firmly supported me and my ambitions, and he has

always made the time to help with whatever advice I need or with whatever project or hobby I have going on. I truly appreciate all the effort and sacrifices my parents have made to give me the best life possible. My brother Keith is a very cool and fun human. I have immensely enjoyed all of our adventures and shenanigans over the years. Thank you for your support and firm confidence in me and my abilities. It has been a joy to watch you grow up into the man you have become, and I cannot believe that you are already graduating from high school. I would also like to thank my grandmother, Wanda Sue Potts, for her support and encouragement.

Lastly, I would like to thank my better half and the love of my life, Joseph (Joey) Mangieri, for his calm demeanor, patience, and endless support to enable me to pursue my goals. I have treasured every minute spent with you from the over four years of long-distance dating to the six years that we have been married so far. I sincerely appreciate you helping me in any way you could over these years, whether it be helping me program in a language you've never used before or waiting in the car way too long for me to finish in lab. Thank you for your unwavering confidence in me and for overall bringing out the best in me.

Table of Contents

Acknowledgements	ii
List of Figures.....	x
List of Abbreviations	xiii
Abstract.....	xvii
Chapters	
I. Introduction.....	1
A. General introduction to sphingolipid metabolism	1
i. S1P functionality.....	3
ii. S1P metabolizing enzymes in viral infection	6
iii. SPL in viral and immune regulation	8
iv. SK2 in viral regulation.....	10
B. Influenza virus basic information	13
i. Influenza A virus proteins and structure.....	13
ii. Influenza A virus replication cycle.....	17
iii. Influenza A virus nonstructural protein 1	19
C. Type I interferon antiviral innate immune responses to RNA viruses.....	22
i. RIG-I-mediated type I IFN innate immune response to infection.....	22
ii. IAV evasion of type I IFN immune responses.....	25
D. Ubiquitin.....	26
i. Protein Degradation	30
II. Influenza A virus NS1 induces degradation of sphingosine 1-phosphate lyase to obstruct the host innate immune response	33
A. Abstract	33
B. Introduction.....	34
C. Materials and methods.....	36

D. Results.....	41
E. Discussion	65
F. Acknowledgements.....	71
G. Data collection and analysis.....	71
III. Host factors are crucial for SK2-mediated enhancement of influenza viral replication	72
A. Abstract	72
B. Introduction	73
C. Materials and methods.....	74
D. Results.....	78
E. Discussion	84
F. Acknowledgements.....	86
G. Data collection and analysis.....	86
IV. PARP1 enhances influenza A virus propagation by facilitating degradation of host type I interferon receptor.....	87
A. Abstract	87
B. Introduction	88
C. Materials and methods.....	90
D. Results.....	94
E. Discussion	103
F. Acknowledgements.....	107
G. Data collection and analysis.....	107
V. Discussion	108
References.....	115
Vita.....	159

List of Figures

<u>Figure</u>	<u>Page</u>
Figure 1. The sphingolipid biosynthesis pathway.	2
Figure 2. The role of sphingosine 1-phosphate.	5
Figure 3. Schematic diagram for the interaction between viruses and S1P-metabolizing enzymes.	7
Figure 4. Diagram of influenza A virus.	16
Figure 5. The type I IFN response to RNA viruses.	24
Figure 6. The Ubiquitin proteasome system.	29
Figure 7. SPL inhibits pH1N1 IAV replication.	42
Figure 8. SPL does not alter the IRF3-mediated induction of type I IFNs.	43
Figure 9. SPL inhibits IAV replication in primary human tracheal epithelial cells.	44
Figure 10. Viral replication is increased in SPL deficient cells during infection with different influenza viruses.	46
Figure 11. SPL impairs measles virus (MV) replication.	47
Figure 12. IAV negatively regulates SPL protein levels.	48
Figure 13. IAV infection induces ubiquitination of SPL.	50
Figure 14. SPL is not downregulated by the type I IFN innate immune response.	52
Figure 15. IAV NS1 induces SPL ubiquitination.	53
Figure 16. IAV NS1 induces both K48 and K63-linked ubiquitination of SPL.	54

Figure 17. Transient IAV NS1 overexpression leads to SPL degradation.	56
Figure 18. NS1-deficient IAV fails to induce the ubiquitination and degradation of SPL.	57
Figure 19. NS1 expression reduces SPL-mediated IKK ϵ activation without affecting their interaction.	59
Figure 20. NS1 inhibits the function of SPL in promoting the type I IFN innate immune response.	60
Figure 21. SYVN1 positively regulates IAV replication.	63
Figure 22. SYVN1 interacts with SPL upon IAV NS1 expression.	64
Figure 23. Influenza A virus NS1 induces degradation of sphingosine 1-phosphate lyase to dampen the host innate immune response.	66
Figure 24. SK2 promotes IAV replication.	79
Figure 25. IAV infection increases SK2 expression and activation.	80
Figure 26. MEK/ERK activation is important for increased SK2/pSK2 levels during IAV infection.	82
Figure 27. SK2 inhibition interferes with p21 expression during IAV infection.	83
Figure 28. Knockdown of PARP1 results in impaired degradation of IFNAR1 during IAV HA expression or IAV infection.	95
Figure 29. Inhibition of PARP1 leads to impaired IFNAR1 downregulation during IAV HA expression.	97
Figure 30. PARP1 localization is altered upon IAV HA expression.	99
Figure 31. PARP1 localization is altered upon IAV infection.	100

Figure 32. PARP1 inhibition suppresses IAV propagation which is dependent on IFNAR1. 102

Figure 33. PARP1 enables IAV HA-induced IFNAR1 degradation. 104

List of Abbreviations

A549.....	Adenocarcinomic human alveolar basal epithelial
ABC.....	ABC294640
BVDV	bovine viral diarrhea virus
CD90.....	Cluster of differentiation 90
CHIKV	Chikungunya virus
COVID-19.....	coronavirus disease 2019
DNA	Deoxyribonucleic acid
Dox.....	Doxycycline
dpi	day(s) post infection
dsRNA.....	double-stranded RNA
eIF-2.....	Eukaryotic translation initiation factor 2
ER	Endoplasmic Reticulum
ERK.....	Extracellular signal-regulated kinase
FTY720	Fingolimod
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HA	Hemagglutinin
HBV.....	hepatitis B virus
HCV	hepatitis C virus
HDAC	Histone deacetylase
HEK293.....	Human Embryonic Kidney 293
HIV	human immunodeficiency virus
hpi	hours post infection
HTEpCs.....	Primary human tracheal epithelial cells
IAV	Influenza A Virus
IBV	Influenza B virus

IFN	Interferon
IFNAR	Interferon α/β Receptor
IFNAR1	Interferon α/β receptor α chain
IKK ϵ	Inhibitor of nuclear factor kappa-B kinase subunit epsilon
IRF	Interferon Regulatory Factor
ISG	Interferon Stimulated Gene
JAK	Janus-Activated Kinase
K	Lysine
KO	Knockout
M1	Matrix Protein 1
M2	Influenza Matrix Protein 2
MAM	Mitochondria-Associated Membrane
MAVS	Mitochondrial Anti-Viral Signaling protein
MDCK	Madin Darby Canine Kidney
MEK	Mitogen-activated protein kinase
MFN2	Mitofusin2
MOI	Multiplicity of Infection
MV	Measles Virus
NA	Neuraminidase
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NP	Nucleoprotein
NS1	Nonstructural protein 1
NS2	Influenza Nonstructural protein 2
OAS-1	2'-5'-oligoadenylate synthetase 1
PA	Polymerase acidic protein
PARP1	Poly ADP-ribose polymerase 1

PB1	Polymerase basic protein 1
PB1-F2	Polymerase basic protein 1-F2
PB2	Polymerase basic protein 2
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Units
PMA	phorbol 12-myristate 13-acetate
pSK2	phospho-SK2
qPCR	Quantitative PCR
RIG-I	Retinoic Acid-Inducible Gene 1
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RSV	Respiratory Syncytial Virus
rt-PCR	reverse transcription PCR
S1P	Sphingosine 1-phosphate
S1PR	S1P Receptor
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SD	Standard Deviation
SDS PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard Error of Mean
SK	Sphingosine Kinase
SK1	Sphingosine Kinase 1
SK2	Sphingosine Kinase 2
SM	Sphingomyelin
SphK2	Sphingosine Kinase 2
SPL	S1P-Lyase
STAT	Signal Transducer and Activator of Transcription

SYVN1	Synoviolin1
TBK1	TANK-Binding Kinase 1
TNF- α	Tumor necrosis factor α
TNKS.....	Tankyrase 1/2 (PARP5)
TRIM25	Tripartite motif-containing protein 25
TRIM6	Tripartite motif-containing protein 6
Ub	Ubiquitin
vRNA.....	Viral RNA
vRNP.....	Viral ribonucleoprotein
WT	Wild type

Abstract

Viral infection is a complex network of interactions involving the host defending itself against the virus and the virus attempting to subdue host defenses and utilize the cellular machinery to promote its replication. Similar to other viruses, influenza A virus (IAV) manipulates and modulates the host cell to maintain control and optimize the cell for viral replication. One of the central obstacles IAV must overcome to further its replication is the type I IFN innate immune response. We have previously shown that sphingosine 1-phosphate (S1P) lyase (SPL) enhances IKK ϵ -mediated type I IFN responses. Here, we show that the nonstructural protein 1 (NS1) of IAV counteracts the SPL-mediated antiviral response by inducing SPL degradation. SPL was ubiquitinated and downregulated upon IAV infection or NS1 expression, and SPL-enhanced IFN production was strikingly inhibited by IAV NS1. Another IAV type I IFN-evasion strategy IAV is viral hemagglutinin (HA) facilitating type I IFN receptor 1 (IFNAR1) degradation. Further defining this, we determined that a cellular protein, poly (ADP-ribose) polymerase 1 (PARP1), plays a critical role in mediating IAV HA-induced degradation of IFNAR1. Knockdown or inhibition of PARP1 rescued IFNAR1 levels during IAV infection or HA expression, and PARP1 was crucial for robust IAV replication, which was associated with regulation of the type I IFN receptor signaling pathway. We have also found that sphingosine kinase 2 (SK2) enhances IAV replication along with multiple host factors. IAV protein synthesis during infection was enhanced by transient overexpression of SK2, and SK2 activity affected p21 expression during IAV infection. IAV infection led to increased SK2 and activated SK2 protein expression, and MEK/ERK activation was found to be important for this increase. Overall, these studies further define IAV-host interactions and reveal novel processes used by IAV to promote its own replication.

Chapter I. Introduction

I-A. General introduction to sphingolipid metabolism

Sphingolipids are a class of lipids that have several significant roles in regulating cellular functions. The sphingolipid family consists of diverse members, including sphingomyelin, ceramide, ceramide 1-phosphate, sphingosine, and sphingosine 1-phosphate (S1P). Sphingolipids were discovered in 1874 by Johann Ludwig Thudichum within fractionally crystallized ethanolic brain extracts. (Kleuser, 2018). He named them after the mythological sphinx due to their enigmatic nature (Thudichum, 1964). Ceramide synthases and ceramides are commonly associated with apoptosis, but sphingosine kinases and S1P are commonly associated with cell survival (Spiegel and Milstien, 2003). The cell tightly controls sphingolipids and their metabolizing enzymes in order to properly maintain homeostasis.

The sphingolipid biosynthesis pathway (Figure 1) begins in the endoplasmic reticulum (ER), where ceramide is formed from non-sphingoid precursors. Ceramidases deacetylate ceramides to form sphingosine. Sphingosine is then phosphorylated by the isoforms of sphingosine kinase, sphingosine kinase 1 (SphK1, SK1) or sphingosine kinase 2 (SphK2, SK2), to make S1P. S1P can either be dephosphorylated by S1P-phosphatases back into sphingosine or irreversibly catabolized by S1P lyase (SPL) into hexadecenal and phosphoethanolamine.

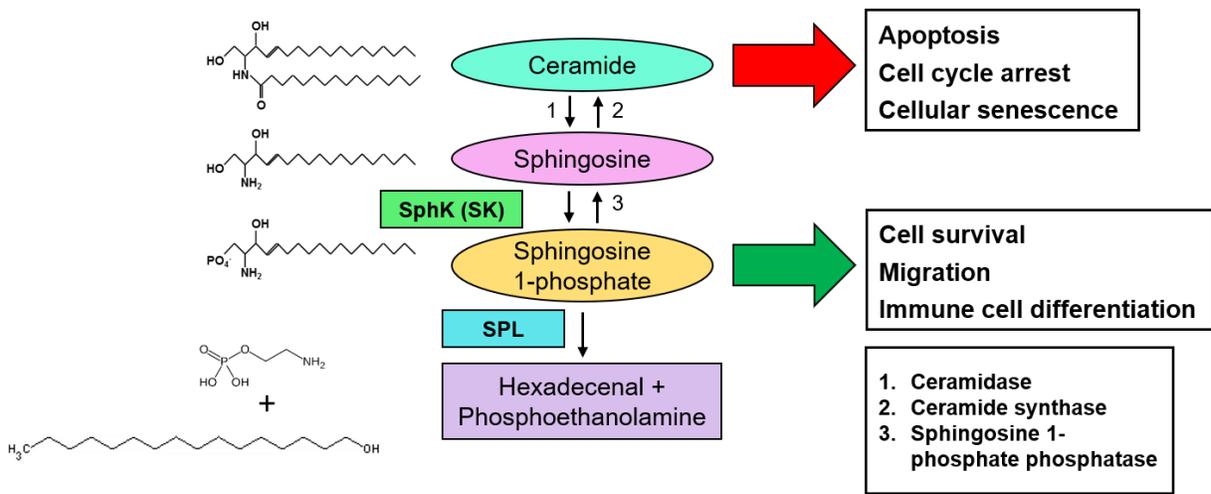


Figure 1: The sphingolipid biosynthesis pathway. Depicted are the main sphingolipid molecules and their key metabolizing enzymes. Sphingosine 1-phosphate lyase (SPL) and sphingosine kinase (SphK, SK) are the primary focus in these studies.

I-Ai. S1P functionality

S1P is known to be an important signaling molecule that modulates multiple mammalian cellular processes, such as proliferation, survival, and migration, through both extracellular receptor-mediated and intracellular mechanisms (Figure 2) (Tiper et al., 2016; Wolf et al., 2019). S1P has been shown to regulate the release of free calcium ions from the endoplasmic reticulum, which has extensive effects on cell proliferation (Mattie et al., 1994; Zhang et al., 1991). Furthermore, S1P binding the S1P receptors (S1PR1-5) on the outer membrane of the cell signals through the Ras/Raf/extracellular-signal-regulated kinase (ERK) pathway, which stimulates DNA synthesis and increases cell growth, promoting cell survival (Cuvillier et al., 1996; Shu et al., 2002). This signaling increases S1P generation by sphingosine kinase (SphK, SK), which in effect creates a positive feedback loop, and blocks the pro-apoptotic functions of ceramide (Cuvillier et al., 1996; Olivera and Spiegel, 1993). In addition, S1P signaling through S1PR1 was shown to promote cell motility in a Rac-dependent manner (Hobson et al., 2001). S1P's functions can also impact the organism as a whole. In several studies, S1P signaling was shown to be crucial for angiogenesis and vascular development, and lack of S1P caused increased vascular leakage (Camerer et al., 2009; Lee et al., 1999; Liu et al., 2000). Finally, S1P has been shown to play a large part in lymphocyte trafficking via the S1P gradient, which has been studied extensively in the functioning of sphingosine analog FTY720 (Brinkmann et al., 2002; Mandala et al., 2002). Both SK and S1P lyase (SPL) have been shown to play important roles in maintaining the S1P gradient, which places these enzymes in a critical role during the host inflammatory response (Schwab et al., 2005). Because S1P has crucial functions in multiple cellular pathways, it has been a large target for curing human diseases. The first oral therapy with the sphingosine analog FTY720 is currently used to treat multiple sclerosis (Brinkmann et al., 2010; OConnor et al., 2009; Ziemssen et al.,

2017). While FTY720 is known to functionally antagonize the S1P receptor to block T cell trafficking and help attenuate autoimmunity, the resultant immune suppressive condition was shown to cause increased susceptibility to viral diseases (Benedetti et al., 2018; Tagawa et al., 2016; Walker and Brew, 2016). It is currently unknown whether the regulation of S1P metabolism, such as targeting S1P-metabolizing enzymes, could relieve the side effects of FTY720-mediated increased viral diseases. Furthermore, SK was shown to be overexpressed in several cancers (Alshaker et al., 2013; Hasanifard et al., 2019; Maceyka et al., 2020; Neubauer et al., 2016; Pyne and Pyne, 2010). Accordingly, SK inhibitors have been evaluated for the treatment of cancers in clinical trials (Alshaker et al., 2013; Britten et al., 2017; French et al., 2003; Lewis et al., 2018). As patients could be persistently infected with viruses or exposed to viruses at any moment, it is imperative to understand how S1P-metabolizing enzymes affect virus pathogenesis and host defense to infections. Importantly, a growing body of studies indicate that many viruses regulate the enzymes in a positive or negative fashion to enhance virus pathogenicity, suggesting that these enzymes play a crucial role during virus infections.

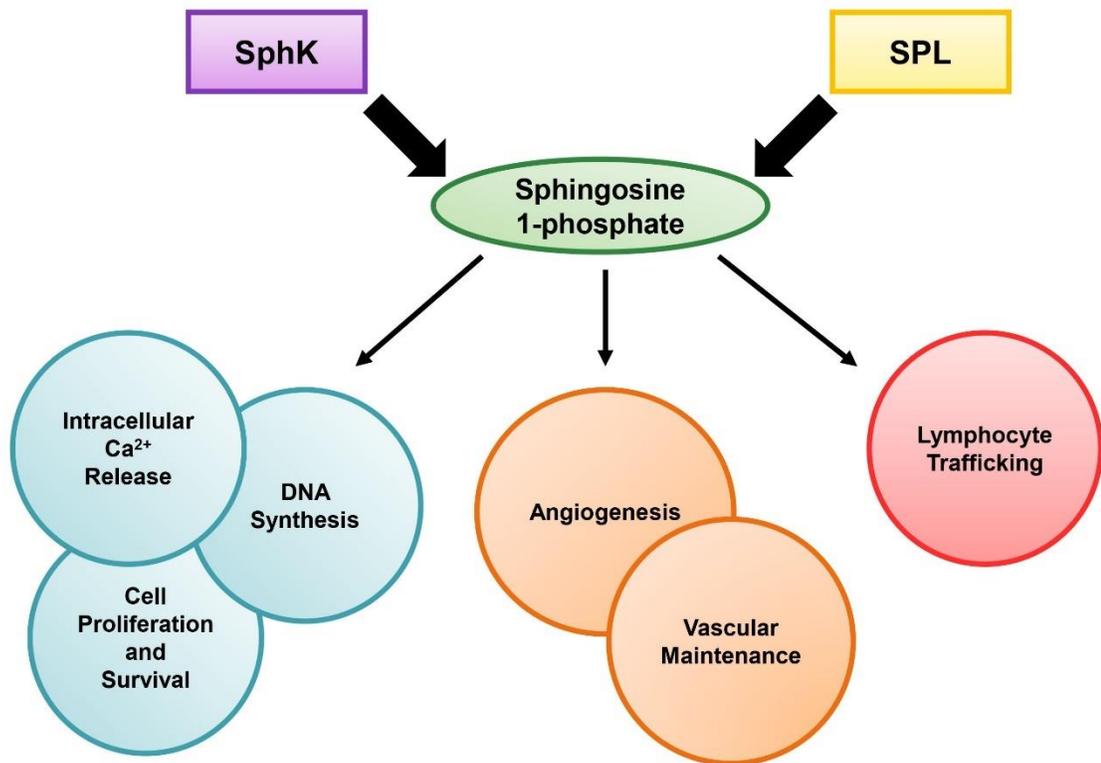


Figure 2. The role of sphingosine 1-phosphate (S1P). S1P levels are regulated by sphingosine kinase (SphK) and S1P lyase (SPL). The diverse functions of S1P on cell survival and host health are shown.

I-Aii. S1P metabolizing enzymes in viral infection

Sphingolipid levels are tightly regulated by sphingolipid-metabolizing enzymes, which are critical for maintaining cell homeostasis. Thus, S1P anabolism from sphingosine is mediated by SK, and sphingosine 1-phosphate lyase (SPL) mediates S1P catabolism into phosphoethanolamine and hexadecenal (Figure 3) (Hait et al., 2007; Spiegel and Milstien, 2003; Wolf et al., 2019). There are two different SK isoforms in humans, sphingosine kinase 1 (SK1) and sphingosine kinase 2 (SK2). The proposed cellular functions of SK1 and SK2 have similarities and distinct differences (Saba and Hla, 2004; Spiegel and Milstien, 2003). Intriguingly, both of the SK isoforms and SPL have all been found to regulate virus propagation (Seo et al., 2010, 2011; Vijayan et al., 2017). Furthermore, the levels and activation of SK have been shown to be affected by several different viruses (Machesky et al., 2008; Monick et al., 2004; Y.-J. J. Seo et al., 2013; Wati et al., 2011; Yamane et al., 2009). Therefore, manipulation of S1P-metabolizing enzymes may represent a virus-induced mechanism for promoting replication.

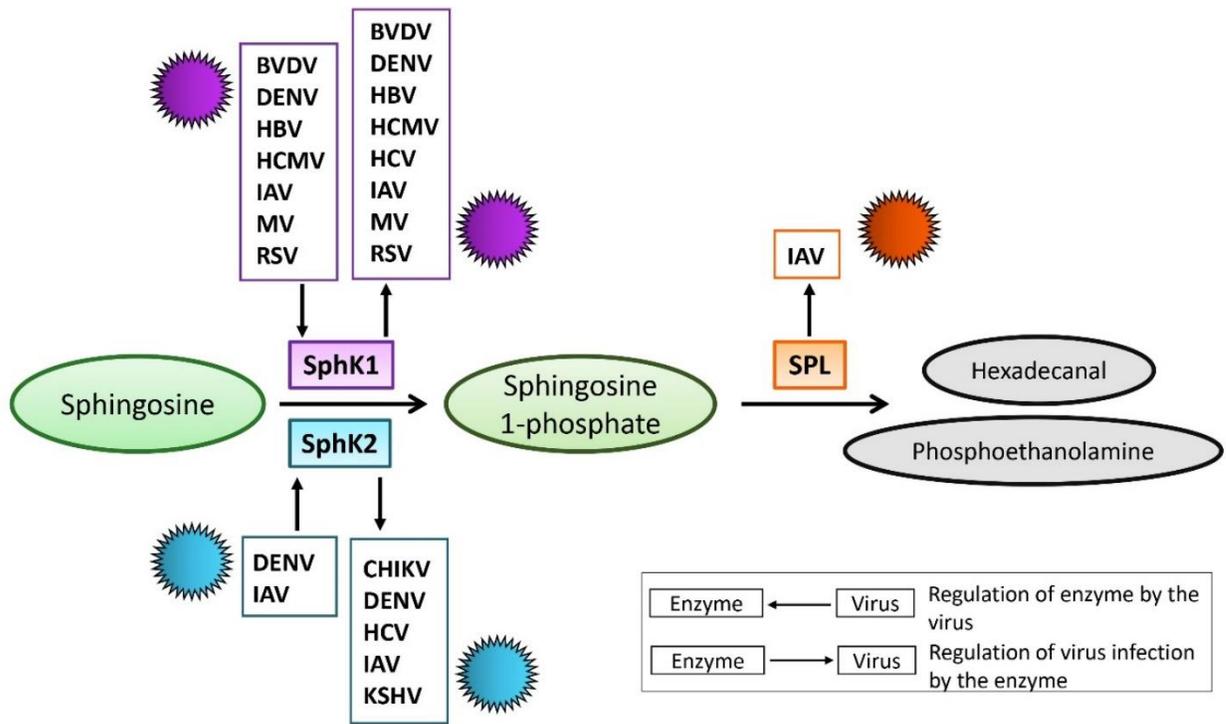


Figure 3. Schematic diagram for the interaction between viruses and S1P-metabolizing enzymes. The enzymatic function of sphingosine kinase (SphK) 1 and 2 is to catalyze the phosphorylation of sphingosine to form sphingosine 1-phosphate (S1P), while S1P lyase (SPL) irreversibly catalyzes the cleavage of S1P into hexadecenal and phosphoethanolamine. Depicted are viruses that were documented to either be affected by or regulate SphK1, SphK2, or SPL.

I-Aiii. SPL in viral and immune regulation

SPL is known for regulating cell migration, differentiation, survival, and complex physiological processes (Serra and Saba, 2010). In the sphingolipid biosynthesis pathway, SPL irreversibly catalyzes the cleavage of S1P to hexadecenal and phosphoethanolamine. SPL is localized to the ER membrane and mitochondrial associated membranes. The catalytic site is in the cytoplasmic compartment, where it can access S1P, while the transmembrane domain anchors it to the membrane. Based on studies performed with cancer and IAV, which are outlined below, SPL shows great research potential for virus–host interactions.

Molecular genetic studies performed in the model organism *Dictyostelium discoideum* discovered that regulation of SPL or SK leads to altered cellular sensitivity to cisplatin, a chemotherapy drug (Li et al., 2000). The finding was recapitulated in human cell lines. For example, SPL-overexpressing human embryonic kidney (HEK) cells were shown to be more sensitive to anti-cancer drugs compared to control cells (Min et al., 2005). This study triggered a follow-up investigation into the possible role of SPL in oncogenesis and cell survival. Thus, SPL was found to be downregulated in colon cancer and prevent colon carcinogenesis (Degagné et al., 2014). SPL has also been shown to have interesting immunologic functions, including the regulation of mature lymphocytes exiting the thymus. Moreover, genetic mutations of SPL were found in humans, linking SPL dysfunction to human diseases, including nephrotic syndrome, immunodeficiency, congenital brain malformation, and primary adrenal insufficiency (Bamborschke et al., 2018; Lovric et al., 2017; Prasad et al., 2017; Settas et al., 2019). The biological functions of SPL in human health have been well-reviewed by Dr. Saba's group (Aguilar and Saba, 2012; Bandhuvula and Saba, 2007; Choi and Saba, 2019; Fyrst and Saba, 2010, 2008; Serra and Saba, 2010).

Unlike the previously described increased sensitivity of SPL-overexpressing cells to the anticancer drug cisplatin, SPL-overexpressing HEK cells were more resistant to cell death when they were infected by influenza A virus (Seo et al., 2010). This altered susceptibility to cell death seemed to be due to overexpressed SPL regulating IAV replication. The antiviral activity of SPL was supported by transient overexpression of SPL in human lung epithelial cells. Furthermore, when cells were engineered to be SPL deficient by utilizing CRISPR/Cas9 genome editing technology, IAV replication and viral propagation substantially increased, confirming the antiviral function of SPL.

Importantly, the underlying mechanism of this resistance (Vijayan et al., 2017) indicates that SPL is a host-protective regulator that enhances the production of type I interferon (IFN) upon IAV infection or cellular recognition of influenza viral RNAs. This mechanism occurs through the interaction of SPL with inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKK ϵ), a kinase important for IFN production. While two kinases, TANK-binding kinase 1 (TBK1) and IKK ϵ , are known to be crucial for the production of type I IFNs, SPL was shown to interact with IKK ϵ but not with TBK1. In support of this observation, SPL increased IKK ϵ activation but not TBK1 activation. Many of the observed effects of SPL modulation can be attributed to the alteration of intracellular S1P levels. However, the pro-IFN function of SPL was independent of the ability of SPL to catalyze S1P cleavage and appeared to be mediated via interaction with IKK ϵ . IKK ϵ was also shown to be critical for SPL's anti-influenza activity in both HEK cells and lung A549 cells.

IKK ϵ gene knockout (KO) mice were reported to be more susceptible to influenza virus infection (Tenover et al., 2007), which demonstrates the antiviral activity of IKK ϵ . Although both IKK ϵ and TBK1 can activate IRF3, a transcription factor crucial for IFN production, the discrete regulatory mechanism of these two kinases remains poorly understood.

The function of SPL has not been widely studied in the context of viral infection. Furthermore, SPL KO mice die when they reach 2 to 4 weeks of age (Fyrst and Saba, 2008), which makes it difficult to use them for studying viral pathogenesis. However, SPL gene (Sgpl1) floxed mice have been created (Degagné et al., 2014; Zamora-Pineda et al., 2016), which will be useful for investigating the role of SPL in antiviral defense and host innate immune signaling in the future.

I-Aiv. SK2 in viral regulation

SK is essential for the production of intracellular and extracellular S1P. Both isoforms of SK, SK1 and SK2, catalyze the phosphorylation of sphingosine to form sphingosine 1-phosphate (S1P) (Oskouian and Saba, 2010; Spiegel and Milstien, 2011). Despite conserved domains and similar enzymatic activity, these isoforms have distinct differences. SK1 is known to promote cell survival and proliferation, but SK2 has been shown to have both pro- and anti-apoptotic properties (Maceyka et al., 2005; J. Min et al., 2007; Min et al., 2005; Stuart M Pitson, 2011). These two isoforms are also located in distinct subcellular compartments. SK1 is in the cytosol and plasma membrane. SK2 localizes primarily in the nucleus but can also be found in the cytosol, endoplasmic reticulum, and lysosomal membrane under certain conditions (Maceyka et al., 2005; J. Min et al., 2007; Schröder et al., 2007; Taha et al., 2006). SK1 has been shown to be critical for tumor necrosis factor-alpha (TNF- α) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signaling during inflammatory responses while both pro- and anti-inflammatory functions of SK2 have been reported (Alvarez et al., 2010; Neubauer and Pitson, 2013; Stuart M. Pitson, 2011). SK2, but not SK1, mediates the phosphorylation of FTY720 (Don et al., 2007; Kharel et al., 2005; Zemmann et al., 2006), an analogue of sphingosine and an immune modulatory drug used clinically for the treatment of multiple sclerosis (Brinkmann et al., 2010; OConnor et al., 2009; Ziemssen

et al., 2017). Since the S1P biosynthesis pathway is crucial to the control of cell survival and apoptosis as well as other functions, both isoforms of SK could be crucial targets for virus replication or inhibition (Figure 2).

A recent study in our lab has indicated that SK2 is beneficial for IAV replication in mice (Xia et al., 2018b). Treatment of IAV-infected mice with ABC294640 (ABC) led to decreased virus titers and increased mouse survival. This study identified a new host target that has therapeutic potential to complement current IAV therapeutics. However, it is not fully understood how IAV utilizes host SK2 to increase viral replication.

SK2 has also been shown to enhance chikungunya virus (CHIKV) infection. In cells, SK2 siRNA decreased the CHIKV viral RNA copy number and chemical SK2 inhibition decreased CHIKV infection (Reid et al., 2015). However, SK1 knockdown did not affect viral replication, which suggests a specificity for SK2 promoting CHIKV infection. SK2 was shown to be recruited to CHIKV viral replication complexes at the plasma membrane. However, greater study is required to clarify the role of SK2 in promoting CHIKV replication during infection.

SK2 has also been shown to function differently in infection with different HCV genotypes. Use of the SK inhibitor, SKI, which the authors stated preferentially inhibits SK2 at the concentrations used, increased the replication of cell-culture adapted genotype 1 HCVs (Yamane et al., 2014). However, SKI treatment did not suppress the replication of a chimeric genotype 2 virus, HJ3-5/GLuc. The authors of this study suggested that SK2 could regulate lipid peroxidation, which has previously been shown to negatively affect the replication of genotype 1b HCV (Huang et al., 2007; Kapadia and Chisari, 2005). SK2 regulating host cellular lipid peroxidation, which can regulate viral replication requires further investigation.

SK2 has been implicated in the modulation of several forms of cancer, which make it unsurprising that SK2 has been shown to play a role in latency for the oncogenic Kaposi's sarcoma-associated herpesvirus (KSHV). In a series of studies, treatment of KSHV-infected patient-derived primary effusion lymphoma (PEL) cell lines with an SK2 inhibitor increased apoptosis in cellular and in murine-graft in vivo experiments (Qin et al., 2014). Subsequent studies that utilized primary human endothelial cell lines showed that SK2 played a positive role in NF- κ B activation, which is known to promote KSHV latency (Dai et al., 2014; Grossmann and Ganem, 2008; Zhu et al., 2011). In this study, SK2 increases the levels of certain viral miRNAs that target KSHV lytic genes, and turning off these genes prevents host cell lysis, allowing for viral latency. SK2 inhibition increased the death of KSHV-infected cells. While the data imply that S1P production by SK2 is crucial viral RNA regulation, a direct mechanism for SK2 regulating viral gene expression remains unknown. These studies show that KSHV may promote viral latency via regulation of host SK2 and reveal a potential therapeutic target for alleviating KSHV-associated tumors.

I-B. Influenza virus basic information

Influenza viruses are negative-sense, single-stranded, segmented RNA viruses in family Orthomyxoviridae, classified as either type A, B, C, or D. Symptoms of influenza infection include fever, chills, muscle ache, cough, headache, congestion, and fatigue. Both Influenza A virus (IAV) and influenza B virus (IBV) are well known for causing seasonal influenza outbreaks annually (Lam et al., 2019), resulting in substantial morbidity and mortality worldwide despite widespread vaccination and preexposure (Iuliano et al., 2018). IAV exhibits remarkable genetic drift and shift that allow IAV to be both a pandemic and zoonotic threat (Brooke, 2017). Recurrent outbreaks of avian influenza add to mounting concerns of the next potential influenza pandemic (Cowling et al., 2013; Fraser et al., 2009; Salvador et al., 2020; Wang and Palese, 2009). An influenza vaccine is available and is reformulated annually (Krammer et al., 2018). However, the high mutation rate of IAV makes it difficult to predict exactly which strain will infect the population. While antiviral drugs that inhibit the function of IAV proteins such as neuraminidase (NA) and the viral polymerase are available, multiple IAV strains have been found to be resistant to these contemporary antivirals (Cheng et al., 2010; Dharan et al., 2009; Hussain et al., 2017; Irwin et al., 2016; Jones et al., 2018; Marjuki et al., 2015; Poland et al., 2009). Therefore, identifying host factors that could be targeted to broadly counteract infection by many different IAV subtypes has great potential as an alternative or complementary therapeutic strategy.

I-Bi. Influenza A virus structure and proteins

Influenza A virus (IAV) has a single-stranded genome made up of 8 segments. The 8 segments encode for 11 viral proteins including nonstructural protein 1 (NS1), nonstructural protein 2 (NS2, also known as nuclear export protein, NEP), matrix protein 1 (M1), matrix protein 2 (M2), hemagglutinin (HA), neuraminidase (NA), nucleoprotein

(NP), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA), and polymerase basic protein 1 – F2 (PB1-F2). HA, NA, and M2 are found in the lipid bilayer of the virion. HA is most well-known for binding to host sialic acid residues for entry into the cell. HA is also responsible for the fusion of the viral envelope with the endosomal membrane once exposed to low pH (Banerjee et al., 2013). NA is known to remove sialic acid from cellular receptors to prevent virus aggregation and enable the release of newly-formed infectious virions (McAuley et al., 2019). M2 acts as an ion channel necessary for fusion of the viral envelope with the host endosome and subsequent release of viral RNPs into the cytoplasm (Pinto and Lamb, 2006). M1 is the matrix protein, which forms a coat inside the viral envelope and mediates encapsidation of viral RNPs, but is also involved in the export of viral RNPs (Shimizu et al., 2011). A single viral RNA gene segment is wrapped around multiple NP proteins, with the conserved promoter regions in the 5' and 3' untranslated regions forming a helical hairpin. The helical hairpin is bound by the viral RNA dependent RNA polymerase, which consists of PB1, PB2, and PA proteins (Dou 2018). The nonstructural proteins of IAV are NS1, NS2, and PB1-F2. NS1 is a highly conserved multifunctional IAV protein (Trigueiro-Louro et al., 2019). While NS1 has a multitude of functions and associates with many host proteins, is most well-known to be a potent interferon agonist (Gack et al., 2009; Gao et al., 2012; Wang et al., 2000). NS2 is involved with the nuclear export of viral RNPs (Shimizu 2011). PB1-F2 is thought to mediate apoptosis of the host infected cells and additionally have some IFN antagonistic functions (Chakrabarti and Pasricha, 2013).

The IAV virion is summarized by Figure 4. The viral envelope consists of a lipid bilayer that contains the viral transmembrane proteins HA, NA, and M2. The lipid bilayer is derived of the host plasma membrane and has been shown to contain carbohydrate and sphingolipid-enriched lipid rafts as well as non-raft lipids. Underneath the viral lipid

membrane is a matrix of M1, which holds viral ribonucleoproteins (vRNPs), as well as small amounts of NS2. There are eight vRNPs, one for each segment of IAV RNA. The vRNPs consist of the viral negative stranded RNAs wrapped around NP as well as the viral RNA polymerase complex (PB1, PB2, and PA).

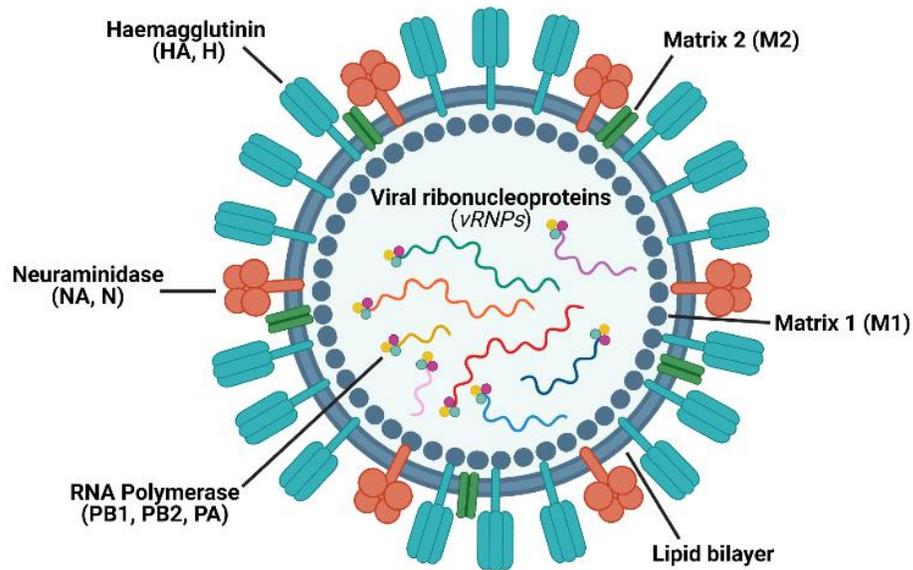


Figure 4: Diagram of influenza A virus. The IAV envelope contains three trans-membrane proteins: hemagglutinin (HA, H), neuraminidase (NA, N), and the matrix 2 (M2) proton channel. Underneath the surface of the viral envelope is the matrix 1 (M1) protein, which associates with the viral ribonucleoprotein complexes (vRNPs). The eight viral RNPs consist of eight negative single-stranded IAV RNA segments, each wrapped around nucleoprotein (NP). Associated with each vRNP is the viral RNA dependent RNA polymerase, which consists of polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA).

I-Bii. Influenza A virus replication cycle

For IAV to enter the host cell, IAV HA binds to host sialic acid residues. This leads to receptor-mediated endocytosis, and the virus enters into the host cell in an endosome. The low pH of the endosome induces a conformational change in HA, which exposes the HA fusion peptide. The fusion peptide then inserts itself into the endosomal membrane, which brings the viral and endosomal membranes together and leads to their fusion. The acidic environment of the endosome also opens the M2 channel, which acidifies the viral core and releases the vRNPs from M1 and into the host cell cytoplasm.

After being released into the host cell cytoplasm, the vRNPs enter the nucleus. The NP, PA, PB1, and PB2 proteins that comprise the vRNP have nuclear localization signals that bind to cellular nuclear import machinery and enable entry into the nucleus. Inside the nucleus, the viral RNAs serve as templates for mRNA synthesis. The viral polymerase obtains primers for viral mRNA transcription through cap snatching. The mRNA is then transported into the cytoplasm for translation. Viral RNA polymerase executes transcription and replication of viral RNAs. The viral mRNA are not complete copies of viral (-) strand RNAs because they are missing sequences from their 5' and 3' ends. To make more viral (-) RNA capable of being packaged into virions, a full length (+) complementary RNA is first transcribed, and then new (-) viral RNA copies are transcribed using the complementary RNA as templates. This process occurs for all 8 segments of IAV RNA.

IAV protein synthesis is entirely dependent on the translation machinery of the host cell. Following nuclear export, viral mRNA translation is divided between cytosolic ribosomes (for PB1, PB2, PA, NP, NS1, NS2, and M1) and endoplasmic reticulum (ER)-associated ribosomes for the membrane proteins HA, NA, and M2. As the HA, NA, and M2 proteins are produced, they are inserted into the membrane of the endoplasmic

reticulum. The proteins are subsequently trafficked through the Golgi to the plasma membrane. As a result, the HA and NA are inserted in the correct direction in the lipid membrane of the cell. After full-length (-) strand viral RNAs are exported to the cytoplasm, they join with the viral polymerase and NP to make RNP. M1 binds to the membrane where HA, NA, and M2 have been inserted, then RNP travels to the site of assembly. The virion then forms by budding, and NA cleaves sialic acid residues so that the newly formed virions can be released.

I-Biii. Influenza A virus nonstructural protein 1

IAV NS1 is expressed at high levels in the cell during infection. It is a nonstructural protein ranging from 215-237 amino acids in length, and its size ranges from 24.5 Da to 27 kDa, with the amino acid length being strain-dependent (Jureka et al., 2020). NS1 has an N-terminal RNA binding domain (amino acid residues 1-73) and a C-terminal effector domain (amino acid residues 74-230). Full-length NS1 is thought to be a homodimer, with different conformational states including “open,” “semi-open,” and “closed” (Carrillo et al., 2014; Nemeroff et al., 1995). The RNA binding domain of NS1 is a symmetrical homodimer, and each monomer was shown to consist of three α -helices (Chien et al., 1997; Liu et al., 1997). It was shown to bind with low affinity to double stranded RNA in vitro in a manner independent of RNA sequence, and dimerization was essential for double-stranded RNA binding (Wang et al., 1999). Two identical helices from each monomer form antiparallel tracks, and these tracks appear to contribute to double-stranded RNA-binding by interacting with the double-stranded RNA polyphosphate backbone (Liu 1997; Yin 2007). NS1 residues mediate this interaction either directly or by improving complex stability (Wang et al., 1999; Yin et al., 2007). The C-terminal effector domain predominantly mediates interactions with host cell proteins, but it can also stabilize the RNA-binding domain. Each monomer of the effector domain consists of seven β -strands and three α -helices (Bornholdt and Prasad, 2006; Hale et al., 2008a). In the monomer, the β -strands form a twisted and crescent-like anti-parallel β -sheet around the central α -helix (Hale et al., 2008b). It has been previously speculated that the C-terminal ~25 amino acids of NS1 could be naturally disordered, only assuming an ordered structure upon the binding of an appropriate ligand (Hale et al., 2008b). This region is involved in strain-specific functions, and disorder would be significant since many NS1 proteins have variable C-terminus length.

C-terminal truncations in NS1 occur naturally (Suarez and Perdue, 1998). For example, while the majority of seasonal IAV strains encode a 230 amino acid NS1, the 2009 pandemic H1N1 virus encodes a 219 amino acid NS1 that has an 11-amino acid C-terminal truncation (Tu et al., 2011). Additionally, NS1 can gain C-terminal amino acid extensions, as sequence analysis showed that NS1 of the IAV subtypes H1N1 and H3N2 gained a seven amino acid C-terminal extension in the 1940s and was retained until the 1980s when the mutation reverted (Lohrmann et al., 2013; Marc, 2014; Nogales et al., 2018). NS1 mRNA is derived from IAV gene segment 8, and splicing results in NS2 synthesis. The N-terminal region of IAV NS1 was found to inhibit IAV segment 8 splicing, and this was later found to be independent of the RNA binding ability of NS1 (Garaigorta and Ortín, 2007; Lu et al., 1994). Furthermore, NS2 mRNA is only 5-10% of the amount of NS1 mRNA produced (Plotch and Krug, 1986).

NS1 is able to localize to and hamper cellular pathways in both the nucleus and the cytoplasm during IAV infection (Rosário-Ferreira et al., 2020). NS1 is directed to the nucleus by a nuclear localization signal. There are either one or two nuclear localization signals in NS1 depending on the strain of IAV (Greenspan et al., 1988; Melén et al., 2012). Nuclear localization signal 1 is part of the RNA-binding domain in the NS1 N-terminus and enables NS1 to migrate to the nucleoplasm, but nuclear localization signal 2 is located in the C-terminal tail and allows NS1 to also localize in the nucleolus (Greenspan et al., 1988). While nuclear localization signal 1 is highly conserved in NS1 proteins, nuclear localization signal 2 is not present in the NS1 proteins of many IAV strains (Melén et al., 2007). The nuclear export signal at the C-terminus, which is in the effector domain, allows NS1 to exit from the nucleus to the cytoplasm (Li et al., 1998).

There has been an abundance of interactions reported between IAV NS1 and a multitude of different host proteins (Hale et al., 2008b; Rosário-Ferreira et al., 2020).

However, many of these interactions have been characterized in vitro, which brings into question their relevance in vivo. Additionally, it has yet to be determined where in the virus life cycle several of these interactions occur as well as their distinct mechanisms. The general trend of these interactions is that they ultimately benefit IAV by enhancing viral replication and weakening host innate immune responses (Nogales et al., 2018). Attenuating host innate immune responses, particularly the type I IFN innate immune response, is considered to be the major function of NS1 (Hale et al., 2010). Therefore, it is unsurprising that NS1 is crucial for robust viral replication and pathogenesis. Several known interactions between IAV NS1 and host proteins that ultimately attenuate host innate immune responses are discussed throughout further sections and chapters. Additionally, several studies have shown that NS1 regulates viral mRNA transcription and translation (Aragón et al., 2000; de la Luna et al., 1995; Falcón et al., 2004; J. Y. Min et al., 2007; Wang and Krug, 1998; Wang et al., 2010). NS1 was also found to upregulate certain virus-specific regulated genes, some of which were previously shown to have important roles in viral replication (Shapira et al., 2009). Host gene expression has been shown to be reduced by NS1 by preventing host mRNA polyadenylation, which would prevent nuclear export of mRNA (Ayllon and García-Sastre, 2015; Nogales et al., 2018). However, this does not affect viral mRNA polyadenylation since that is mediated by the viral polymerase (Robertson et al., 1981). It has been speculated that by preventing host mRNA polyadenylation, pre-mRNA can build up in the nucleus, leading to a pool of host mRNA that can be used for cap-snatching purposes. Additionally, NS1 is also thought to inhibit cellular pre-mRNA splicing (Kuo et al., 2016). By affecting many different aspects of the host cell, NS1 ultimately makes the cell more conducive to IAV replication.

I-C. Type I interferon

In the early stages of infection, host cells must defend themselves to ensure their survival. Fortunately, cells possess an intricate defense system that specifically responds to pathogens. Interferons (IFNs) are a group of signaling proteins produced and secreted by virtually all vertebrate host cells in response to the presence of several viruses (Pestka et al., 2004; Swiecki and Colonna, 2011). IFNs belong to a large class of proteins known as cytokines. Cytokines are molecules used for communications between cells which ultimately trigger the immune system to eliminate pathogens.

I-Ci. RIG-I-mediated type I IFN innate immune response to infection

Type I IFN was first found to have anti-influenza viral activity in 1957 by Isaacs and Lindenmann, and was named due to its broad interference in viral replication (Isaacs and Lindenmann, 1957). The type I IFN innate immune response is among the first lines of defense against viral infections (Figure 5) (Hoffmann et al., 2015; Seo and Hahm, 2010). Host retinoic acid inducible gene I (RIG-I) senses IAV RNA products by recognizing the 5'-ppp moiety on IAV RNA, which activates RIG-I (Goubau et al., 2013; Killip et al., 2015; Loo et al., 2008; Pichlmair et al., 2006). Activated RIG-I then interacts with downstream adaptor protein mitochondrial antiviral signaling adaptor (MAVS), which subsequently recruits and activates inhibitor of nuclear factor $\kappa\beta$ kinase subunit ϵ (IKK ϵ) and TANK binding kinase 1 (TBK1). Both IKK ϵ and TBK1 activate the transcription factor interferon regulatory transcription factor 3 (IRF3) as well as interferon regulatory transcription factor 7 (IRF7) later after its production (Gale and Foy, 2005; Sharma et al., 2003). The activation of these transcription factors leads to IFN- α and IFN- β production (Sato et al., 2000). The resultant IFN- α and IFN- β molecules are secreted from the host cell and sensed by the producer cell as well as the cells surrounding it. IFNs then bind to their cognate receptor, interferon α/β receptor (IFNAR). IFNARs are then endocytosed and trigger the activation

of the JAK-STAT signaling pathway (Platanias, 2005). Janus kinase 1 (JAK1) and non-receptor tyrosine-protein kinase (TYK2) phosphorylate signal transducer and activator of transcription 1/2 (STAT1/STAT2), which complexes with interferon regulatory factor 9 (IRF9) to form the transcriptionally active interferon stimulated gene factor 3 (ISGF3) complex. The ISGF3 complex then translocates to the nucleus and induces the expression of hundreds of IFN-stimulated genes (ISGs) by binding to Interferon Stimulated Response Elements, which are found in ISG promoter regions (Aaronson and Horvath, 2002; Gale and Foy, 2005). ISGs act to inhibit virus replication by promoting an antiviral cellular state (Li et al., 2018; Schoggins, 2014).

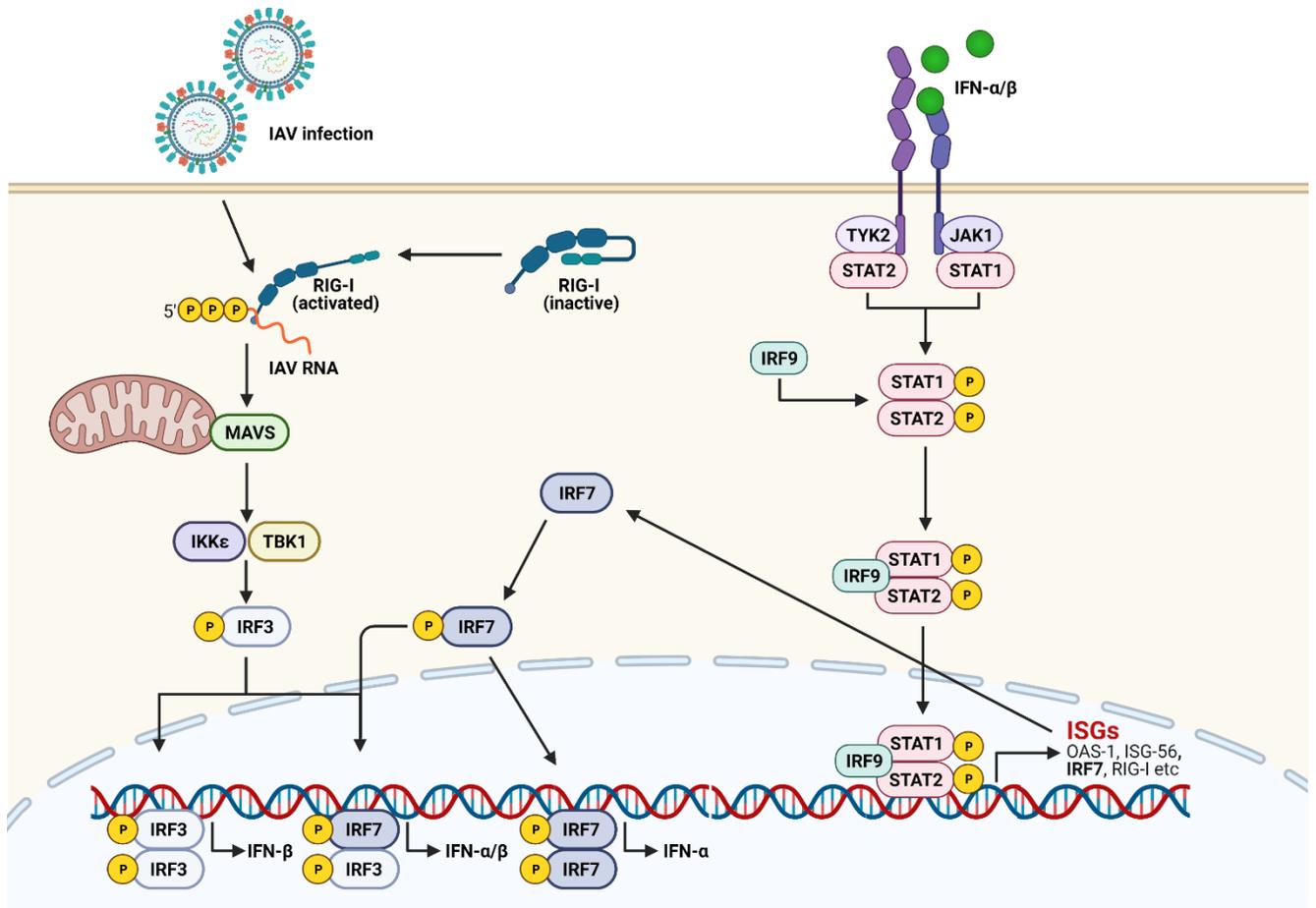


Figure 5: The RIG-I-mediated type I IFN response to IAV. Following IAV infection, RIG-I initiates an antiviral response by recognizing and binding IAV 5'-triphosphate RNA. RIG-I then activates downstream adaptor protein MAVS. MAVS acts as scaffold protein to recruit downstream kinases TBK1 and IKK ϵ . These two kinases then phosphorylate IRF3 and later IRF7, both of which are transcription factors that can lead to IFN production. IRF3 and IRF7 either homodimerize or heterodimerize and translocate to the nucleus, where the dimer complexes with coactivators and binds to its target DNA sequence in type I IFN genes and lead to the production of IFN- β and IFN- α . The secreted type I IFNs act by binding to the cognate IFNAR and initiate the JAK/STAT pathway that leads to induction of ISGs. JAK1 and TYK2 phosphorylate STAT1/STAT2, which then complexes with IRF9, forming the ISGF3 complex. The ISGF3 complex then translocates to the nucleus and induces the expression of hundreds of IFN-stimulated genes (ISGs).

I-Cii. IAV evasion of RIG-I-mediated type I IFN immune responses

Despite being well-equipped to counteract viral pathogens, the innate immune response is not always successful in preventing viral infections. While the host innate immune system has developed many defenses against viral pathogens, IAV has adapted to overcome the type I IFN innate immune response and facilitate their replication. IAV must counteract host antiviral activities, especially IFN production and the activity of ISGs, in order to replicate efficiently (Muñoz-Moreno et al., 2020). Influenza viral PB2 and PB1-F2 proteins were reported to inhibit induction of type I IFNs by binding to MAVS, which decreased mitochondrial membrane potential (Varga et al., 2012). HA was also shown to induce downregulation of IFNAR1, which reduced the type I IFN innate immune response (Xia et al., 2016). IAV NS1 protein is considered to be the main antagonist of the type I IFN innate immune response. Influenza virus is known to inhibit the host type I IFN response by NS1 binding to double-stranded RNA (dsRNA) (Min and Krug, 2006; Wang et al., 1999). NS1 was reported to directly limit the antiviral state by inhibiting OAS-1 activation, outcompeting OAS for interaction with dsRNA (Min and Krug, 2006). The binding of NS1 to dsRNA also prevents activation of dsRNA-activated protein kinase (PKR) (Bergmann et al., 2000). This leads to phosphorylation of the alpha subunit of eukaryotic translation initiation factor 2 (eIF-2 α), leading to a decrease in the rate of initiation of translation. Additionally, NS1 blocks the TRIM25 ubiquitin E3 ligase-mediated K63-linked ubiquitination of the viral RNA sensor RIG-I (Gack et al., 2009), which is required for optimal downstream signaling of the type I IFN response. NS1 has also been reported to interact with RIPLET to prevent RIG-I ubiquitination (Rajsbaum et al., 2012) and directly bind to RIG-I to prevent RIG-I-mediated induction of the type I IFN innate immune response (Mibayashi et al., 2007).

I-D. Ubiquitination

Ubiquitination is an essential post-translational modification that controls the stability and functionality of proteins. The ubiquitin system is vital for the functioning of many cellular processes, including protein quality control, protein trafficking, signal transduction, gene expression, DNA repair, cell differentiation, and cell division (Foot et al., 2017; Oh et al., 2018; Pohl and Dikic, 2019; Rape, 2018; Schwertman et al., 2016). Ubiquitin is a small 8.6 kDa regulatory protein, named for being ubiquitously found in most tissues of eukaryotic organisms. The discovery of ubiquitin occurred in the field of immunology by Gideon Goldstein in 1975 while he was searching for thymopoetin, a protein involved in the induction of CD90 (Cluster of Differentiation 90) in the thymus (Goldstein et al., 1975). Ubiquitin was further characterized throughout the remainder of the 1970s and the 1980s, and more information continues to be discovered to this day. Ubiquitin can be conjugated with its C-terminus to lysine residues in substrate proteins. Conjugation of ubiquitin to the substrate protein requires three classes of enzymes: a ubiquitin activating (E1) enzyme, a ubiquitin conjugating (E2) enzyme, and a ubiquitin ligase (E3) (Figure 7). Additional ubiquitin can be added to the first using one of the 7 lysine residues found in ubiquitin itself (K6, K11, K27, K29, K33, K48, K63), which make polyubiquitin chains. Polyubiquitin chains can be homotypic or heterotypic. Homotypic chains are linked uniformly through the same acceptor site of ubiquitin, while heterotypic chains contain multiple linkage types. Heterotypic chains can be further classified into either mixed or branched. Mixed ubiquitin chains have ubiquitin subunits that are modified, but only on a single acceptor site. However, branched ubiquitin chains contain at least one ubiquitin subunit that is simultaneously modified on multiple acceptor sites. The message given by the ubiquitin chain depends on the chain's linkage type and the chain type. The most well-studied ubiquitin chains are homotypic K48-linked and K63-linked ubiquitin

chains. Homotypic K48-linked and K63-linked ubiquitin chains generally lead to proteasomal degradation or activation of the substrate, respectively. However, the structures and differential functions of branched ubiquitin chains have only recently emerged, which greatly increases the complexity of ubiquitin signaling, and much remains undefined (French et al., 2021). For example, branched K48/K63 ubiquitin chains have been recently shown to lead to proteasomal degradation of a host protein, mediated by separate host E3 ubiquitin ligases (Ohtake et al., 2018). Furthermore, ubiquitin chains can undergo deubiquitination by deubiquitinating enzymes. Deubiquitination is also known to signal various cellular activities. For example, K63 chain cleavage from the substrate by a proteasomal deubiquitinase can stimulate autophagy-dependent aggresome clearance, which is critical for protein aggregate removal (Hao et al., 2013).

Ubiquitin is an important regulating factor for host cell functions, which makes it unsurprising that viruses have evolved diverse ways to manipulate the ubiquitin system. Influenza virus has been shown to manipulate the ubiquitin system in several ways to enhance its replication. Unanchored ubiquitin chains, which are packaged into IAV virions, can facilitate viral uncoating by recruiting histone deacetylase 6 (HDAC6) to viral fusion sites through the HDAC6 zinc-finger ubiquitin binding domain. RNA segments of IAV are then transported towards the nucleus via microtubule movement and released into the nuclear pore (Banerjee et al., 2014). Additionally, E3 ubiquitin ligase Itch was shown to be crucial for the release of IAV RNPs from endosomes by ubiquitination of IAV M1 protein (Su et al., 2013). IAV NS1 was shown to destabilize Mdm2 E3 ligase, leading to more robust viral replication (Pizzorno et al., 2018). All three subunits of IAV RNA-dependent RNA polymerase are ubiquitinated, which enhances IAV genome replication (Kirui et al., 2016). A previous study also revealed that the cytoplasmic domain of IAV M2 protein is ubiquitinated, which was crucial for infectious viral particle production (Su et al., 2017).

Ubiquitination allows the host to regulate cellular processes by altering the function, abundance, or subcellular distribution of proteins, which has a strong presence in the regulation of innate immunity. Influenza A virus has been also shown to manipulate the ubiquitin system to evade innate immune mechanisms against its infection such as the type I IFN innate immune response. For example, IAV NS1 binds to the coiled coil domain of E3 ubiquitin ligase TRIM25, which interferes with K63-linked ubiquitination of RIG-I and subsequent innate immune signaling (Gack et al., 2009). IAV NS1 has also been shown to interact with RIPLET to prevent RIG-I ubiquitination (Rajsbaum et al., 2012).

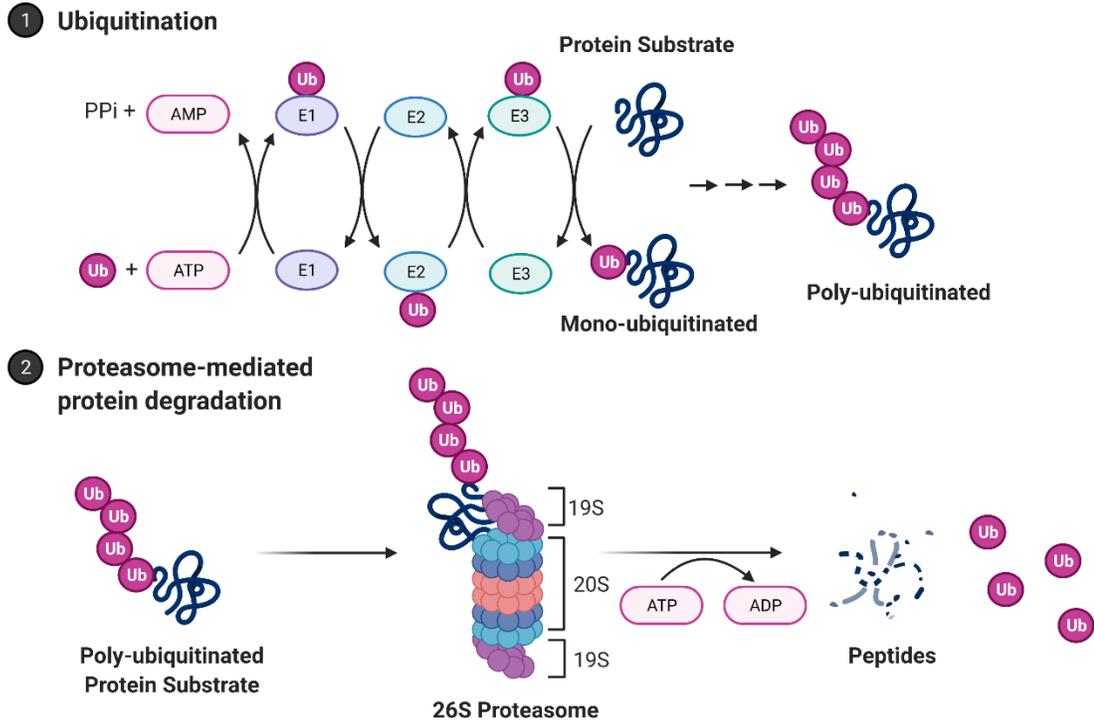


Figure 6: The ubiquitin proteasome system. (1) Ubiquitination is carried out by a cascade of enzymatic reactions. E1 activating enzyme uses ATP to activate the C-terminal carboxyl group of ubiquitin, which then forms a thioester bond with the cysteine residue in the active site of an E2 ubiquitin conjugating enzyme. In the final step, an E3ubiquitin ligase transfers the ubiquitin from the E2-Ub to a specific substrate. (2) After being targeted for degradation by ubiquitination, the protein binds to the 19S cap of the 26S proteasome. The 19S particle then cleaves off the ubiquitin chain and linearizes the protein in an ATP-dependent manner. The linear protein then enters the 20S barrel, where it is digested into peptides. Figure made using Biorender (cite).

I-Di. Protein degradation

Protein levels are tightly regulated within the cell by both transcription and protein degradation. Protein degradation is essential for controlling both quality and quantity of proteins. Generally, the proteasome degrades individual proteins in a highly targeted manner, and lysosomes degrade cytoplasmic components including protein aggregates, defective or surplus organelles through autophagy, and some individual proteins (Wang and Robbins, 2014). Both proteasomal and lysosomal degradation are essential for cellular proteostasis. The majority of proteins in all tissues are targeted for degradation by the ubiquitin-proteasome pathway (Rock et al., 1994). The main specificity factor in the ubiquitin-proteasome pathway is the E3 ligase. There are over 600 estimated E3 ubiquitin ligase in humans (George et al., 2018), and they link ubiquitin to proteins in a highly regulated manner. After being targeted for degradation by ubiquitination, most cell proteins are degraded by the 26S proteasome, which is summarized in Figure 6. The 26S proteasome is found in the cytosol and nucleus of all cells. It consists of a central barrel-shaped 20S proteasome that contains the unique proteolytic sites that break peptide bonds as well as a 19S regulatory particle at either or both ends that cleaves off the ubiquitin chain and uses ATPases to unfold the protein substrate and translocate it to the 20S core for degradation (Voges et al., 1999). After the ubiquitinated protein binds to the 19S compartment, the polyubiquitin chain is cleaved off of the substrate and disassembled. The protein is unfolded by the ATPases in the particle. After linearization, the protein is translocated through the pore of the 20S gated entry channel and into the 20S proteasome. The protein is then digested into small peptides and released by the proteasome into the cell, where they are quickly digested by cytoplasmic peptidases into amino acids (Lecker et al., 2006). The amino acids are then either reutilized by the cell to

make new proteins or are metabolized for energy (Saric et al., 2004). Unsurprisingly, the ubiquitin-proteasome system is extremely important for IAV replication. For example, IAV RNA synthesis has been shown to be dependent on the ubiquitin proteasome system, and inhibition of the proteasome attenuated IAV replication at a post-fusion step of virus replication (Widjaja et al., 2010). Additionally, IAV M2 was revealed to interact with and downregulate tetherin via the proteasomal degradation pathway (Hu et al., 2017).

The other major cellular protein degradation pathway is lysosomal degradation. Lysosomes are membrane-bound spherical organelles that contain many different digestive enzymes. The interior of a lysosome is acidic (~4.5-5) compared to the cytosol (pH 7.2), which is optimal for the hydrolytic enzymes contained within (Cooper, 2000). In order to be degraded by the lysosome, proteins must first be taken up by one. One pathway for this is autophagy, where small areas of cytoplasm or cytoplasmic organelles are enclosed in ER-derived membranes. These vesicles, named autophagosomes, then fuse with lysosomes and the contents are digested. A major function of lysosomes is the digestion of outside materials taken up via endocytosis. Another major route of lysosomal-mediated degradation is autophagy. Autophagy plays a role in the clearance of misfolded proteins and protein aggregates. Ubiquitin tagging can signal proteins for lysosomal degradation as well as tag organelles for disposal. K63 ubiquitination in particular has been shown to promote the induction of autophagy in response to stressed conditions or accelerates autophagy initiation through feedback mechanisms (Chen et al., 2019; Tan et al., 2008). IAV also takes advantage of lysosomal degradation to efficiently replicate. For example, IAV infection led to the lysosomal degradation of eukaryotic initiation factor 4b (eIF4B), which was mediated by IAV NS1 protein. NS1-mediated downregulation of eIF4B enhanced IAV replication by suppressing interferon-induced transmembrane protein 3 (IFITM3) expression (Wang et al., 2014a). Additionally, autophagy has been shown to

dampen the early IFN response to IAV (Perot et al., 2018), and several viral proteins including NS1, HA, and M2, have shown to be involved with autophagy upregulation (Gannagé et al., 2009; Zhirnov and Klenk, 2013).

Chapter II. Influenza A virus NS1 induces degradation of sphingosine 1-phosphate lyase to obstruct the host innate immune response

II-A. Abstract

The type I interferon (IFN)-mediated innate immune response is one of the central obstacles influenza A virus (IAV) must overcome in order to successfully replicate within the host. We have previously shown that sphingosine 1-phosphate (S1P) lyase (SPL) enhances IKK ϵ -mediated type I IFN responses. Here, we build upon those studies to further characterize the antiviral activity of SPL, and we also demonstrate that the nonstructural protein 1 (NS1) of IAV counteracts the SPL-mediated antiviral response by inducing degradation of SPL. SPL was ubiquitinated and downregulated upon IAV infection or NS1 expression, whereas NS1-deficient IAV failed to elicit SPL ubiquitination or downregulation. Transiently overexpressed SPL increased phosphorylation of IKK ϵ , resulting in enhanced expression of type I IFNs. However, this induction was markedly inhibited by IAV NS1. A prediction analysis revealed synoviolin 1 (SYVN1) as a potential E3 ubiquitin ligase for SPL, and SYVN1 was found to interact with SPL during transient overexpression of IAV NS1. Additionally, chemical inhibition or knockdown of SYVN1 during IAV infection led to reduced IAV protein expression compared to the control. Collectively, this study reveals a novel strategy employed by IAV to subvert the type I IFN response, providing new insights into the interplay between IAV and host innate immunity.

II-B. Introduction

Influenza A virus (IAV) is a negative sense segmented RNA virus in family Orthomyxoviridae. IAV and influenza B virus (IBV) are well known for causing seasonal influenza outbreaks annually (Lam et al., 2019), resulting in substantial morbidity and mortality worldwide despite widespread vaccination and preexposure (Iuliano et al., 2018). IAV exhibits remarkable genetic and genomic diversity that allows IAV to be both a pandemic and zoonotic threat (Brooke, 2017). Recurrent outbreaks of avian influenza add to mounting concerns of the next potential influenza pandemic (Cowling et al., 2013; Fraser et al., 2009; Salvador et al., 2020; Wang and Palese, 2009). Vaccines targeting influenza viruses must be reformulated annually (Krammer et al., 2018). While antiviral drugs that inhibit the function of IAV proteins such as neuraminidase (NA) and the viral polymerase are available, multiple IAV strains have been found to be resistant to these contemporary antivirals (Cheng et al., 2010; Dharan et al., 2009; Hussain et al., 2017; Irwin et al., 2016; Jones et al., 2018; Marjuki et al., 2015; Poland et al., 2009). Therefore, identifying host factors that could be targeted to broadly counteract infection by many different IAV subtypes has great potential as a therapeutic strategy.

One of the host's first lines of defense against IAV infection is the powerful type I interferon (IFN)-mediated innate immune response (Hoffmann et al., 2015; Seo and Hahm, 2010). Briefly, host RIG-I senses IAV RNA products by recognizing the 5'-ppp moiety that is present on IAV RNA (Goubau et al., 2013; Killip et al., 2015; Loo et al., 2008). The activation of RIG-I leads to its interaction with the downstream adaptor protein MAVS and subsequent recruitment and activation of IKK ϵ and TBK1, both of which activate transcription factors IRF3 and IRF7 (Sharma et al., 2003). This leads to the production and secretion of IFN- α and IFN- β molecules (Sato et al., 2000). IFNs then bind to their cognate receptor, triggering the activation of the JAK-STAT signaling pathway and

ultimately inducing the transcription of IFN-stimulated genes (ISGs) (Aaronson and Horvath, 2002). ISG products promote a cellular antiviral state by inhibiting virus replication (Schoggins, 2014).

Sphingosine 1-phosphate (S1P) lyase (SPL) is a host enzyme that mediates the catabolism of the bioactive lipid S1P into hexadecenal and phosphoethanolamine (Aguilar and Saba, 2012; Bourquin et al., 2010). Due to its ability to metabolize S1P, SPL has been implicated in a diverse array of mammalian cellular processes and diseases, such as cell proliferation, cell survival, cell development, host immunity, and cancer (Bandhuvula and Saba, 2007; Chi, 2011; Fyrst and Saba, 2008; Herr et al., 2003; Kumar et al., 2004; Li et al., 2000; J. Min et al., 2007; Serra and Saba, 2010). Recessive SPL mutations have been found to lead to SPL Insufficiency Syndrome, which can involve symptoms including nephrosis and immunodeficiency among others (Lovric et al., 2017). However, we have previously shown that SPL has antiviral activity during IAV infection (Seo et al., 2010). SPL enhanced the type I IFN response to IAV independently of its ability to metabolize S1P by interacting with IKK ϵ but not TBK1 (Vijayan et al., 2017), ultimately resulting in the production of more IFN molecules and subsequently more ISGs.

In this study, we expand upon our previous findings to further characterize the antiviral activity of SPL, and we report that IAV infection leads to the ubiquitination and subsequent downregulation of SPL via IAV nonstructural protein 1 (NS1). IAV NS1 reduced the previously observed SPL-mediated activation of IKK ϵ , which resulted in reduced IFN responses. This study reveals a novel strategy used by IAV to subvert the type I IFN innate immune response.

II-C. Materials and Methods

Viruses and Cells

Influenza A/Puerto Rico/8/34 (H1N1) virus (gift from Adolfo Garcia Sastre), influenza A/Puerto Rico/8/34 (H1N1) virus deficient in NS1 (Δ NS1) (gift from Dr. Adolfo Garcia Sastre), influenza A/Hong Kong/8/68 (H3N2 VR-1679) virus (ATCC), 2009 pandemic influenza A/CA/04/09 (H1N1) virus (gift from Dr. Wenjun Ma) (Lee et al., 2017; Xia et al., 2018b), and influenza B/Lee/40 (IBV) virus (ATCC VR-1535), were used as previously described. In experiments, IAV denotes Influenza A/Puerto Rico/8/34 virus unless stated otherwise. Viruses used in this study were amplified either on Madin-Darby Canine Kidney (MDCK) cells or in chicken eggs as described previously (Eisfeld et al., 2014; Neumann et al., 1999; Seo et al., 2010; Varble et al., 2014). Briefly, for amplification of viruses on MDCK cells, cells were incubated with virus for 1 hour (h). The cells were then washed with PBS and incubated with fetal bovine serum (FBS) free medium containing 0.3% bovine serum albumin (BSA) and TPCK-trypsin (1 μ g/mL) for amplification. For viral amplification in chicken eggs, serum pathogen-free fertilized chicken eggs were candled then inoculated with virus diluted in 1x phosphate buffered saline via the allantoic route. Infected eggs were incubated for 48 hours without turning at 37°C and ~60% humidity. Eggs were then chilled and the allantoic fluid was collected and centrifuged to remove debris. Virus titers were determined by plaque assay. Briefly, supernatants containing viruses were harvested, serially diluted, and then were adsorbed onto 1 x 10⁶ MDCK cells/well in a 6-well plate for at least 1 h. Cells were then overlaid and incubated with 2X EMEM (Gibco) containing 0.6% BSA and 2 ug/mL TPCK-trypsin mixed with an equal portion of 1% agarose (Seakem ME). Cells were fixed using 25% formalin and stained with 1X crystal violet prior to assessing viral titer. Sources of human embryonic kidney (HEK) 293 cells, MDCK cells, SPL-knockout (KO) cells, and human lung

epithelial A549 cells have been previously described (J. Min et al., 2007; Seo et al., 2012; Vijayan et al., 2017, 2014). HEK293 cells, SPL-KO cells, and A549 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco), while the MDCK cells were cultured in Minimum Essential Medium Eagle (MEM, Gibco) as previously described (Y.-J. J. Seo et al., 2013; Vijayan et al., 2017, 2014; Xia et al., 2018b, 2016). All cells were cultured in a CO₂ incubator at 37°C. All media to culture immortalized cell lines were supplemented with 10% FBS (HyClone) and 1% penicillin (100 U/mL)/streptomycin (100 µg/mL) (Invitrogen) unless stated otherwise. Undifferentiated primary human tracheal epithelial cells (HTEpCs) derived from excess tissue of lungs donated for transplant from human donors were expanded as previously described (Dickinson et al., 2018) with PneumaCult-Ex Plus medium (StemCell) supplemented with 0.1% hydrocortisone stock solution (96 µg/mL) (StemCell), 1% penicillin (100 U/mL)/streptomycin (100 µg/mL) (Invitrogen), 0.1% amphotericin B (250 µg/mL) (Fungizone), and 2% PneumaCult-Ex Plus 50x supplement (StemCell).

Plasmids and Transfection

Mammalian expression plasmids encoding IKK ϵ , Flag-tagged SPL, wild type SPL, HA-tagged ubiquitin, and IAV neuraminidase were used as described previously (Vijayan et al., 2017; Xia et al., 2016). Plasmid encoding IAV NS1 was a gift from Dr. Adolfo Garcia-Sastre (Mt Sinai). Plasmids encoding short hairpin RNA (shRNA) against human SPL (shSPL) in PLKO.1 from Addgene and scramble control shRNA were gifts from Dr. Julie D. Saba (University of California San Francisco, Children's Hospital Oakland Research Institute). pINDUCER reagents were a gift from Dr. David Pintel (University of Missouri). Lentivirus containing shSPL, human SPL, and shSCR was generated as previously described (Meerbrey et al., 2011) and lentivirus-containing supernatants were concentrated using lenti concentrator (OriGene, TR30025) according to manufacturer's

instructions. For transfection of cultured cells, cells were seeded onto 6-well plates or 24-well plates at densities of 1×10^6 cells/well or 2.5×10^5 cells/well respectively 24 hours prior to transfection. Cells were then transfected with the indicated plasmids using Lipofectamine2000 Transfection Reagent (Thermo Scientific) or LipoD293 transfection reagent (Signagen) at 80%-90% confluency following the protocols recommended by the manufacturers. A concentration of 500 ng/mL DNA was used for the transfection experiments unless specifically indicated. Empty vector plasmids were used as a control in all transfection experiments to ensure that each transfection sample received the same amount of total DNA.

Reagents and Antibodies

Anti-DYKDDDDK (FLAG) G1 Affinity Resin (GenScript), IP lysis buffer (Thermo Scientific), protease inhibitor PMSF (Gold Bio), and cycloheximide (CHX, Sigma-Aldrich) were purchased from the indicated manufacturers. Antibodies against IBV NP (B017) and IAV M1 (GA2B) were purchased from Abcam; antibodies against p-IKK ϵ (Ser172, D1B7), IKK ϵ (D20G4), FLAG tag (9A3), HA tag (C29F4), human GAPDH (D1GH11), RIG-I (D14G6), and ubiquitin (P4D1) were purchased from Cell Signaling Technology; antibodies against SPL (H-300), IAV NS1 (NS1-23-1), and IAV M2 (vi-19) were purchased from Santa Cruz Biotechnology; antibody against IAV H5N1 NA (GTX127984) was purchased from Genetex. Fluorophore-labeled IR-Dye secondary antibodies against mouse and rabbit IgG were purchased from LI-COR.

Denatured immunoprecipitation (denatured-IP), and western blotting

293T cells, seeded in 6-well plates, were transiently transfected or co-transfected with the indicated plasmids (1 μ g DNA in total). For detection of ubiquitination of SPL during IAV infection, cells were infected with IAV with an MOI of 1. Cells were harvested and lysed 24 hours post-transfection in 1 mL IP lysis buffer (48 h post-transfection for

infection experiments). Denatured IP was performed as previously described (Xia et al., 2016). Briefly, cell lysates were boiled at 95°C for 5 minutes, were chilled on ice, and were centrifuged to clear lysates of cell debris. A small amount of lysate was reserved for analysis via western blotting, and the remainder of the samples were incubated with 20 µl of anti-DYKDDDDK (FLAG) G1 affinity resin (GenScript) overnight with rotation at 4°C. The beads were washed three times with 1 mL IP lysis buffer, and precipitates and lysates were analyzed by western blot analysis. For co-IP experiments, cell lysates containing protease inhibitor (PMSF, 1 mM) were incubated with 20 µl of anti-DYKDDDDK (FLAG) G1 affinity resin overnight with rotation at 4°C. The beads were washed three times with 1 mL IP lysis buffer, and precipitates were analyzed by western blot analysis. Western blotting was performed as previously described. Briefly, cells were lysed by addition of 2x sample buffer containing β-mercaptoethanol and boiled at 95°C for 10 min. The denatured polypeptides from cell lysates or IP were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked then incubated with the indicated primary antibodies overnight at 4°C. Membrane-bound antibodies were detected using IRDye secondary antibodies (LI-COR). Bound antibodies were visualized and imaged using an Odyssey Fc (LI-COR), and the resulting data were analyzed using Image Studio software V5.2 (LI-COR). Similar results were obtained from at least two independent experiments.

Real-time quantitative PCR

Total cellular RNA was extracted and purified by using Tri Reagent (Sigma-Aldrich) according to the manufacturer's instructions. Purified RNA was reverse transcribed with random primers (Invitrogen), and the resulting cDNA was used as a template for real-time quantitative PCR (RT qPCR) using gene-specific primers. Primers for human SPL (5'-

GGA TGA AGA TTG TGC GGG TC-3' and 5'-GAA CAG ACG AGC ATG GCA GT-3'), human OAS-1 (5'-GAT CTC AGA AAT ACC CCA GCC A-3' and 5'-AGC TAC CTC GGA AGC ACC TT-3'), human IFN- β (5'-CGC CGC ATT GAC CAT CTA-3' and 5'-GAC ATT AGC CAG GAG GTT CTC A-3'), human IFN- α (5'-GTG AGG AAA TAC TTC CAA AGA ATC AC-3' and 5'-TCT CAT GAT TTC TGC TCT GAC AA-3'), and human GAPDH (5' - TCA CCA CCA TGG AGA AGG -3' and 5' -GAT AAG CAG TTG GTG GTG CA -3') were used. After cDNA synthesis, qPCR reactions were performed with Power SYBR green PCR Master Mix (Applied Biosystems) using a Step One real-time PCR instrument (Applied Biosystems), and cDNA quantities were normalized to quantities of GAPDH RNA measured from the same samples.

Statistical analysis

Shapiro-Wilk test and Bartlett's test of homogeneity of variances were used to determine if the assumptions were met to analyze using a one-way ANOVA. Data were then analyzed and compared pairwise using either one-way ANOVA with Tukey's range test as post hoc or Kruskal-Wallis with Conover-Iman test of multiple comparisons as post hoc. One-way ANOVA, Tukey's range test, and Kruskal-Wallis were performed using JASP (JASP Team, 2020). Statistical analysis was conducted in R (R Core Team, 2020), with the indicated packages for the following: Conover-Iman test of multiple comparisons (conover.test package (Alexis Dinno, 2015)), Shapiro-Wilk normality test (dplyr package (Wickham et al., 2021)), and Bartlett's test of homogeneity of variances (stats package (R Core Team, 2020)). Error bars represent mean \pm either standard error of the mean (SEM) or standard deviation (SD) where indicated. Data are representative of at least two independent experimental repetitions.

II-D. Results

SPL has antiviral activity against IAV

We have previously determined that sphingosine 1-phosphate lyase (SPL) has antiviral capabilities against influenza A virus (IAV) infection by enhancing the type I IFN innate immune response. However, it had yet to be confirmed with a more modern influenza virus. To assess this, we infected either wild type or SPL-deficient cells with pandemic influenza A/CA/04/09 IAV (pH1N1). In support of our lab's previous findings, SPL-deficient cells had greater amounts of viral protein expression (Figure 7A) and viral titers (Figure 7B) during infection compared to the wild type control cells. While it is known that IKK ϵ , but not TBK1, is required for SPL-mediated antiviral activity, it remained yet to be determined if SPL alone could alter IRF3-mediated induction of type I IFNs. To determine this, relative levels of *IFN- α* and *IFN- β* mRNA molecules were measured using reverse transcription followed by qPCR. As expected, transient overexpression of IRF3 led to greater expression of *IFN- α* and *IFN- β* mRNA, and transient overexpression of both IRF3 and SPL did not lead to a distinct change in relative *IFN* mRNA expression (Figure 8A and 8B). This shows that increased SPL and IRF3 alone does not alter IRF3-mediated induction of type I IFN, which implicates the necessity of IKK ϵ for SPL antiviral activity.

To investigate if SPL had antiviral activity in a more relevant cell line, SPL was either overexpressed (Figure 9A) or knocked down (Figure 9B) in primary human tracheal epithelial cells (HTEpCs) using lentiviral transduction, then infected with IAV. As previously observed in other cell lines, SPL overexpression resulted in reduced IAV replication, and SPL knockdown led to increased IAV replication.

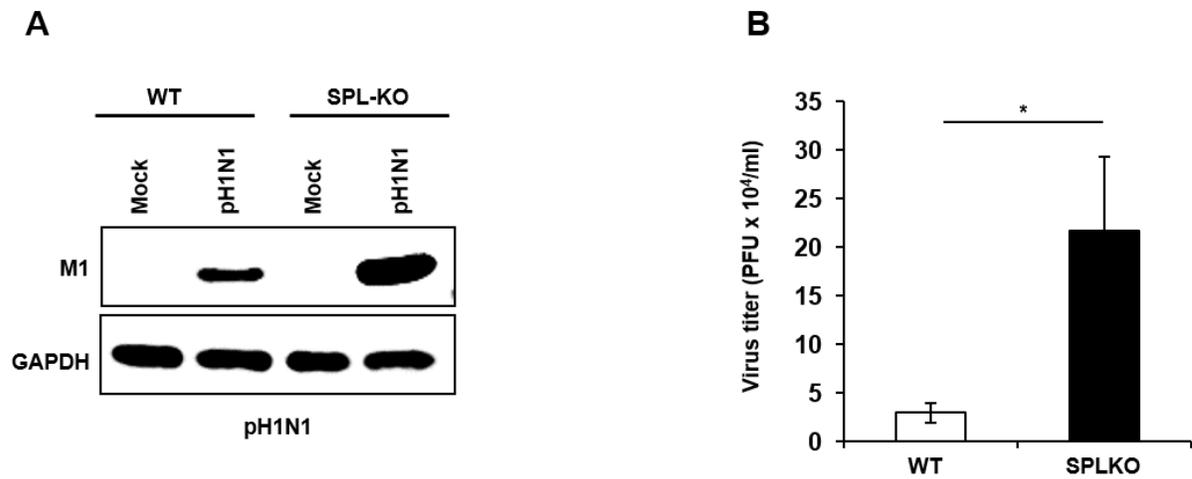


Figure 7. SPL inhibits pH1N1 IAV replication. (A) WT or SPL-KO cells were infected with pandemic influenza A/CA/04/09 (pH1N1) virus at an MOI of 1 and harvested at 24 hpi. Viral protein levels were assessed using western blotting. **(B)** WT or SPL-KO cells were infected with pH1N1 IAV at an MOI of 0.01. Viral supernatants were collected 48 hpi, and titers were measured using plaque assay. Data represent mean values \pm SD (n = 3 per group; *, p < 0.05).

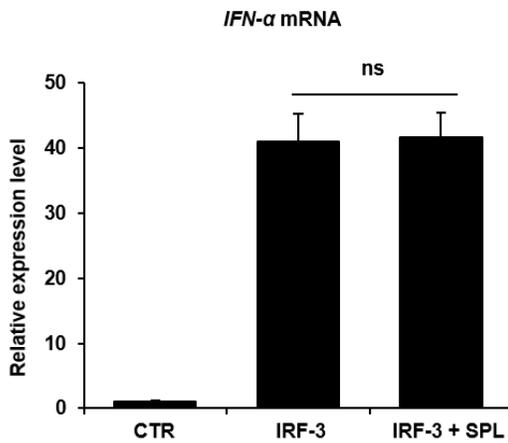
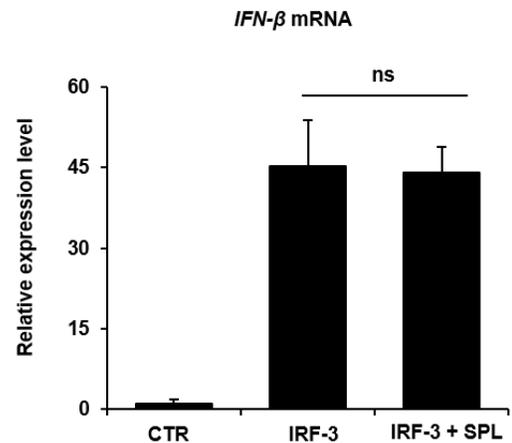
A**B**

Figure 8. SPL does not alter the IRF3-mediated induction of type I IFNs. 293T cells were co-transfected with empty vector plasmid (CTR) or plasmids encoding IRF-3 and SPL, and cells were harvested 1 day post transfection. The relative mRNA levels of *IFN-α* (A) and *IFN-β* (B) were analyzed by real-time qPCR. Data represent mean values \pm SD (n = 3 per group; *, ns = not significant).

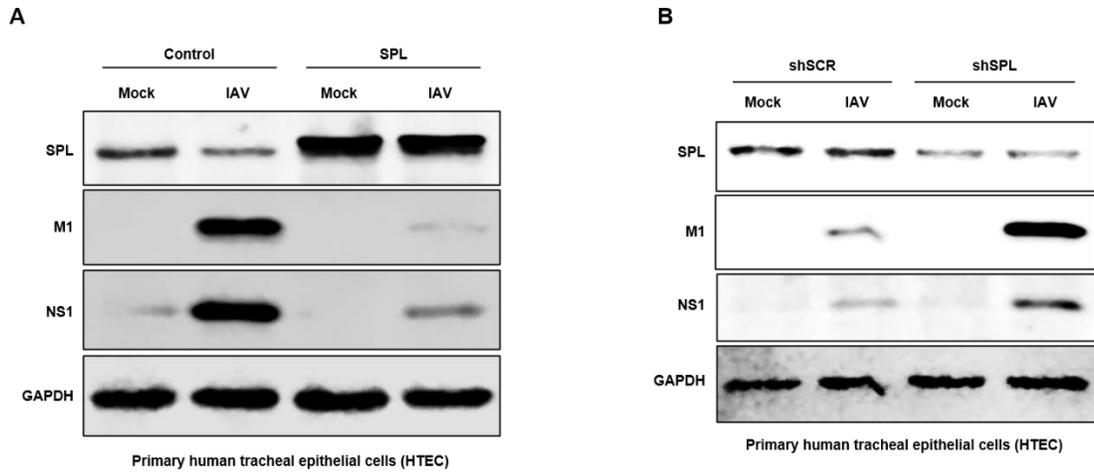


Figure 9. SPL inhibits IAV replication in primary human tracheal epithelial cells. (A) Primary human tracheal epithelial cells (HTEpCs) from donors were transduced with lentivirus that overexpressed SPL during dox treatment. “Control” was not treated with dox, and “SPL” was treated with dox to overexpress SPL. Transduced cells were infected with IAV, then viral protein levels were assessed using western blotting **(B)** HTEpCs were transduced with lentivirus that either downregulated SPL with short hairpin RNA (shSPL), or a scrambled control (shSCR). Transduced cells were infected with IAV, then viral protein levels were assessed using western blotting.

While we had assessed the ability of SPL to reduce IAV H1N1 replication, we had yet to determine if SPL could inhibit replication of other influenza viruses. To determine if the anti-influenza activity of SPL was limited to IAV H1N1 infection, SPL knockout cells or control wild type cells were infected with either IAV A/WSN/1933 (H1N1), influenza A/Hong Kong/8/68 (H3N2), or influenza B/Lee/40 virus (IBV), and viral protein levels were assessed using western blot. Compared to the infected wild type cells, viral protein levels were increased in the SPL knockout cells when infected by any of the tested influenza viruses (Figure 10A, 10B, and 10C). To investigate if SPL could have antiviral activity against other respiratory RNA viruses, we next tested if SPL could affect measles virus (MV) replication. Transient SPL overexpression resulted in decreased MV protein expression (data not shown). MV protein expression was higher in SPL knockout cells compared to infected wild type cells (Figure 11). Based on these results, we can conclude that SPL has antiviral capabilities against influenza viruses as well as measles virus.

IAV negatively regulates SPL protein levels

While the antiviral effects of SPL are known, it had yet to be determined if SPL levels could change during IAV infection, either as a response by the host to infection or as a result of the viral infection itself. To investigate if SPL levels change during IAV infection, SPL protein levels were assessed by western blotting at 2, 4, 6, 12, and 24 hours post infection (hpi) with pH1N1 in A549 cells. Following infection, SPL protein levels decreased progressively over time as viral protein levels, represented by IAV nonstructural protein 1 (NS1), increased (Figure 12A). Furthermore, HTEpCs were infected with either influenza A/Puerto Rico/8/34 (H1N1) (Figure 12B) or pH1N1 IAV (Figure 12C), and SPL protein levels were assessed by western blotting. Similar to infection in A549 cells, SPL levels decreased in the IAV-infected HTEpCs (Figure 12B and 12C).

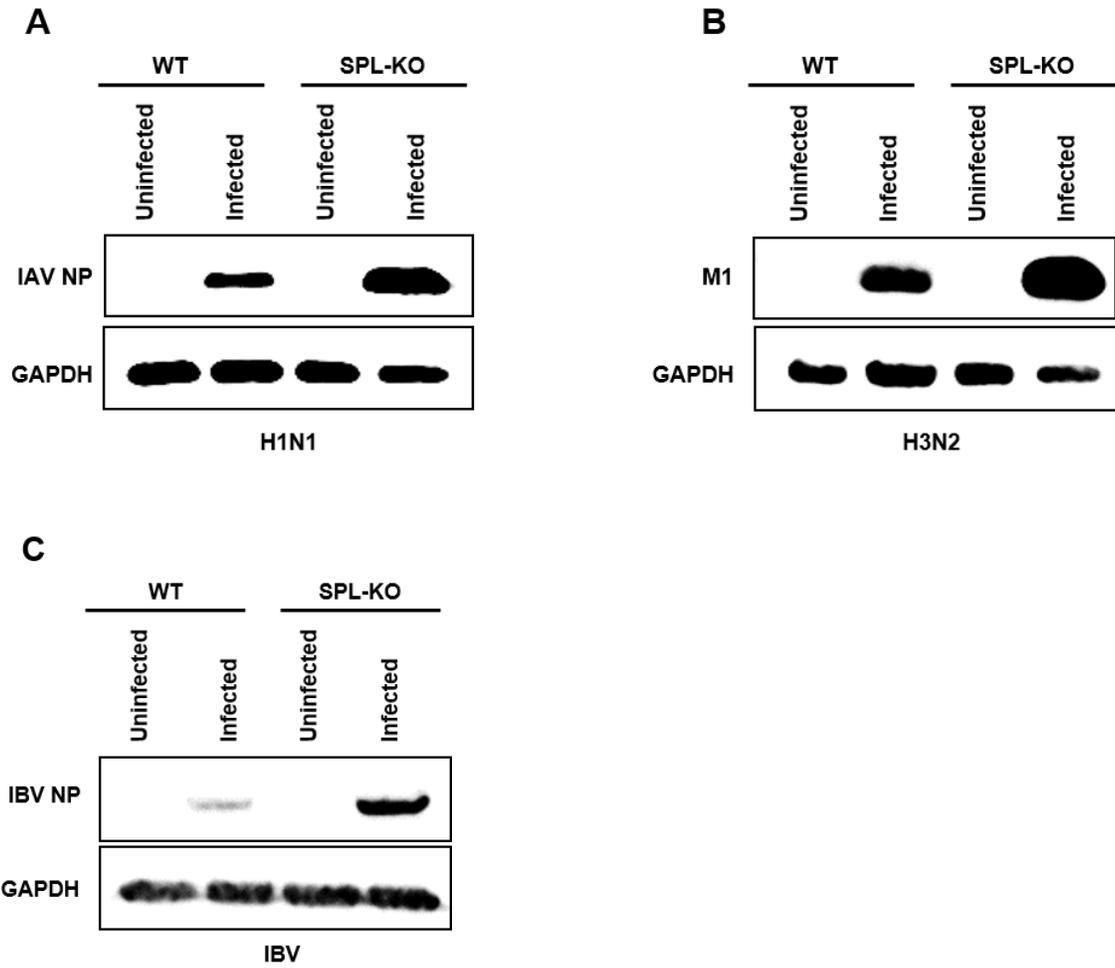


Figure 10. Viral replication is increased in SPL deficient cells during infection with different influenza viruses. WT or SPL-KO cells were infected with an MOI of 1 with either influenza A/Puerto Rico/8/34 (H1N1) virus **(A)**, influenza A/Hong Kong/8/68 (H3N2) virus **(B)**, or influenza B/Lee/40 virus (IBV) **(C)**. Viral protein levels were measured using western blotting.

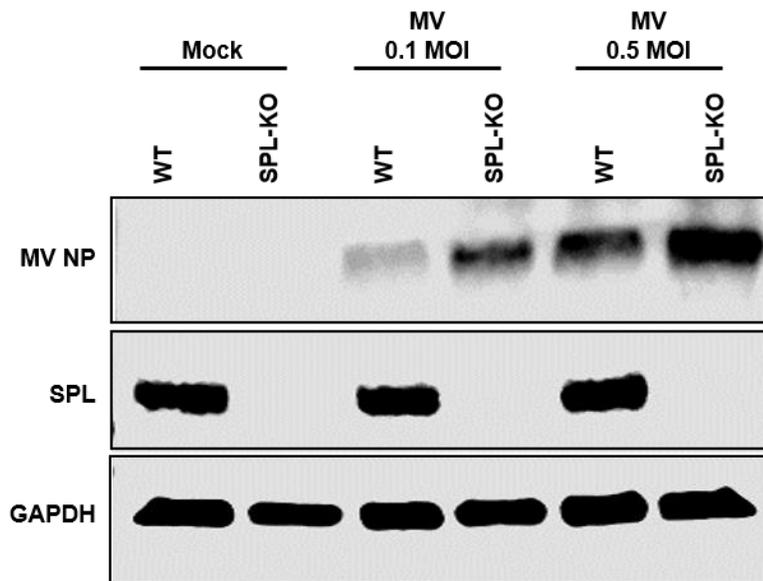


Figure 11. SPL impairs measles virus (MV) replication. WT or SPL-KO cells were infected with either 0.1 MOI or 0.5 MOI Edmonston strain MV, and viral protein levels were assessed using western blotting.

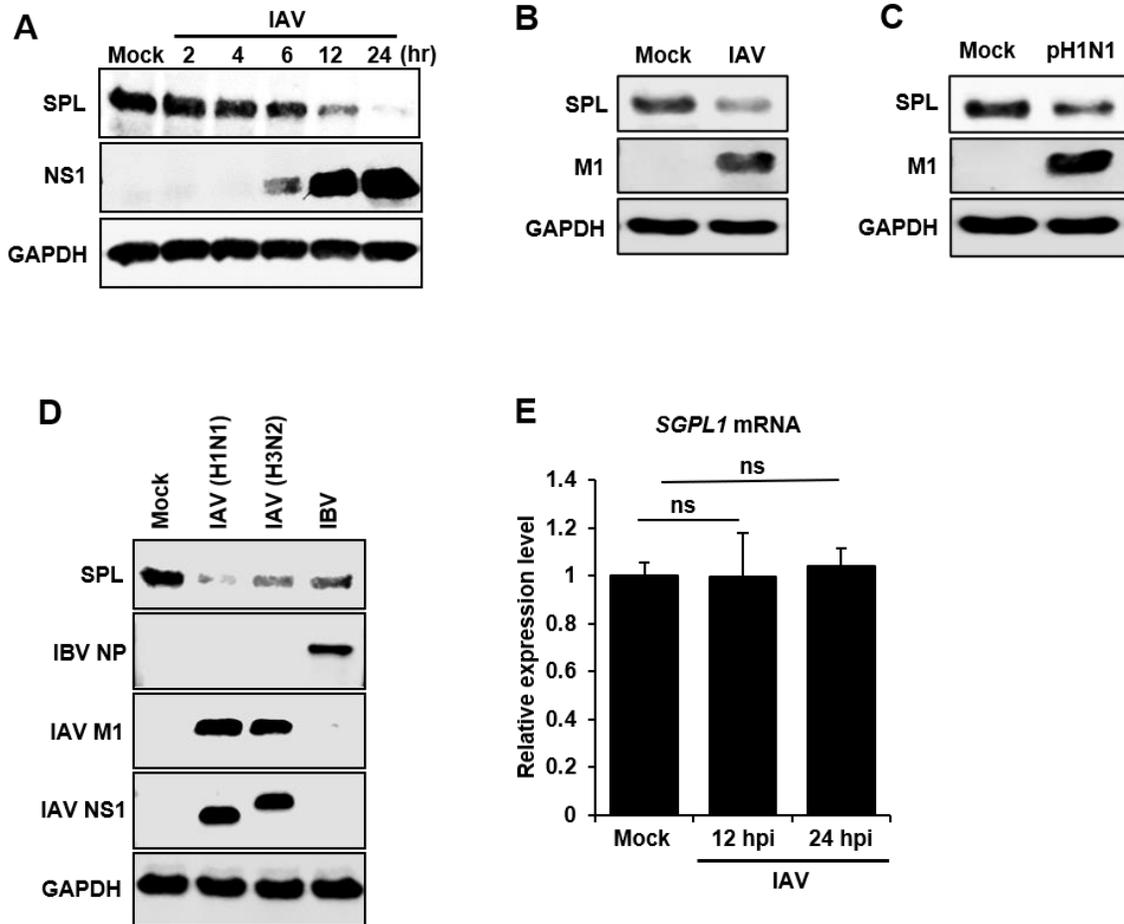


Figure 12. IAV negatively regulates SPL protein levels. (A) A549 cells were infected with influenza A virus (IAV), pandemic influenza A/CA/04/09 (pH1N1) virus, at an MOI of 1. Protein levels of SPL, IAV NS1, and GAPDH were detected respectively at the indicated time points post infection. (B and C) Primary human tracheal epithelial cells (HTEpCs) were infected with IAV, either influenza A/Puerto Rico/8/34 (H1N1) (B) or pH1N1 virus (C), and levels of SPL, M1, and GAPDH were detected. (D) A549 cells were infected for 24 hours with either pH1N1 virus (IAV H1N1) or influenza A/Hong Kong/8/68 virus (IAV H3N2), or influenza B/Lee/40 virus (IBV) (MOI = 1). Levels of SPL, IBV NP, IAV M1, IAV NS1, and GAPDH were detected using western blot analysis. (E) A549 cells were infected with pH1N1 IAV at an MOI of 1 and were harvested at the indicated timepoints. The relative mRNA levels of SGPL1 were analyzed using real time qPCR. Data represent mean \pm SEM. Pairwise comparison was performed using one-way ANOVA followed by Tukey post hoc ($n = 4$ per group; ns = not significant).

To determine if this reduction of SPL protein was specific to IAV H1N1 infection, A549 cells were infected with either IAV pH1N1, H3N2, or IBV, and SPL protein levels were assessed using western blot analysis. Compared to the uninfected control, SPL protein levels were reduced when cells were infected by any of the tested influenza viruses (Figure 12D). Next, to assess whether this downregulation was occurring at a transcriptional level, SPL mRNA levels in A549 cells were measured using reverse transcription followed by real-time quantitative PCR (qPCR) at 12 hpi and 24 hpi with IAV. Interestingly, IAV infection did not alter SPL mRNA expression significantly at either timepoint (Figure 12E), indicating post-transcriptional downregulation of SPL. Collectively, these data demonstrate that IAV infection negatively regulates SPL levels at the post-transcriptional stage.

IAV infection induces ubiquitination of SPL

To further characterize the effects of IAV infection on SPL, denatured immunoprecipitation (IP) assays were performed. Cells were co-transfected with FLAG-SPL and HA-tagged-ubiquitin expressing plasmids and were subsequently infected with IAV. Denatured IP was performed on the IAV infected and uninfected control cells, and relative ubiquitination of FLAG-SPL was subsequently assessed using western blotting. IAV infection strongly increased ubiquitination of FLAG-SPL compared to the uninfected controls (Figure 13A). Consistent with these findings, we observed enhanced endogenous ubiquitination of FLAG-SPL upon IAV infection in the absence of ubiquitin-overexpressing plasmid (Figure 13B). These results led us to conclude that SPL ubiquitination is induced during IAV infection, which could lead to the previously observed decrease in SPL protein levels.

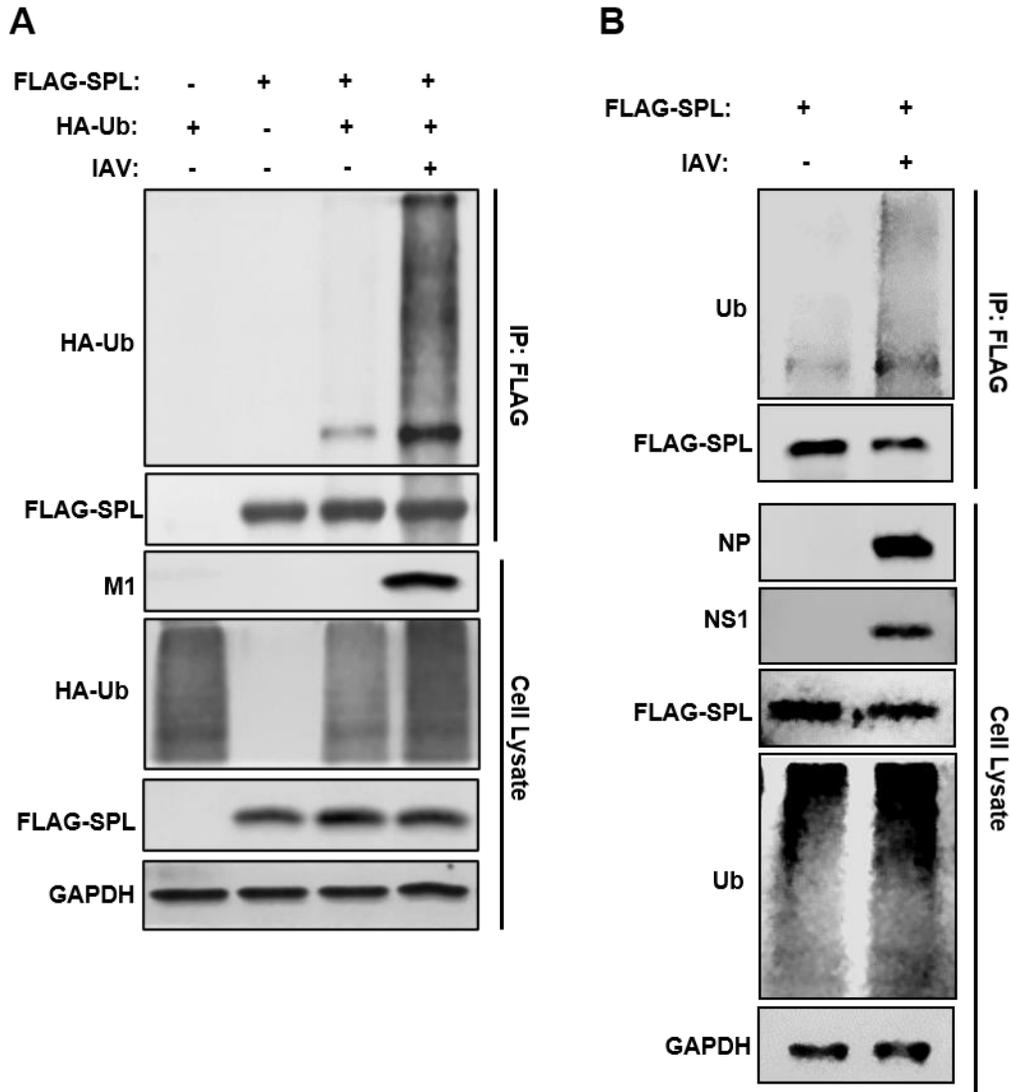


Figure 13. IAV infection induces ubiquitination of SPL. **(A)** HEK293 cells were co-transfected with plasmid DNA encoding HA-tagged ubiquitin (HA-Ub) and FLAG-tagged SPL (FLAG-SPL) and at 24 hours post transfection were subsequently infected with IAV (MOI = 1). Denatured IP experiments were performed at 1 day post infection (dpi) to detect the ubiquitination of SPL. **(B)** HEK293 cells were transfected with FLAG-SPL and were infected with IAV (MOI = 1) 24 hours post-transfection. Denatured IP was performed, and endogenous ubiquitin of FLAG-SPL was detected.

IAV NS1 protein is responsible for ubiquitination and downregulation of SPL

To determine which component of IAV infection induces the ubiquitination and subsequent downregulation of SPL, we looked at the effects of both host and viral components of infection. First, because SPL is a newly identified pro-IFN factor, we assessed the effect of the type I IFN response on SPL protein level in cells. To this end, western blotting was performed after induction of the type I IFN innate immune response with human IFN- α or IAV viral RNA (vRNA). SPL protein levels remained relatively unchanged after treatment with human IFN- α (Figure 14A) or IAV vRNA (Figure 14B) compared to the uninduced controls, indicating that SPL downregulation was not a result of the type I IFN innate immune response to IAV infection. Because IAV nonstructural protein 1 (NS1) is known to regulate host type I IFN responses (Kochs et al., 2007; Krug, 2015; Muñoz-Moreno et al., 2020), we next tested if NS1 could be responsible for the ubiquitination of SPL. Viral proteins were assessed for their abilities to ubiquitinate SPL by transiently overexpressing either NS1 or neuraminidase (NA) as a negative viral protein control and performing denatured IP followed by western blotting to measure relative ubiquitination of FLAG-SPL. Relative to the controls, NS1, but not NA, was determined to induce ubiquitination of SPL (Figure 15A and 15B). Hemagglutinin (HA) was also tested and found to not induce SPL ubiquitination (data not shown), and NS1 was also found to not induce IFNAR1 ubiquitination (data not shown). To elucidate which type of ubiquitination SPL could be undergoing during NS1 expression, K48 or K63-linked ubiquitin was transiently overexpressed in addition to FLAG-SPL and IAV NS1, and denatured IP was performed. Interestingly, SPL was found to undergo both K48 and K63-linked ubiquitination during NS1 expression (Figure 16).

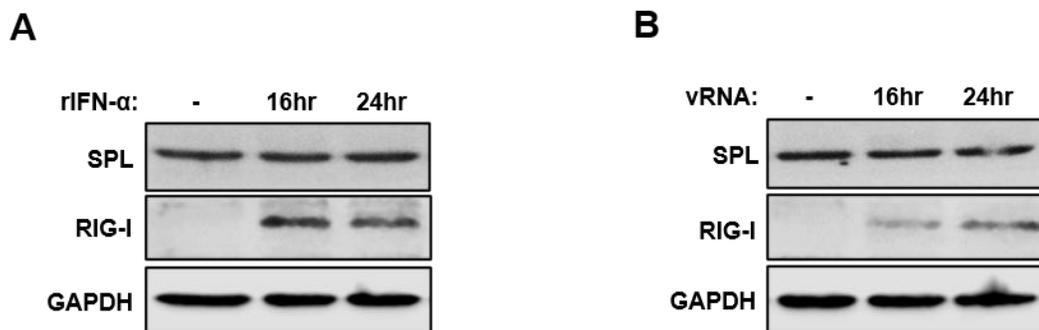


Figure 14. SPL is not downregulated by the type I IFN innate immune response. The type I IFN innate immune response was stimulated in HEK293 cells either by treatment with 1000 U/mL recombinant IFN- α (rIFN- α) **(A)** or by transfection with 2.5 μ g/mL IAV RNA (vRNA) or RNA isolated from uninfected cells (-) **(B)**, and levels of SPL, RIG-I, and GAPDH were measured using western blotting.

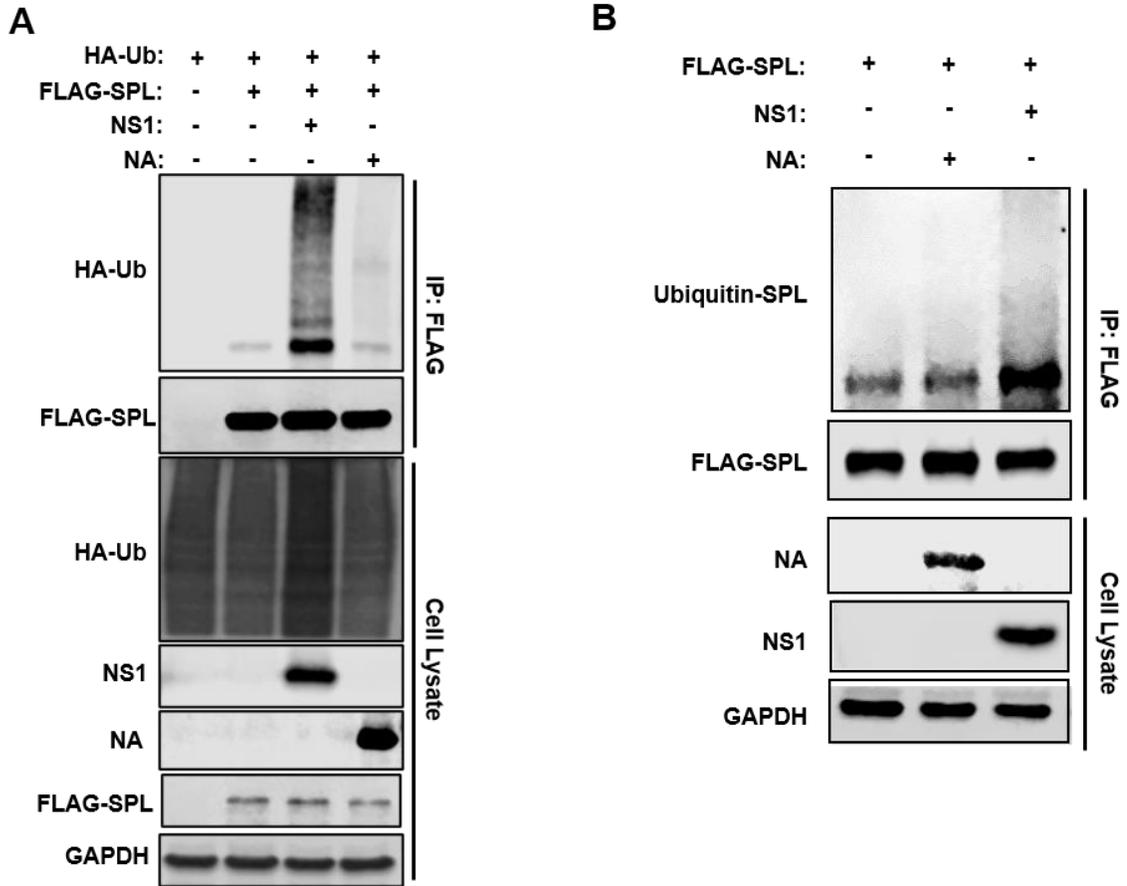


Figure 15. IAV NS1 induces SPL ubiquitination. (A) HEK293 cells were co-transfected with plasmids expressing HA-tagged ubiquitin (HA-Ub) and FLAG-tagged SPL (FLAG-SPL), together with plasmids expressing viral NS1 or NA as indicated. Denatured IP experiments were performed to detect the ubiquitination of SPL. **(B)** HEK293 cells were co-transfected with plasmids expressing FLAG-SPL, NS1, or NA. Denatured IP and subsequent western blotting was performed to determine endogenous ubiquitination of SPL.

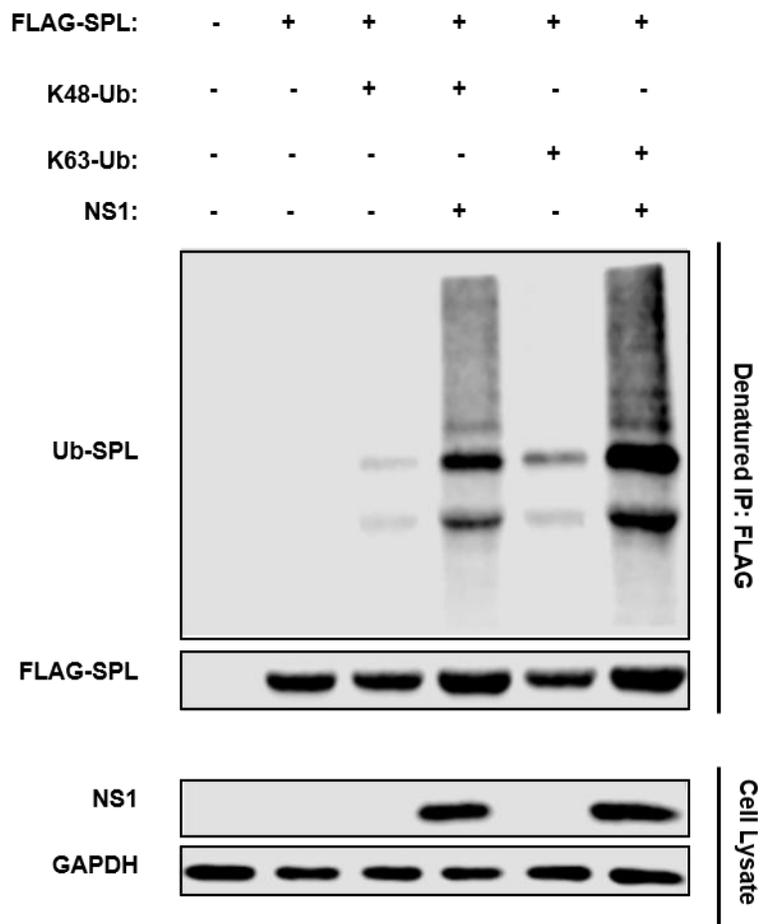


Figure 16. IAV NS1 induces both K48 and K63-linked ubiquitination of SPL. HEK293 cells were co-transfected with plasmids expressing FLAG-tagged SPL, IAV NS1, and either HA-tagged K48 or K63-linked ubiquitin. Denatured IP and subsequent western blotting were performed, and levels of K48 and K63-linked ubiquitination of FLAG-SPL were assessed.

Furthermore, NS1 was found to directly instigate SPL downregulation (Figure 17A). To eliminate the possibility of protein translation affecting total SPL levels, cycloheximide (CHX) treatment was used to stop new protein synthesis in conjunction with NS1 overexpression; with these conditions, we observed a more dramatic reduction of SPL levels in the presence of NS1 (Figure 17A). Typically, post-transcriptional downregulation of proteins can be attributed to the proteasomal and/or lysosomal degradation pathway. In order to assess whether IAV NS1 was downregulating SPL via these pathways, we used proteasomal inhibitor MG132 or lysosomal inhibitor NH₄Cl in conjunction with cycloheximide treatment. Treatment with MG132 or NH₄Cl prevented the downregulation of SPL, demonstrating that NS1-mediated SPL downregulation utilizes both the proteasomal and lysosomal degradation pathways (Figure 17B).

SPL degradation during IAV infection is dependent upon NS1

To confirm that IAV NS1 is a pivotal factor necessary for triggering SPL ubiquitination and downregulation during IAV infection, we next tested the effect of infection with NS1-deficient (Δ NS1) IAV (IAV- Δ NS1) on SPL ubiquitination and downregulation. Denatured IP of transiently overexpressed FLAG-SPL and subsequent western blotting were used to assess SPL ubiquitination during infection with either IAV or IAV- Δ NS1. Infecting with increasing MOIs of IAV likewise increased ubiquitination of SPL. However, infecting with increasing MOIs of IAV- Δ NS1 at even higher MOIs than used in wild type infection did not show discernible ubiquitination of SPL compared to the uninfected control (Figure 18A). Next, to assess whether NS1 was important for endogenous SPL protein downregulation during infection, cellular SPL protein levels were measured by western blotting with either IAV (MOI = 1) or IAV- Δ NS1 (MOI = 3). While IAV

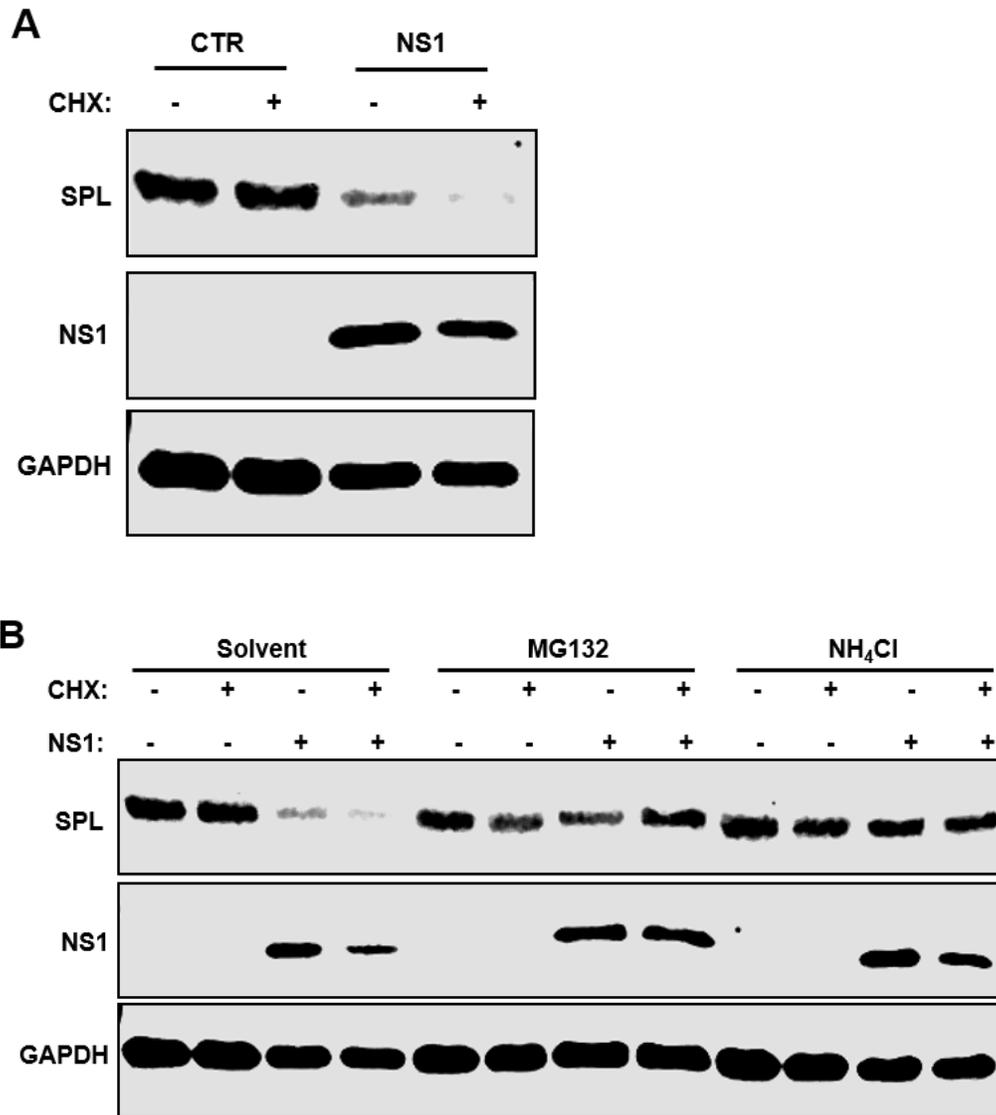


Figure 17. Transient IAV NS1 overexpression leads to SPL degradation. (A) A549 cells were transfected with IAV NS1 expressing plasmid for 16 hours and were then subsequently treated for 6 hours with 30 ug/mL cycloheximide (CHX) or DMSO (Solvent) for the duration of the 6-hour treatment to inhibit protein synthesis. SPL downregulation was then assessed using western blotting. **(B)** A549 cells were transfected with IAV NS1 expressing plasmid for 16 hours and were then subsequently treated for 6 hours with DMSO (Solvent), 20 μ M MG132, or 20 mM NH₄Cl. Samples were also treated with 30 μ g/mL cycloheximide (CHX) or DMSO (Solvent) for the duration of the 6-hour treatment to inhibit protein synthesis. Western blotting was then used to detect levels of SPL, NS1, and GAPDH.

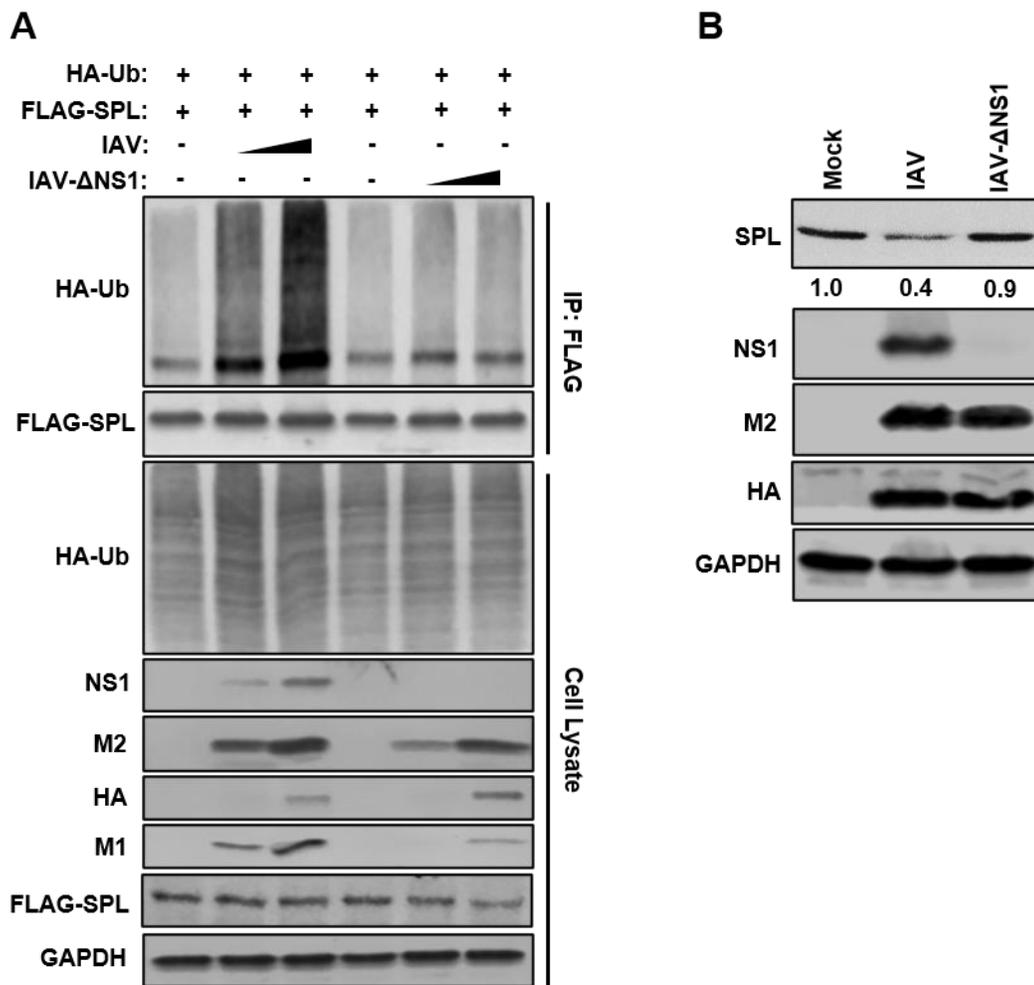


Figure 18. NS1-deficient IAV fails to induce the ubiquitination and degradation of SPL. (A) HEK293 cells were co-transfected with HA-Ub and FLAG-SPL. At 1 dpi, cells were infected with IAV or NS1-deficient IAV (IAV-ΔNS1) at MOIs of 0.1 or 1. Denatured IP experiments were performed to detect the ubiquitination of SPL. (B) HEK293 cells were infected with IAV or IAV-ΔNS1 at 1 MOI or 3 MOI respectively. The levels of SPL, NS1, M2, HA, and GAPDH were analyzed with western blotting at 1 dpi. The relative intensity of each band of SPL was determined using densitometry based on the GAPDH level of the sample and is depicted below each blot. The relative level of SPL from the mock-infected sample was set at 1.0.

infection led to endogenous SPL downregulation, IAV- Δ NS1 infection did not lead to significant SPL downregulation despite being infected with a higher MOI (Figure 18B). Collectively, these data demonstrate that IAV NS1 is essential for IAV-mediated ubiquitination and downregulation of SPL.

NS1 inhibits SPL-mediated activation of IKK ϵ and production of type I IFNs

Our previous study revealed a new role for SPL, in which SPL interacts with IKK ϵ , increasing IKK ϵ activation (phosphorylation of IKK ϵ) as well as subsequent production of IFNs and ISGs. However, it remained to be determined whether IAV could inhibit this SPL-mediated type I IFN innate immune response. To this end, western blotting was performed to assess protein levels of phosphorylated IKK ϵ (pIKK ϵ) 16 hours post transfection with plasmids expressing IKK ϵ , SPL, and NS1. As we have previously observed, transiently overexpressed IKK ϵ increased pIKK ϵ due to autophosphorylation, and transient overexpression of both SPL and IKK ϵ resulted in a larger increase of pIKK ϵ than IKK ϵ overexpression alone. However, transient overexpression of NS1, SPL, and IKK ϵ decreased pIKK ϵ levels compared to the controls (Figure 19A). While NS1 decreased pIKK ϵ levels, it did not disrupt the association between IKK ϵ and SPL (Figure 19B). To further assess whether the type I IFN response was subsequently impacted by those conditions, relative levels of IFN- α and IFN- β mRNA molecules were measured using reverse transcription followed by qPCR. Similar to our previous findings, IKK ϵ autophosphorylation-induced expression of IFN- α and IFN- β mRNAs was greatly increased by SPL transient overexpression. However, the expression of IFN- α and IFN- β mRNA transcripts was greatly reduced when NS1 was transiently expressed in addition to IKK ϵ and SPL (Figure 20A and 20B). ISGs are the ultimate antiviral effectors of the type I IFN innate immune response to IAV infection. In order to determine whether the NS1-

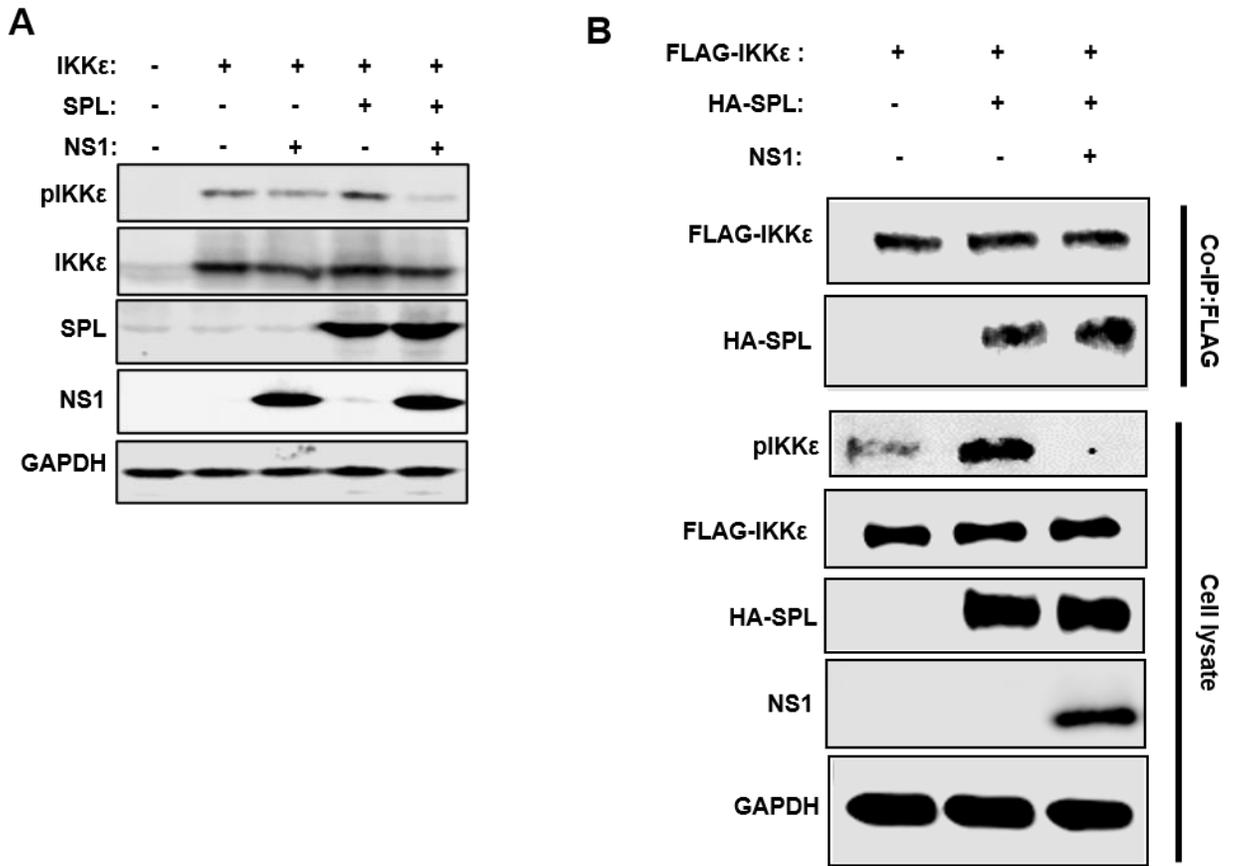


Figure 19. NS1 expression reduces SPL-mediated IKK ϵ activation without affecting their interaction. 293T cells were co-transfected with the indicated plasmids, and cells were harvested 1 day post transfection. The levels of phosphorylated IKK ϵ (pIKK ϵ), IKK ϵ , SPL, NS1, and GAPDH were analyzed by western blotting to assess levels of pIKK ϵ (**A**), and co-IP was used to assess the interaction between SPL and IKK ϵ (**B**).

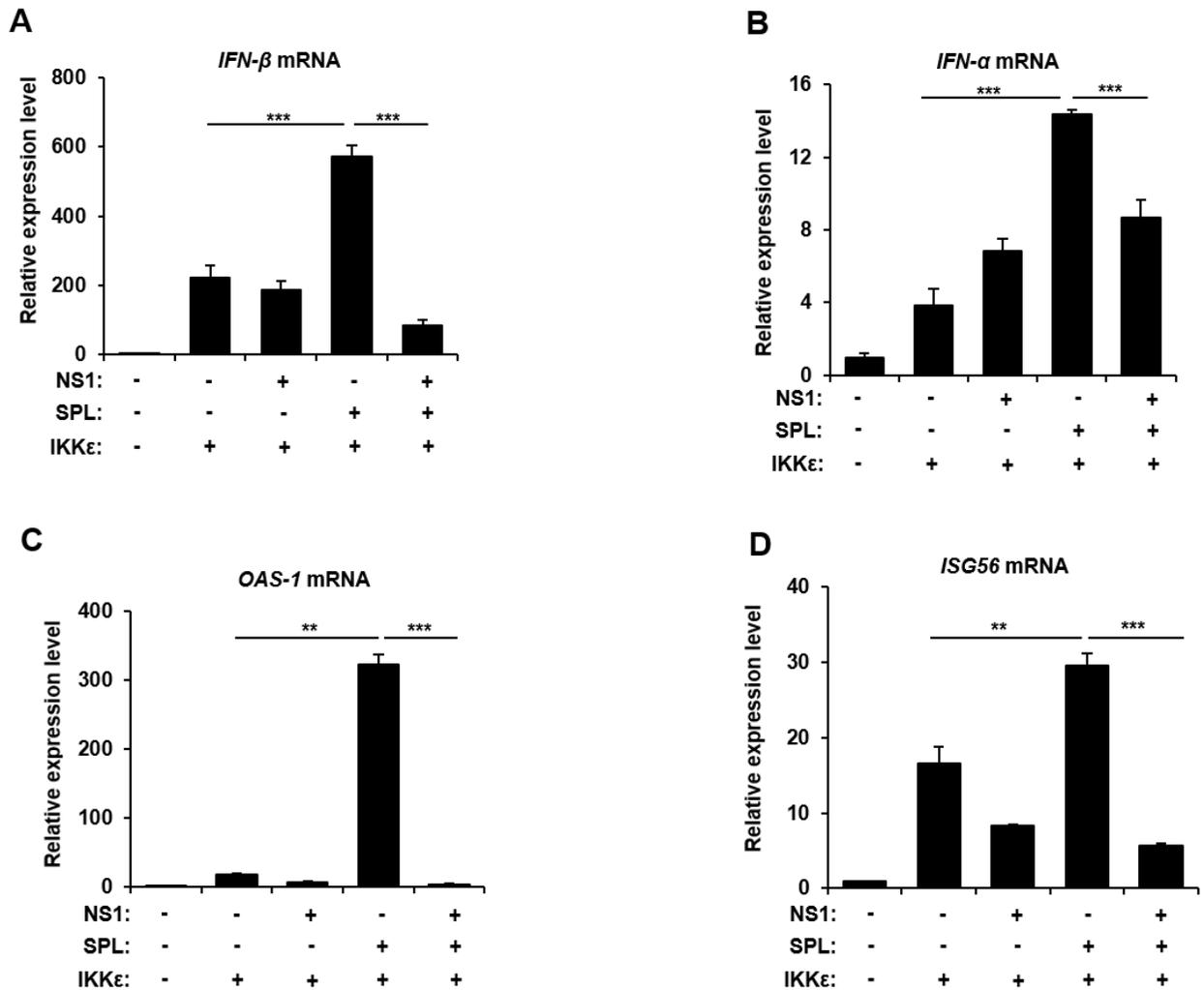


Figure 20. NS1 inhibits the function of SPL in promoting the type I IFN innate immune response. 293T cells were co-transfected with the indicated plasmids, and cells were harvested 1 day post transfection. The relative mRNA levels of IFN- β (A), IFN- α (B), OAS-1 (C), and ISG56 (D) were analyzed by real-time qPCR. Data represent mean \pm SEM. One-way ANOVA with Tukey's range test (A, B, and D) or Kruskal-Wallis with Conover-Iman test (C) were used for pairwise comparisons. (n = 3 per group; **, $P \leq 0.01$; ***, $P \leq 0.001$).

mediated inhibition of the SPL-enhanced type I IFN innate immune response impacts the production of ISGs, OAS-1 and ISG56 mRNA transcript levels were subsequently measured with reverse transcription and qPCR. Strikingly, OAS-1 transcription was increased by over 300-fold in conditions where both SPL and IKK ϵ were transiently overexpressed, compared to a 20-fold increase during transient overexpression of IKK ϵ alone. However, the enhanced OAS-1 transcription seen in those conditions was mitigated by the addition of transiently overexpressed NS1 (Figure 20C). Similarly, ISG56 transcription was increased in conditions where both SPL and IKK ϵ were transiently overexpressed, but the enhanced ISG56 transcription was diminished by also overexpressing NS1 (Figure 20D). Collectively, these data suggest that IAV NS1 obstructs the pro-IFN function of SPL, enabling IAV to evade the SPL-mediated type I IFN innate immune response during infection.

Implications of SYVN1 in NS1-mediated SPL downregulation

To predict which E3 ubiquitin ligases were most likely to ubiquitinate SPL, we used a bioinformatics tool called UbiBrowser (Li et al., 2017), which can investigate and predict E3 ubiquitin ligase-substrate interactions. From this analysis, Synoviolin1 (SYVN1) was predicted to be an E3 ubiquitin ligase that interacted with SPL. SYVN1 had the highest predicted confidence score and likelihood ratio of any of the other candidates, with 5 inferred E3 recognizing motif sites to the SPL amino acid sequence. SYVN1 is a RING-finger E3 ubiquitin ligase which resides in the membrane of the endoplasmic reticulum (ER) of cells and is known for ER-associated degradation of unfolded proteins. Notably, SPL is also localized in the ER membrane. If SYVN1 is involved in IAV NS1-induced ubiquitination of SPL, it would likely enhance IAV replication. To test this, we used either chemical inhibitor of SYVN1, LS-102 (Figure 21A), or siRNA-mediated knockdown of SYVN1 (Figure 21B) during infection with IAV, and viral proteins were measured using

western blotting. Both chemical inhibition and siRNA knockdown of SYVN1 resulted in decreased viral protein levels, indicating SYVN1 enhances IAV replication. Next, to determine if SYVN1 could have involvement with IAV NS1-mediated ubiquitination of SPL, we performed co-IP for FLAG-SPL with or without transiently overexpressed IAV NS1 and observed SYVN1 levels using western blotting. Interestingly, SYVN1 was found to interact with SPL when IAV NS1 was transiently overexpressed (Figure 22), which could indicate involvement with NS1-mediated ubiquitination and degradation of SPL. Preliminary data also indicate that SYVN1 could mediate ubiquitination of SPL (data not shown) as well as downregulation of SPL during IAV infection (data not shown). Collectively, these data indicate that SYVN1 could be involved in SPL ubiquitination.

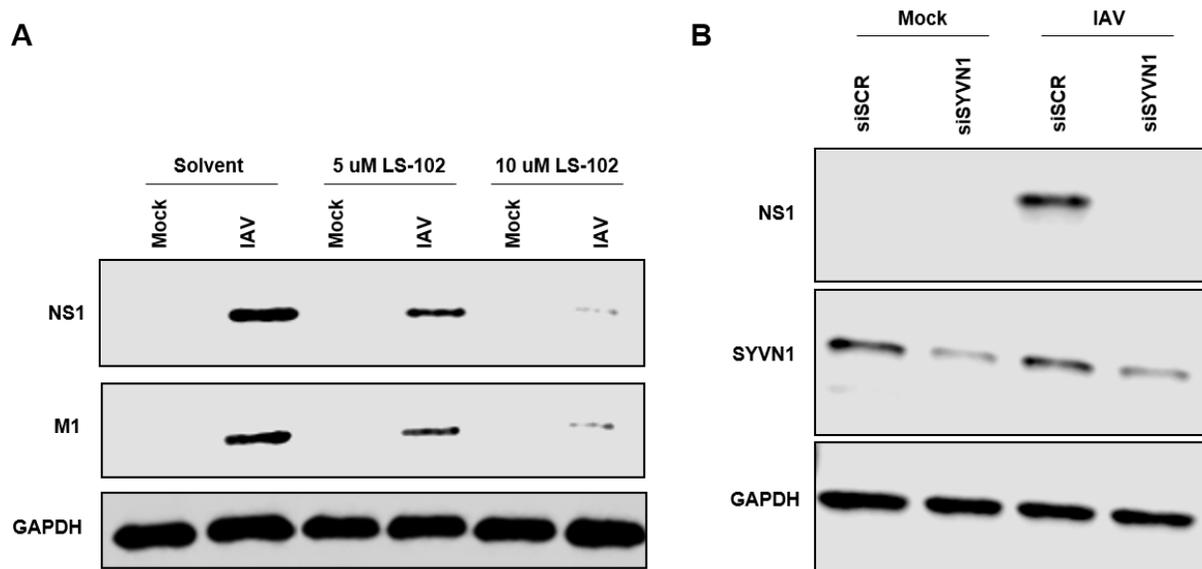


Figure 21. SYVN1 positively regulates IAV replication. (A) A549 cells were treated with either DMSO (Solvent) or LS-102 at the indicated concentration and were subject to either mock or IAV infection (MOI = 5). The cells were harvested at 12 hpi and western blotting was used to determine viral protein levels. **(B)** A549 cells were transfected with control siRNA (SCR) or SYVN1 siRNA and were then either mock or IAV infected (MOI = 0.01). The cells were harvested at 9 hpi, and western blotting was performed to assess viral protein levels.

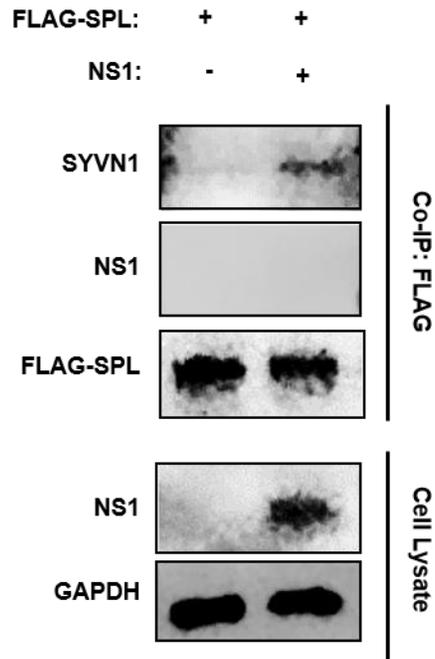


Figure 22. SYVN1 interacts with SPL upon IAV NS1 expression. HEK293 cells were co-transfected with plasmids expressing FLAG-SPL and IAV NS1. Cells were harvested 1 day post transfection, and co-immunoprecipitation was performed to determine the potential interaction between FLAG-SPL, IAV NS1, and SYVN1.

II-E. Discussion

Our study further clarifies the antiviral function of SPL as well as demonstrates that IAV NS1 induces the ubiquitination and subsequent downregulation of host SPL. This downregulation allows IAV to subvert the SPL-mediated type I IFN innate immune response to IAV infection (Figure 23). Preliminary data indicate that SYVN1 could have involvement with IAV-mediated SPL downregulation.

While SPL is known to have many functions in the host, our lab was the first to show its antiviral function in the type I IFN innate immune response. Interestingly, we observed that SPL had antiviral activity against IAV H1N1 and H3N2 strains, IBV, as well as measles virus, which are all single-stranded negative-sense RNA viruses that infect the respiratory tract. This indicates SPL could have antiviral activity against several respiratory RNA viruses. It could be interesting to determine if SPL has antiviral effects against other viruses as well.

The IAV NS1 protein has several functions during influenza virus replication (Rosário-Ferreira et al., 2020). NS1 is largely responsible for hindering host IFN antiviral responses, ensuring more effective IAV replication (Kochs et al., 2007; Krug, 2015; Muñoz-Moreno et al., 2020). NS1 was reported to directly limit the antiviral state by inhibiting OAS-1 activation, outcompeting OAS for interaction with double-stranded RNA (dsRNA) (Min and Krug, 2006; Wang et al., 1999). NS1 binding to dsRNA also prevents the activation of dsRNA-activated protein kinase (PKR) (Bergmann et al., 2000). Since the experimental conditions of Figure 20C did not use dsRNA or IAV infection, NS1's impairment of the SPL-mediated pro-IFN function is not due to this previously known mechanism. Additionally, NS1 inhibits RIG-I activation by blocking the TRIM25 ubiquitin E3 ligase-mediated K63-linked ubiquitination of RIG-I (Gack et al., 2009), by binding to RIPLET and preventing its ability to ubiquitinate and activate RIG-I

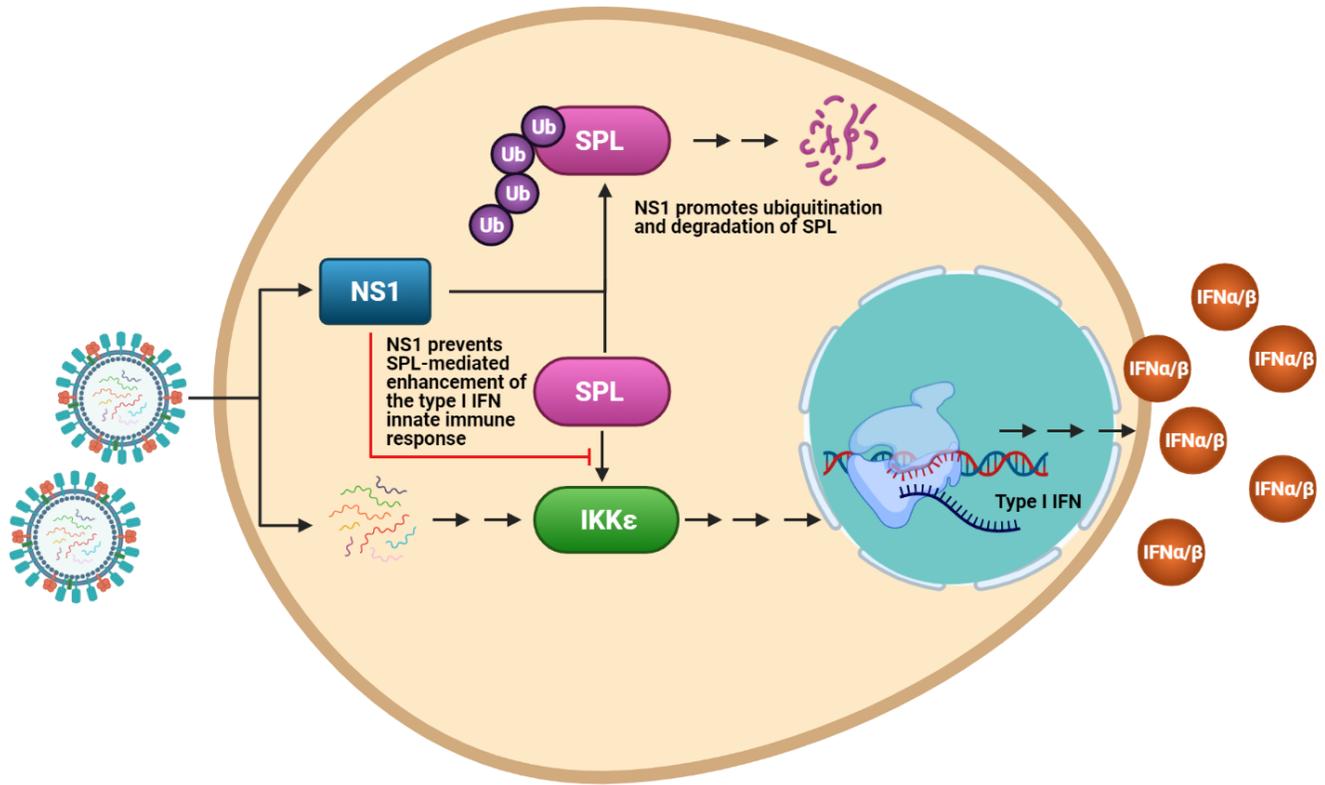


Figure 23. Influenza A virus NS1 induces degradation of sphingosine 1-phosphate lyase to dampen the host innate immune response. SPL functions as a positive regulator of IKK ϵ to enable the induction of a robust type I IFN response. However, IAV NS1 protein dampens the type I IFN response by downregulating SPL, allowing IAV to replicate more efficiently.

(Rajsbaum et al., 2012), or by directly binding to RIG-I to prevent downstream activation of IRF3 (Mibayashi et al., 2007). Our findings of IAV NS1 preventing SPL from promoting IKK ϵ phosphorylation is a novel method utilized by IAV NS1 to dampen host innate immunity.

The underlying mechanism of NS1 triggering the ubiquitination and degradation of SPL remains unknown. It is possible that another host protein, such as a host ubiquitin ligase, is somehow activated by NS1 to mediate SPL's ubiquitination and subsequent destruction. IAV infection has been previously shown to direct host ubiquitin ligase NEDD4 to target interferon-induced transmembrane protein 3 (IFITM3), a protein involved in the immune defense against several RNA viruses, for ubiquitination and subsequent lysosomal degradation (Chesarino et al., 2015). Investigating how IAV NS1 induces the ubiquitination and degradation of SPL is an exciting avenue of research which remains to be explored.

Viruses must counteract host antiviral activities, especially IFN production and the activity of ISGs, in order to replicate efficiently. We have shown that IAV mediates the ubiquitination and subsequent proteasomal and lysosomal degradation of host SPL. We have previously found that IAV infection induces ubiquitination and subsequent degradation of type I IFN receptor 1 (IFNAR1) in order to dampen the type I IFN innate immune response (Xia et al., 2016). Comparable to our findings, IAV infection was found to mediate both proteasomal and lysosomal degradation of IFNAR1. A previous study determined that OTUB1, which can regulate RIG-I dependent innate immune signaling, is targeted for proteasomal degradation by IAV NS1 (Jahan et al., 2020). IAV NS1 has also been previously shown to mediate the ubiquitination and lysosomal degradation of eukaryotic translation initiation factor 4B (eIF4B) protein (Wang et al., 2014b). While eIF4B is a key component in regulating the initiation of mRNA translation, it could also regulate

the IFN-induced expression of IFITM3. Indeed, several viruses are known to hijack the host ubiquitin system in order to subvert or evade IFN responses. For example, human rotavirus nonstructural protein NSP1 stifles IFN expression by inducing ubiquitination-dependent degradation of interferon regulatory factor 3 (IRF3), a key component in the induction of the IFN α/β cascade (Barro and Patton, 2005). It is likely that IAV also utilizes the host ubiquitin system in order to induce degradation of SPL and further evade host IFN responses.

SPL was found to undergo both K48 and K63-linked ubiquitination upon the expression of IAV NS1. We were surprised to see both, as K48-linked ubiquitination is primarily known to be associated with protein degradation by the proteasome, while K63-linked ubiquitination is commonly associated with proteasome-independent pathways. However, ubiquitin is capable of sending diverse signals. For example, both K48 and K63-linked ubiquitination have been shown to be capable of signaling lysosomal degradation (Zhang et al., 2013). Additionally, K63 ubiquitin chains have also been shown to facilitate assembly of K48/K63 branched ubiquitin chains through recruitment of ubiquitin ligases assembling K48 ubiquitin chains, which facilitate proteasomal degradation (Ohtake et al., 2018). Thus, observing both K48 and K63-linked ubiquitination of SPL during NS1 expression is consistent with our findings of SPL undergoing both proteasomal and lysosomal degradation.

Sphingolipid-metabolizing enzymes have been shown to affect the IAV replication cycle. Sphingosine kinase 1 and 2 (SK1 and SK2) enhance IAV replication in cultured cells, and IAV infection increases the expression and phosphorylation of SK1 and SK2 (Xia et al., 2018). SK1 and SK2 are also known to regulate the replication of other viruses. For example, measles virus (MV) infection elevates SK1 and pSK1 levels (Vijayan et al., 2014). Inhibition of SK impairs MV replication and suppresses NF- κ B activation. These

results, among others, demonstrate that SK impacts viral replication and could be a promising therapeutic target in some instances. Our previous studies have also indicated that SPL has a pro-IFN function upon influenza virus infection (Vijayan et al., 2017). However, here we have shown that IAV NS1 protein counteracts this SPL-mediated enhanced type I IFN response to infection. Determining whether these effects are applicable to other viruses is a promising area of research.

Our studies have indicated that SYVN1 enhances IAV replication. Preliminary data suggests that SYVN1 may be involved with the NS1-mediated degradation of SPL. SYVN1 is an E3 ubiquitin ligase which is involved with unfolded or misfolded protein removal in the ER stress response. SYVN1 resides in the ER membrane. It has an interesting structure, with both the N and C termini residing in the cytoplasm, and is anchored in the ER membrane with 6 transmembrane domains which allow SYVN1 to span the ER membrane more than once (Kaneko et al., 2016). SYVN1 has been shown to be upregulated during the ER stress response (Kaneko et al., 2002), and a previous study in mice showed that IAV infection led to increased *Syvn1* mRNA transcription in the lungs (Singhania et al., 2019). SYVN1 has also been shown to facilitate both proteasomal (Wang et al., 2011) and autophagy-lysosomal degradation (Feng et al., 2017) of the Z variant of protein α -1-antitrypsin.

We observed that expression of IAV NS1 led to an interaction between SYVN1 and SPL that was not present in the control. It has been previously shown in a published study that MAVS and IKK ϵ interact, with MAVS recruiting IKK ϵ to the mitochondria, when both are transiently overexpressed. In the same study, Sendai Virus infection led to MAVS recruiting IKK ϵ to the mitochondria as well as MAVS interaction with IKK ϵ (Paz et al., 2009). Our lab has previously determined using coimmunoprecipitation analysis that MAVS can interact with the SPL-IKK ϵ complex when SPL and IKK ϵ are transiently

overexpressed together (unpublished, data not shown). While SPL may not interact with MAVS directly, they could be present together in a complex. MAVS is a tail-anchored membrane protein. In addition to mitochondrial and peroxisomal membranes, MAVS also localizes to mitochondrial associated membranes (MAMs) (Vazquez and Horner, 2015). MAMs are composed of membrane fragments of both the ER and outer mitochondrial membrane, and they create junctions between the ER and mitochondria. While SPL is known to be localized to the ER membrane, it has also been shown to be localized to MAMs (Aguilar and Saba, 2012; Rolando et al., 2016). A recently published study determined that viral infection during corticosterone-induced cell stress led to mitofusin 2 (MFN2) interacting with MAVS to recruit SYVN1, promoting polyubiquitination and subsequent proteasomal degradation of MAVS, and subsequently attenuating the type I IFN innate immune response (Luo et al., 2020). Interestingly, MFN2 has been shown to be enriched in MAMs and is also localized on the ER (de Brito and Scorrano, 2009). If SYVN1 is confirmed to promote NS1-mediated degradation of SPL, it may be worthwhile to investigate whether MFN2 could be involved as well. The authors also determined that SYVN1 knockdown attenuated IAV replication and increased levels of MAVS during corticosterone-induced cell stress (Luo et al., 2020). Further studies into the potential role of SYVN1 in the IAV-NS1 mediated downregulation of SPL could yield further insight and clarify the interaction.

This study sheds light on the intricate struggle between influenza virus and the host immune defense and confirms the important role of SPL in the innate immune response to influenza virus infection. Understanding the precise mechanism of how IAV NS1 targets SPL for ubiquitination and degradation could yield potential new therapeutic targets for the treatment of IAV infection.

II-F. Acknowledgments

This work was supported by the Department of Surgery at University of Missouri and NIH/NIAID AI091797 (B.H.). J.J.W. and C.J.S. were supported by Life Sciences Fellowships from the University of Missouri. H.N. was supported by the McNair Scholars program at University of Missouri. We would like to thank Adolfo Garcia-Sastre (Icahn School of Medicine at Mount Sinai), Julie D. Saba (University of California San Francisco), David Pintel (University of Missouri), Marc Johnson (University of Missouri), and Wenjun Ma (University of Missouri) for their kind provision of research reagents as described in the Materials and Methods section. We would also like to thank Paige Williams for her work in lab making and maintaining lab reagents, Regina Wamsley for her work in the lab, and Joseph Mangieri for his help with learning to use R. Part of the figures and text from this dissertation chapter was published in *Virology* (Wolf et al., 2021). Elsevier permits authors to include their published articles in full or in part in a dissertation for non-commercial purposes.

II-G. Data collection and analysis

Jennifer J. Wolf: Investigation, Validation, Formal analysis, Writing-Original draft preparation, Writing - Review & Editing, Conceptualization, Visualization. **Chuan Xia:** Investigation, Validation, Formal analysis, Conceptualization, Visualization. **Caleb J Studstill:** Investigation, Conceptualization. **Ngo H:** Investigation. **Steven L. Brody:** Resources. **Paul E. Anderson:** Resources. **Hahm B:** Funding acquisition, Project administration, Supervision, Writing - Review & Editing, Conceptualization, Visualization.

Chapter III. Host Factors are crucial for SK2-mediated enhancement of influenza viral replication

III-A. Abstract

Influenza A virus (IAV) is widely known for causing seasonal and pandemic influenza, continuing to be an annual threat. The characterization of host factors which promote IAV replication is crucial for the development of novel therapeutics to counteract IAV. Here, we report that sphingosine kinase 2 (SphK2, SK2) promotes IAV replication in cooperation with multiple host factors. IAV protein synthesis during infection was enhanced upon transient overexpression of SK2 and was reduced with siRNA-mediated knockdown of SK2. IAV infection led to increased SK2 and activated SK2 protein expression. MEK/ERK activation was found to be important for increased SK2 and phosphorylated-SK2 protein levels during IAV infection. Treatment with SK2 inhibitor led to decreased p21 expression upon IAV infection. These studies provide novel insight into the effects of host factors on SK2-enhanced IAV replication.

III-B. Introduction

Seasonal influenza infections lead to severe morbidity and mortality annually. The potential severity of pandemic influenza A virus (IAV) infection can be exemplified by the 1918 H1N1 influenza pandemic, which killed an estimated 50-100 million people (Morens and Fauci, 2007). The severity of the ongoing COVID-19 pandemic (Piroth et al., 2021) and the 2009 IAV pandemic (Fraser et al., 2009; Wang and Palese, 2009) as well as the recurrent outbreaks of highly pathogenic avian influenza (Hobbelen et al., 2020; Lai et al., 2016; Peiris et al., 2007) have all contributed to mounting concern of the next potential influenza pandemic. Vaccines targeting the predicted most prevalent strains of influenza A and influenza B viruses must be reformulated annually. Antiviral drugs that target IAV proteins such as neuraminidase (NA) and viral polymerase are available. However, multiple viral strains quickly developed resistance to these contemporary antivirals (Cheng et al., 2010; Dharan et al., 2009; Hussain et al., 2017; Irwin et al., 2016; Jones et al., 2018; Marjuki et al., 2015; Poland et al., 2009), which illustrates the vital need for novel therapeutic targets able to broadly counteract infection, such as host factors.

Sphingosine kinase (SK) is a host lipid kinase that produces intracellular and extracellular sphingosine 1-phosphate (S1P) by ATP-dependent phosphorylation of sphingosine. There are two SK isoforms, sphingosine kinase 1 (SK1) and sphingosine kinase 2 (SK2). Both SK isoforms have conserved domains and similar enzymatic activity, yet also have distinct differences. SK1 is known to promote cell survival and proliferation, and is also critical for tumor necrosis factor-alpha (TNF- α) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signaling during inflammatory responses. In contrast, SK2 has been shown to have both pro- and anti-apoptotic properties as well as both pro- and anti-inflammatory functions. SK1 is localized to the plasma membrane and cytosol, while SK2 is primarily localized to the nucleus, but is also localized to the cytosol

or endoplasmic reticulum under certain conditions (Igarashi et al., 2003; Maceyka et al., 2005; Taha et al., 2006). Both SK1 and SK2 have been previously shown to affect the replication of several different viruses. However, little research has been conducted regarding the role of SK2 in IAV replication.

In this study, we show that SK2 enhances IAV replication. Inducible SK2 overexpression increased the production of IAV proteins, while SK2 inhibition or knockdown decreased IAV protein levels. Furthermore, IAV infection increased SK2 levels. MEK/ERK activation was important for increased SK2 and pSK2 levels during IAV infection, and inhibition of SK2 was found to interfere with p21 expression during IAV infection. This study provides novel insight into the role of SK2 in IAV infection.

III-C. Materials and methods

Viruses and Cells

Influenza A/Puerto Rico/8/34 (H1N1) virus (gift from Adolfo Garcia Sastre), influenza A/WSN/33 (H1N1) virus was initially provided by Yoshihiro Kawaoka (University of Wisconsin-Madison), pandemic influenza A/CA/04/09 (H1N1) virus was a gift from Wenjun Ma (Kansas State University) (Lee et al., 2017; Xia et al., 2018b). In experiments, IAV denotes Influenza A/Puerto Rico/8/34 virus unless stated otherwise. Viruses used in this study were amplified either on Madin-Darby Canine Kidney (MDCK) cells or in chicken eggs as described previously (Eisfeld et al., 2014; Neumann et al., 1999; Seo et al., 2010; Varble et al., 2014). Briefly, for amplification of viruses on MDCK cells, cells were incubated with virus for 1 hour (h). The cells were then washed with PBS and incubated with fetal bovine serum (FBS) free medium containing 0.3% bovine serum albumin (BSA) and TPCK-trypsin (1 µg/mL) for amplification. For viral amplification in chicken eggs, serum

pathogen-free fertilized chicken eggs were candled then inoculated with virus diluted in 1x phosphate buffered saline via the allantoic route. Infected eggs were incubated for 48 hours without turning at 37°C and ~60% humidity. Eggs were then chilled and the allantoic fluid was collected and centrifuged to remove debris. Virus titers were determined by plaque assay. Briefly, supernatants containing viruses were harvested, serially diluted, and then were adsorbed onto 1×10^6 MDCK cells/well in a 6-well plate for at least 1 h. Cells were then overlaid and incubated with 2X EMEM (Gibco) containing 0.6% BSA and 2 ug/mL TPCK-trypsin mixed with an equal portion of 1% agarose (Seakem ME). Cells were fixed using 25% formalin and stained with 1X crystal violet prior to assessing viral titer. Sources of human embryonic kidney (HEK) 293 cells, MDCK cells, and human lung epithelial A549 cells have been previously described (J. Min et al., 2007; Seo et al., 2012; Vijayan et al., 2014). HEK293 cells and A549 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco), while the MDCK cells were cultured in Minimum Essential Medium Eagle (MEM, Gibco) as previously described (Y.-J. J. Seo et al., 2013; Vijayan et al., 2017, 2014; Xia et al., 2018b, 2016). All cells were cultured in a CO2 incubator at 37°C. All media to culture immortalized cell lines were supplemented with 10% FBS (HyClone) and 1% penicillin (100 U/mL)/streptomycin (100 µg/mL) (Invitrogen) unless stated otherwise.

Construct and Transfection

Doxycycline (DOX) inducible expression plasmid encoding SK2 was generated by PCR from pVB201 (provided by Stephen Alexander, University of Missouri-Columbia) (J. Min et al., 2007) with primers 5' -GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT ACC ACC ATG GGG GGT TCT CAT CAT CAT- 3' and 5' -GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG TCA GGC TTG TGG CTT TTG ACC TGC AGG- 3'. The amplified murine SK2-encoding fragment was cloned into pINDUCER20 vector using BP and LR

clonase kits (Invitrogen) according to the manufacturer's instructions. The pINDUCER20 reagents were a gift from David Pintel at University of Missouri-Columbia (Adeyemi et al., 2014; Meerbrey et al., 2011). For transient expression, HEK293 cells (2×10^5 /well) were seeded in a 24-well plate one day before transfection. Cells were then transfected with a plasmid encoding SK2 (250 ng/well) using LipoD293 transfection reagent (SignaGen) and protocols recommended by the manufacturer. DOX (100 ng/ml, MP Biomedical) was added to the cell culture 24 h post transfection to induce the transient expression of SK2.

Western Blotting

Western blotting was performed as described previously (Seo et al., 2010; Y.-J. J. Seo et al., 2013; Vijayan et al., 2014; Xia et al., 2018c, 2016) . Briefly, cells were lysed in $2 \times$ sample buffer containing β -mercaptoethanol and heated at 95 °C for 10 min. Equal amounts of protein samples were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a nitrocellulose membrane (Bio-Rad). Membrane bound antibodies were detected using IRDye secondary antibodies (IRDye 800CW Goat anti-Mouse IgG and Goat anti-Rabbit IgG; LI-COR). The signals were imaged by Odyssey Fc (LI-COR), and data were analyzed using Image Studio V5.2 (LI-COR). Data are representative of at least two repeat experiments.

Antibodies and Reagents

SK2 inhibitor ABC294640 (Opaganib) (Orr Gandy and Obeid, 2013) was purchased from MedKoo. Antibodies against human SK2, influenza A viral NP, and M1 were purchased from Abcam; the antibodies against p21 and influenza A viral NS1 was purchased from Santa Cruz; the antibodies against human GAPDH (glyceraldehyde-3-phosphate dehydrogenase), ERK 1/2 (extracellular signal-related kinase), and phospho-

ERK 1/2 was purchased from Cell Signaling Technology; the antibody against human phospho-SK2 was purchased from ECM Biosciences.

RNA interference

ON-TARGETplus Human SK2 siRNA (si-SK2) and universal scrambled negative control siRNA (SCR) were purchased from Dharmacon. All siRNAs were used at a final concentration of 20 nM to transfect A549 cells. The cells (2×10^5 /well) were transfected with siRNA by reverse transfection using Lipofectamine RNAiMax reagent according to the manufacturer's instructions. The transfected cells were then seeded in a 24-well plate. One day later, cells were infected with IAV and then harvested at one day post-infection (dpi). The knockdown of SK2 was confirmed by Western blot analysis.

Statistical analysis

Densitometric analysis was performed using Image Studio V5.2 (LI-COR). Data were analyzed and compared using a bidirectional, unpaired Student t-test (Pritzl et al., 2015; Xia et al., 2018c). Error bars represent means \pm standard error of the mean (SEM).

******, $p \leq 0.01$;

III-D. Results

SK2 promotes IAV replication

We had previously shown that SK isoform SK1 served as a pro-influenza viral factor during infection (Y.-J. J. Seo et al., 2013), but the effects of SK2 during IAV infection remained unknown. To determine the effect of SK2 on IAV replication, cells were transfected with either a scrambled control siRNA or siRNA targeting SK2 and were subsequently infected with IAV. SK2 knockdown reduced IAV protein expression levels compared to the infected control (Figure 24A), suggesting that SK2 is important for robust IAV replication. To confirm that SK2 would enhance IAV replication, cells were transfected with a doxycycline (dox)-inducible SK2-encoding plasmid, followed by subsequent infection with or without dox. SK2 overexpression during IAV infection resulted in more robust viral protein expression compared to the uninduced controls (Figure 24B), strengthening the conclusion that SK2 promotes IAV replication. Collectively, these results indicate that SK2 functions as a pro-influenza viral host factor, crucial for robust IAV replication.

IAV infection increases SK2 expression

Next, to observe whether IAV infection could impact SK2 protein expression, we measured relative protein levels of SK2 and activated (phosphorylated) SK2 (pSK2) in cells infected with IAV. IAV infection increased relative expression levels of both SK2 and pSK2 (Figure 25). Densitometric analysis confirmed that IAV infected cells increased SK2 protein levels by about twofold compared to the mock-infected control (Figure 25).

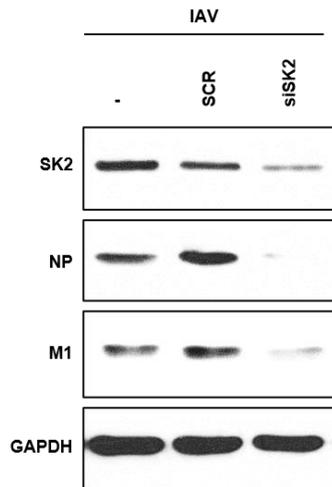
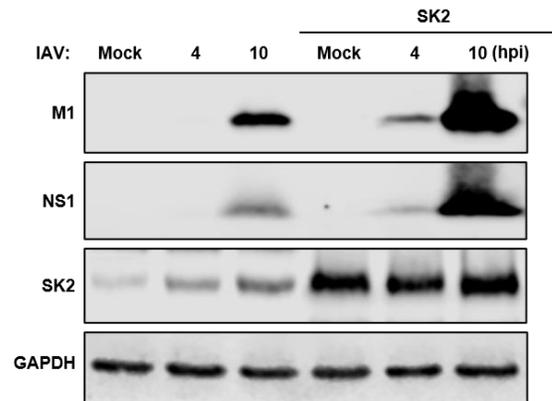
A**B**

Figure 24. SK2 promotes IAV replication. (A) A549 cells were left untransfected (-), transfected with scrambled control siRNA (SCR) or siRNA targeting human SK2 (si-SK2). At 24 hours post-transfection, cells were infected with IAV at an MOI of 1. At 24 hpi, the levels of SK2, NP, NS1, M1, and GAPDH were analyzed using western blotting. **(B)** A549 cells were transfected with an inducible SK2-encoding plasmid. 24 hours post-transfection, cells were infected with IAV at an MOI of 1 without (-) or with (+) the treatment of Dox (100 ng/mL). The levels of viral NS1, viral M1, SK2, and GAPDH were analyzed at 4 hpi or 10 hpi using western blotting.

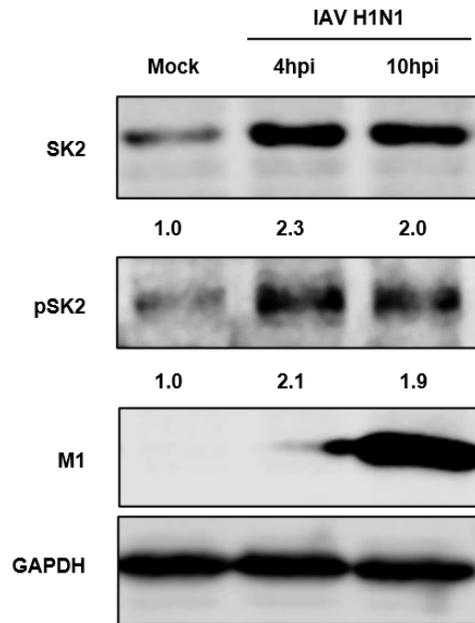


Figure 25. IAV infection increases SK2 expression and activation. A549 cells were infected with influenza A/WSN/33 (H1N1) virus at an MOI of 1. The levels of SK2, pSK2, viral M1, viral NP, and GAPDH were analyzed by Western blotting at 4 hours post-infection (hpi) or 10 hpi. The relative intensity of each band of SK2 and pSK2 compared to the mock-infected control was determined using densitometry based on the GAPDH level of the sample and is depicted below each blot. The relative levels of SK2 and pSK2 from the mock infected sample was set as 1.0.

MEK-ERK activation is important for increased SK2 expression

SK2 has been previously shown to be phosphorylated by ERK 1/2 under certain cellular conditions (Hait et al., 2007). Furthermore, ERK has been reported to be important for IAV infection through experiments that utilized the inhibitor U0126, which blocks MEK/ERK activation (Marjuki et al., 2011; Pleschka et al., 2001). To determine if ERK-mediated activation could impact the IAV-induced increase of SK2 and pSK2, U0126 was used during IAV infection. Inhibition of ERK prior to infection interfered with the IAV-induced increase of SK2 and pSK2 throughout infection (Figure 26). This result suggests that MEK/ERK activation is important for IAV-induced upregulation of SK2/pSK2.

SK2 inhibition interferes with p21 expression during IAV infection

SK2 has been previously described as interacting with histone deacetylase (HDAC) 1/2 in the nucleus upon phorbol 12-myristate 13-acetate (PMA) stimulation, subsequently regulating the expression of genes such as p21 (Hait et al., 2009). IAV infection has also been shown to upregulate p21 and cause temporal cell cycle arrest in the G0/G1 phase, maximizing viral replication (He et al., 2010). Therefore, we tested whether SK2 could impact p21 expression during IAV infection by measuring relative levels of p21 after infection with IAV and treatment with SK2 inhibitor ABC294640 (ABC). Interestingly, SK2 inhibition impaired p21 expression specifically during IAV infection (Figure 27). This suggests that SK2 could regulate p21 during IAV infection, which has implications in mediating IAV-induced cell cycle arrest.

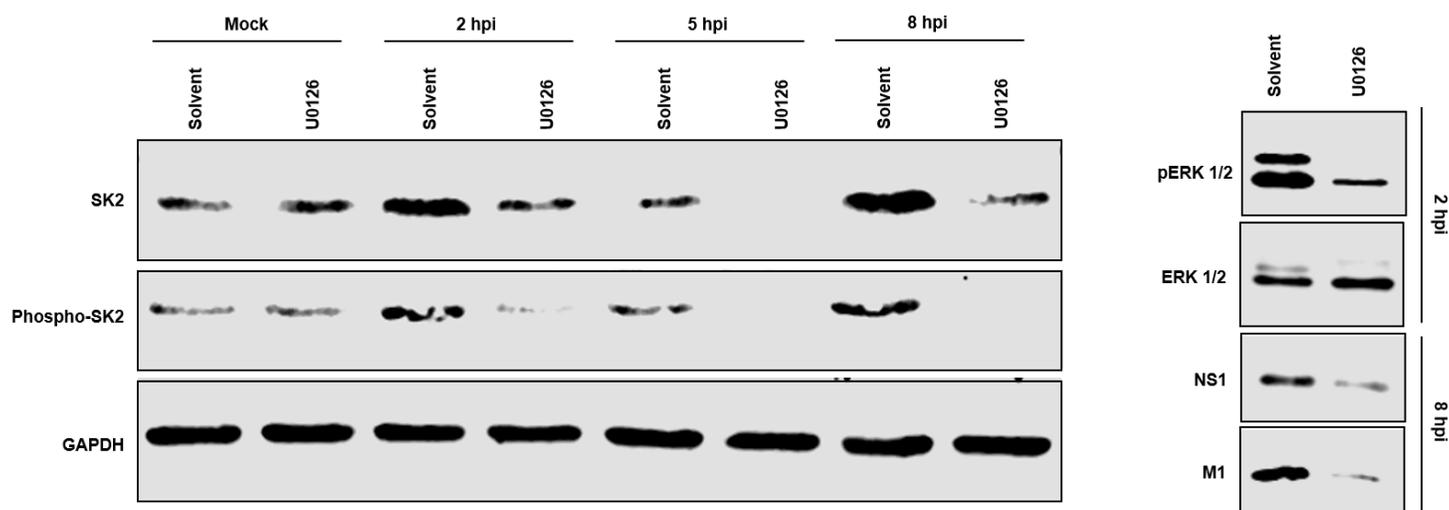


Figure 26. MEK/ERK activation is important for increased SK2/pSK2 levels during IAV infection. A549 cells were pre-treated with DMSO (Solvent) as a control or 15 μ M U0126 and were then infected with IAV (MOI=5). The levels of pSK2, SK2, pERK, ERK, GAPDH, and viral M1 were assessed using western blotting.

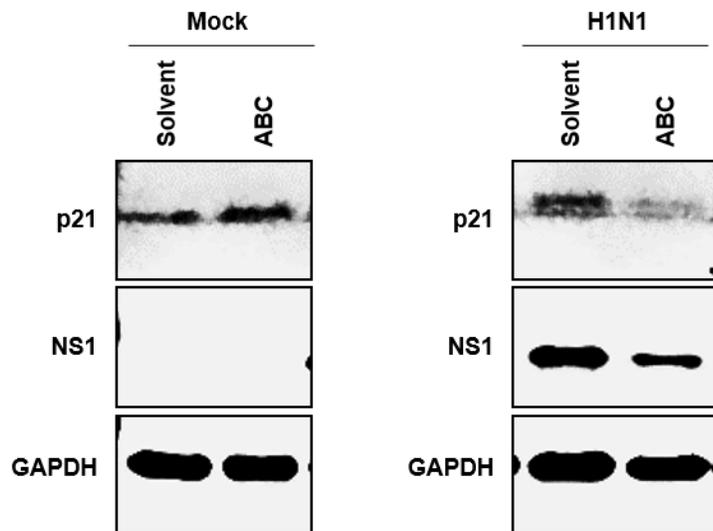


Figure 27. SK2 inhibition interferes with p21 expression during IAV infection. A549 cells were mock or IAV-infected, and at 1hpi were treated with either 50% PEG 5% DMSO (Solvent) or 100 μ M ABC294640 (ABC). Levels of p21 and NS1 were assessed using western blotting. Mock and H1N1 blots depicted are from the same blot.

III-E. Discussion

This investigation shows that SK2 promotes IAV infection. SK2 knockdown or inhibition resulted in reduced IAV protein levels, while transient SK2 overexpression increased IAV protein levels. Likewise, IAV infection increased SK2 expression, and the enhanced SK2 expression was mediated by ERK activation. SK2 inhibition decreased p21 expression, which suggests potential SK2 involvement with mediating IAV-induced cell-cycle arrest.

Due to SK2 enhancing IAV replication, it is logical to assume that targeting SK2 as an IAV therapeutic could be advantageous. A high mutation rate allows IAV to rapidly develop resistance to antiviral drugs that target IAV proteins. However, targeting host cell factors that enhance IAV replication may prove to be an effective antiviral strategy. An SK2 inhibitor is currently being tested as a therapeutic for patients with COVID-19 (McGowan et al., 2020), but the role of SK2 in virus-host interaction remains unclear. Greater study is necessary to ascertain the mechanism of SK2 promoting IAV replication. Combining antiviral drugs that target IAV proteins along with host-targeted therapeutics that display minimal cytotoxicity has great potential as a therapeutic strategy to treat influenza (Davidson, 2018). Further research is required to determine whether ABC294640 could be synergistic with current antivirals.

Inhibition of ERK activation was shown to interfere with the IAV-induced increase of SK2. However, previous work has described that inhibition of ERK activation reduces IAV replication (Marjuki et al., 2006; Pleschka et al., 2001). Therefore, it is unclear whether the reduced SK2 levels observed in the IAV-infected, ERK inhibitor-treated cells at 8 hpi are due to ERK inhibition or the reduced viral protein expression. It could be interesting to determine if a viral component could be involved with increased SK2 expression during

infection or if ERK could be interacting with SK2. Further study is required to more fully assess how ERK activation leads to the IAV infection-induced increase of SK2 levels.

Inhibition of SK2 during IAV infection resulted in decreased p21 protein levels compared to the uninfected control treated with SK2 inhibitor. Therefore, it is possible that SK2 could modulate the expression of genes important for IAV replication. It is known that SK2 regulates gene expression and DNA synthesis in cancer cells (Alvarez et al., 2010; Igarashi et al., 2003), which suggests that SK2 could regulate gene expression or viral replication in the nucleus. IAV infection has been previously shown to upregulate p21, leading to temporal cell cycle arrest in the G0/G1 phase and maximizing viral replication (He et al., 2010). Determining the involvement of SK2 in IAV-mediated cell cycle arrest could provide greater insight into the observed results.

SK2 is a promising target to counteract IAV infection. This work increases our understanding of host-IAV interactions. Further investigation is required to clarify how SK2 enhances IAV replication and how IAV increases SK2 levels.

III-F. Acknowledgments We thank Yoshihiro Kawaoka (University of Wisconsin-Madison), Adolfo Garcia-Sastre (Mount Sinai School of Medicine), Wenjun Ma (Kansas State University), Stephen Alexander (University of Missouri-Columbia), and David Pintel (University of Missouri-Columbia) for their kind provision of research reagents as described in Materials and Methods. This work was supported by the Department of Surgery at University of Missouri, and NIH/NIAID grant R21AI127404 (B.H.). J.J.W. and C.J.S. were supported by Life Sciences Fellowships from the University of Missouri. Part of the figures and text from this dissertation chapter was published in Antiviral Research (Xia et al., 2018b). Elsevier permits authors to include their published articles in full or in part in a dissertation for non-commercial purposes.

III-G. Data collection and analysis

Jennifer J. Wolf: Investigation, Validation, Formal analysis, Writing-Original draft preparation, Writing - Review & Editing, Conceptualization, Visualization. **Chuan Xia:** Investigation, Validation, Formal analysis, Writing-Original draft preparation, Conceptualization, Visualization. **Caleb J Studstill:** Investigation, Conceptualization. **Hahm B:** Funding acquisition, Project administration, Supervision, Writing - Review & Editing, Conceptualization, Visualization.

Chapter IV. PARP1 enhances influenza A virus propagation by facilitating degradation of host type I interferon receptor

IV-A. Abstract

Influenza A virus (IAV) utilizes multiple strategies to confront or evade host type I interferon (IFN)-mediated antiviral responses in order to enhance its own propagation within the host. One known strategy is viral hemagglutinin (HA) mediating the induction of type I IFN receptor 1 (IFNAR1) degradation. However, the molecular mechanism behind this process remains undefined. Here, we report that a cellular protein, poly (ADP-ribose) polymerase 1 (PARP1), plays a critical role in mediating IAV HA induced degradation of IFNAR1. We identified PARP1 as an interacting partner for IAV HA through mass spectrometry analysis. Knockdown or inhibition of PARP1 rescued IFNAR1 levels during IAV infection or HA expression, exemplifying the importance of PARP1 for IAV-induced reduction of IFNAR1. Furthermore, confocal fluorescence microscopy showed altered localization of endogenous PARP1 upon transient IAV HA expression or during IAV infection. Notably, PARP1 was crucial for robust IAV replication, which was associated with regulation of the type I IFN receptor signaling pathway. These results indicate that PARP1 promotes IAV replication by controlling viral HA-induced degradation of host type I IFN receptor. Altogether, these findings provide novel insight into interactions between influenza virus and the host innate immune response and reveal a new function for PARP1 during influenza virus infection.

IV-B. Introduction

Influenza A viruses (IAV) cause seasonal and pandemic influenza, which leads to unpredictably severe morbidity and mortality worldwide (Blanton et al., 2019; Cowling et al., 2013). The type I interferon (IFN) innate immune response acts as one of the first lines of host defense against IAV infection (García-Sastre, 2011; Grandvaux et al., 2002; Pitha and Kunzi, 2007; Seo and Hahm, 2010). Influenza viruses are known to utilize multiple tactics to evade host innate immune responses. The most well-characterized strategies aim to inhibit synthesis of type I IFNs (Gack et al., 2009; Liedmann et al., 2014; Patel et al., 2013). However, during IAV infection, IFN- α/β molecules are still produced to some extent. These molecules bind to their cognate IFN receptor (IFNAR), which activates the JAK/STAT pathway, generating antiviral responses. The continued production of IFN- α/β suggests that IAV could overcome IFN receptor-mediated antiviral innate responses by utilizing a method that does not target type I IFN molecule synthesis.

Our previous findings have shown that upon infection, IAV promotes the degradation of subunit 1 of the type I and type II IFN receptors, IFNAR1 and IFNGR1 respectively, using viral hemagglutinin (HA) (Xia et al., 2018c, 2016). Viral HA induces the phosphorylation of IFNAR1 and IFNGR1, which is followed by poly-ubiquitination of these receptors. Ubiquitinated IFNAR1 and IFNGR1 are subsequently degraded, resulting in both decreased IFN receptor levels on the cell surface and decreased IFN sensitivity for that cell (Xia et al., 2018c, 2016). Receptor degradation results in a broad-spectrum downregulation of receptor-mediated interferon responses, creating more optimal conditions for IAV survival and replication. However, the molecular mechanism for IAV HA triggering IFN receptor degradation remains unknown. Strikingly, HA-mediated IFNAR1 degradation does not follow known cellular mechanisms of IFNAR1 degradation, such as PERK-mediated ER stress or high levels of IFN molecules (Bhattacharya et al., 2011; Liu

et al., 2009a; Xia et al., 2018c, 2018a, 2016; H. Zheng et al., 2011; Hui Zheng et al., 2011). This led us to hypothesize that there could be an unknown cellular factor(s) that mediates this receptor degradation process. Utilizing mass spectrometry, we identified poly (ADP-ribose) polymerase 1 (PARP1) as a host protein that binds to IAV HA.

PARP1, a member of the PARP family, regulates various critical cellular processes such as cellular proliferation and differentiation (Chaitanya et al., 2010; Du et al., 2016; Gerö et al., 2014; Liu and Yu, 2015; Rossi et al., 2009). Of note, PARP1 is well-known as a regulator during tumor development (Du et al., 2016), and PARP1 inhibitors are currently approved as therapeutic options for BRCA-mutated ovarian and breast cancers (Slade, 2020). PARP1 as well as PARP13.1 have been shown to regulate influenza A virus polymerase activity, which in turn affects IAV replication (Bortz et al., 2011; Liu et al., 2015; Westera et al., 2019). Additionally, PARP1 depletion in human cancer cells induces interferon-stimulated gene (ISG) expression, which suggests potential PARP1 involvement in IFN signaling (Ghosh et al., 2018). However, the exact role of PARP1 in regulating the host type I IFN signaling pathway and its resultant effects on viral replication remain unknown.

In this study, we identified PARP1 as an IAV HA interacting partner during IAV infection using mass spectrometry. Furthermore, PARP1 was proven to be important for mediating IAV HA-induced degradation of IFNAR1, regulating host type I IFN responses, and consequently promoting IAV propagation. These findings reveal novel insight into host-influenza interactions as well as highlight a promising therapeutic target to more effectively control influenza virus infection.

IV-C. Materials and methods

Viruses and Cells

Influenza A/WSN/33 (H1N1) virus, pandemic influenza A/CA/04/09 (H1N1) virus, and influenza A/Puerto Rico/8/34 (H1N1) virus were used as previously reported (Ma et al., 2011; Xia et al., 2018c, 2018b, 2016). Viruses were amplified on Madin-Darby Canine Kidney (MDCK) cells as previously described (Xia et al., 2018d, 2018b, 2016). Briefly, cells were incubated with virus at indicated multiplicity of infection (MOI) for 1 hour (h). The cells were then washed with phosphate-buffered saline (PBS) and incubated with fetal bovine serum (FBS) free medium containing 0.3% BSA and TPCK-trypsin (1µg/ml) for indicated time periods optimal for amplification. Titration of virus was performed using plaque assay. Briefly, supernatants containing viruses were harvested and diluted into serial dilutions. The diluted supernatants were then adsorbed onto 4×10^5 MDCK cells/well in a 6-well plate for at least 1 hour. Cells were then incubated with 2 X EMEM (Gibco) mixed with an equal portion of 1% agarose (Seakem ME). Human embryonic kidney (HEK) 293 cells, human lung epithelial A549 cells, and African green monkey kidney epithelial cells (Vero cells) have been previously reported (Y.-J. J. Seo et al., 2013; Y. J. Seo et al., 2013; Vijayan et al., 2014; Xia et al., 2018d, 2016). The HEK293 cells, A549 cells, and Vero cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco), while the MDCK cells were cultured in Minimum Essential Medium Eagle (MEM, Gibco) as previously described (Seo et al., 2010; Vijayan et al., 2014; Xia et al., 2018c, 2016). All the cells were cultured in a CO₂ incubator at 37°C, and all media were supplemented with 10% FBS (HyClone) and penicillin (100 U/ml)/streptomycin (100 µg/ml) (Invitrogen).

Constructs and Transfection

The plasmid encoding FLAG-tagged human PARP1 was purchased from GenScript; the plasmid encoding influenza viral hemagglutinin (HA) from the A/New Caledonia/20/99 (H1N1) has been previously described and was provided by the NIH Vaccine Research Center (Wei et al., 2008; Xia et al., 2018d, 2016). To construct the FLAG-tagged HA, the coding sequences of full-length HA was amplified by PCR from the DNA of full-length HA of influenza A/New Caledonia/20/99 (H1N1) using the primers 5'-CGG AAT TCG ATG AAG GCC AAA CTG CTG -3' and 5'-GGG GTA CCC GTC AGA TAC AGA TCC TGC ACT GCA -3'; 5'-CGG AAT TCG ATG AAG GCC AAA CTG CTG -3' and 5'-GGG GTA CCC GTC ATC TGC TCT GGA TGC TAG GGA -3', respectively. For transfection of cultured cells, cells were seeded onto 6-well plates or 24-well plates at densities of 10^6 cells/well or 2.5×10^5 cells/well 24 h prior to transfection. Cells were then transfected with the indicated plasmids using Lipofectamine2000 Transfection Reagent (Thermo Scientific) at 80%-90% confluency following the protocols recommended by the manufacturer. A concentration of 500 ng/ml DNA was used for the transfection experiments unless specifically indicated. Empty vector plasmids were used as a control in all transfection experiments to ensure that each transfection sample received the same amount of total DNA.

Reagents and Antibodies

PARP1 inhibitor ABT-888 (Cayman) (Clark et al., 2012; Du et al., 2016) and TNKS inhibitor XAV939 (Cayman) were purchased from the indicated manufacturers. The antibodies against influenza viral HA (H1N1) were purchased from GeneTex and Santa Cruz; antibodies against human IFNAR1 and influenza viral M1 were purchased from Abcam; antibodies against human PARP1, human GAPDH, phospho-p38, and Myc-tag were

purchased from Cell Signaling Technology. Fluorophore-labeled secondary antibodies against mouse and rabbit IgG were purchased from Invitrogen.

Confocal microscopy and image analysis

A549 cells were seeded in 24 well plates on poly L-lysine coated coverslips (Neuvitro) and were either transfected with IAV HA-encoding plasmid or were infected with influenza A/Puerto Rico/8/34 (H1N1) virus. Cells were fixed with 4% paraformaldehyde (Alfa Aesar), then were permeabilized, blocked with 10% FBS 1% BSA for 1 hour, and incubated with primary antibodies overnight at appropriate concentrations in 1% BSA, i.e., anti-IAV HA antibody (Santa Cruz) or anti-Myc tag antibody (Cell Signaling Technology), and anti-PARP1 antibody (Cell Signaling Technology). After washing, samples were incubated with the appropriate secondary antibodies such as Alexa Fluor 488 goat anti-mouse (Invitrogen) or Alexa Fluor 546 goat anti-rabbit (Invitrogen) for one hour, then washed. Cell nuclei were then stained using Draq5 according to manufacturer's instructions (ThermoFisher), then mounted on glass slides with Prolong Gold antifade mountant (ThermoFisher). Confocal images of thousands of cells were acquired at the University of Missouri Molecular Cytology Core facility using a Leica SP8 TCP confocal microscope. For quantification purposes, imaging was performed using a 40x objective, capturing 8-10 random images per condition per experiment. Quantification was performed using Fiji (Schindelin et al., 2012) of ImageJ (Rueden et al., 2017). Levels of cytoplasmic PARP1 were determined by using the ImageJ "Intensity Ratio Nuclei Cytoplasm Tool" (Wan and Hopper, 2018; Zhong et al., 2018) where Draq5 was used as the nuclear stain. The threshold, select area, and ROI (Region of Interest) manager functions of ImageJ were used to restrict measurements of the Intensity Ratio Nuclei Cytoplasm Tool to cells that only expressed HA. The Manders (M1) Coefficient (MC) between HA and PARP1 was determined using the ImageJ plugin "JACoP" (Bolte and Cordelières, 2006).

Western blotting

Western blotting was performed as previously described (Seo et al., 2010; Vijayan et al., 2014; Xia et al., 2018c, 2016). Briefly, the denatured polypeptides from cell lysates or co-IP were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to nitrocellulose membranes (Bio-Rad). Membrane-bound antibodies were detected using IRDye secondary antibodies (LI-COR). The signals were imaged using an Odyssey Fc (LI-COR), and the resulting data were analyzed using Image Studio software V5.2 (LI-COR). Similar results were obtained from at least two independent experiments.

RNA interference

SMARTpool: ON-TARGETplus siRNA targeting human PARP1 (L-006656-03-0005) was purchased from Dharmacon. siRNA targeting human IFNAR1 was purchased from Ribobio. 27mer universal scrambled negative control siRNA duplex (SCR) was purchased from Origene. All siRNAs were used at a final concentration of 20 nM to transfect A549 cells using Lipofectamine RNAiMax transfection reagent (Thermo Scientific) according to the manufacturer's instructions. Cells were harvested at 48 h post-transfection and the knockdown of PARP1 was confirmed by Western blot analysis.

Statistical analysis

Data were analyzed and compared using a bidirectional, unpaired Student's t-test. All error bars represent mean \pm standard deviations (SD). Data are representative of at least two independent experimental repetitions.

IV-D. Results

PARP1 is an IAV HA interacting protein

To investigate the molecular mechanisms behind the HA-induced degradation of IFNAR1, we sought to identify involved cellular proteins by employing a mass spectrometric screening approach. MS analysis of the co-immunoprecipitation (co-IP) precipitates revealed the presence of viral HA-associated host proteins including poly (ADP-ribose) polymerase 1 (PARP1) protein. Since PARP1 is known to regulate multiple cellular signaling events, we further investigated the interplay between PARP1 and viral HA. We confirmed this interaction by performing reciprocal co-IP experiments (data not shown).

Knockdown or inhibition of PARP1 results in impaired IAV HA-mediated degradation of IFNAR1

Given that IFNAR1 expression is essential for the type I IFN-mediated innate immune response, we hypothesized that PARP1 could promote IAV replication in cells by modifying IFNAR1 levels. To test this, we utilized a knockdown approach using an siRNA targeting human PARP1. During PARP1 knockdown, HA-induced downregulation of IFNAR1 was strongly inhibited (Figure 28A). In support of this, PARP1 knockdown also inhibited IFNAR1 downregulation during IAV infection (Figure 28B). Notably, PARP1 knockdown also reduced IAV protein expression during infection compared to the control siRNA transfected group (Figure 28B). Virus infection triggers the production of type I IFNs, which in turn, is known to decrease IFNAR1 levels through the ligand-dependent pathway when present in high amounts (Kumar et al., 2003; Suresh Kumar et al., 2004). To eliminate the possibility that PARP1 downregulation could be indirectly affecting

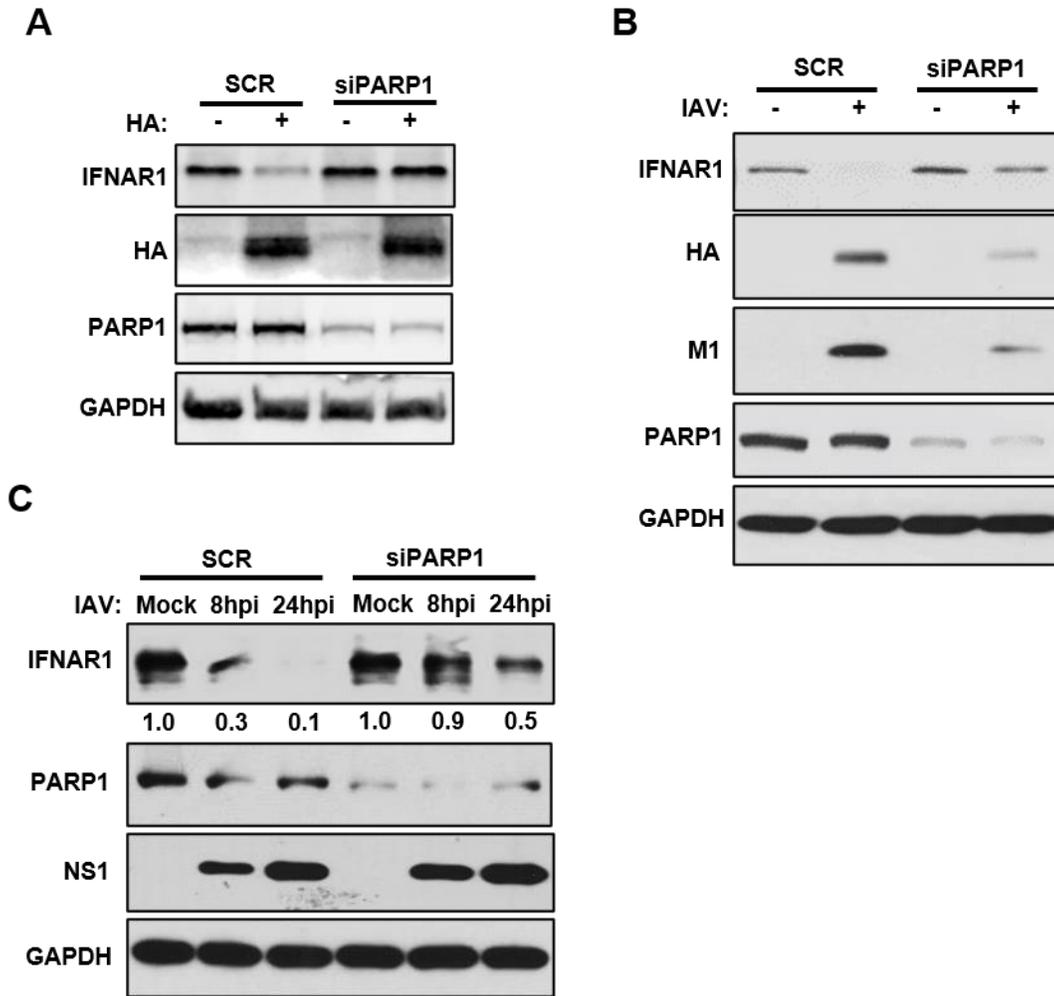


Figure 28: Knockdown of PARP1 results in impaired degradation of IFNAR1 during IAV HA expression or IAV infection. (A) A549 cells were transfected with either PARP1 siRNA or nonspecific scrambled control siRNA (SCR). After 24 h post transfection, the cells were transfected with a control vector (-) or plasmids encoding HA (+). Western blotting was performed 24 h after HA transfection, and the levels of IFNAR1, HA, PARP1, and GAPDH were detected. **(B)** A549 cells were transfected with either SCR or si-PARP1. At 24 h post transfection, cells were either mock infected (-) or infected with IAV (+) at an MOI of 1. Cells were harvested at 24 hpi, and the levels of IFNAR1, PARP1, and viral HA and M1 proteins were detected by Western blotting. **(C)** Vero cells were transfected with SCR or siPARP1. At 24 h post transfection, cells were left uninfected (Mock) or infected with IAV at an MOI of 1 for the indicated time periods. The levels of IFNAR1, PARP1, NS1, and GAPDH were analyzed by Western blotting. The relative intensity of each band of IFNAR1 was determined by densitometry based on the GAPDH level of the sample and is depicted below each blot. The relative level of IFNAR1 from the mock-infected sample was set as 1.0.

IFNAR1 downregulation by inhibiting viral replication, we utilized Vero cells, which have type I IFN receptors but are unable to synthesize type I IFNs. IAV infection strongly induced IFNAR1 downregulation in Vero cells (Figure 28C), and knockdown of PARP1 partially rescued IFNAR1 levels at both 8 hpi and 24 hpi in Vero cells (Figure 28C). This indicates that PARP1 regulates IFNAR1 levels independently of type I IFN production or signaling during IAV infection.

In order to further investigate the regulatory effect of PARP1 on IFNAR1 degradation, cells were pretreated with a PARP1 inhibitor (ABT-888) (Clark et al., 2012; Du et al., 2016) followed by transient overexpression of HA. Strikingly, PARP1 inhibition resulted in almost no downregulation of IFNAR1 in the presence of HA, while levels of HA were not affected by the inhibitor (Figure 29A). To further clarify the inhibition specificity, we utilized XAV-939, which is a compound known to inhibit PARP family member Tankyrase 1/2 (PARP5) but not PARP1 (Voronkov et al., 2013). Upon HA expression, PARP5 inhibition did not alter IFNAR1 levels, whereas PARP1 inhibition strongly suppressed IFNAR1 downregulation (Figure 29B). Notably, both inhibitors did not affect p38 MAP kinase activation, which is known to regulate IFNAR1 degradation during the host PERK-dependent ER-stress response (Bhattacharya et al., 2011). Collectively, these data suggest that host factor PARP1 is crucial in mediating IAV HA-induced IFNAR1 degradation.

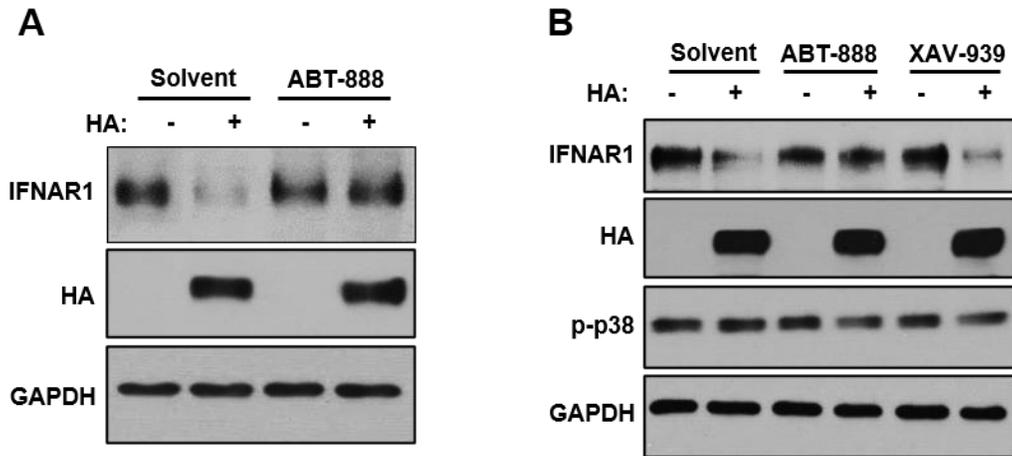


Figure 29: Inhibition of PARP1 leads to impaired IFNAR1 downregulation during IAV HA expression. (A) HEK293T cells were transfected with a control vector (-) or plasmids encoding HA. The cells were then treated with DMSO (Solvent) or a PARP1 inhibitor (ABT-888) at a concentration of 20 μ M. At 24 h post transfection, the levels of IFNAR1, HA, and GAPDH were detected by Western blotting. **(B)** HEK293T cells were transfected with a control vector (-) or plasmids encoding HA. Cells were then treated with the indicated reagents for a time period of 24 h. The levels of IFNAR1, HA, p-p38, and GAPDH were detected by Western blotting.

IAV HA changes PARP1 localization.

PARP1 is a well-known DNA damage response protein which localizes predominantly within the nucleus of human cells (Vyas et al., 2013). However, IAV HA is located within the cytoplasm and plasma membrane of host cells. These contrasting localization patterns of PARP1 and HA led us to ask if viral HA expression could affect endogenous PARP1 localization. To determine if PARP1 localization changes upon IAV HA expression, IAV HA was transiently expressed in A549 cells and immunofluorescence and confocal microscopy were performed for PARP1 and IAV HA. Endogenous PARP1 localization was nuclear in control cells but was strikingly nuclear-cytoplasmic in cells expressing IAV HA (Figure 30A). Quantification of PARP1 signal in HA-expressing A549 cells indicates that over 20% of quantified PARP1 was cytoplasmic compared to the control (Figure 30B). Colocalization between overexpressed HA and endogenous PARP1 was determined by Manders (M1) Coefficient (MC). The MC between overexpressed HA and endogenous PARP1 was 0.3 (Figure 30C), indicating 30% of HA also colocalized with PARP1. This positive correlation was significantly higher than the control. To ensure that this localization could also occur during IAV infection, we next performed immunofluorescence on infected cells. Immunofluorescence of IAV HA and PARP1 during infection revealed a similar nuclear-cytoplasmic localization of endogenous PARP1 in A549 cells (Figure 31A). Quantification of PARP1 cytoplasmic signal as described above in IAV-infected A549 cells revealed a higher percentage of cytoplasmic PARP1 compared to the uninfected control, similar to the cells overexpressing HA (Figure 31B). MC measurements of infected cells also determined positive levels of colocalization between IAV HA and endogenous PARP1 which were similar to the levels in the HA transfected samples (Figure 31C). These data indicate that IAV HA induces PARP1 localization to the cytoplasm in addition to the nucleus.

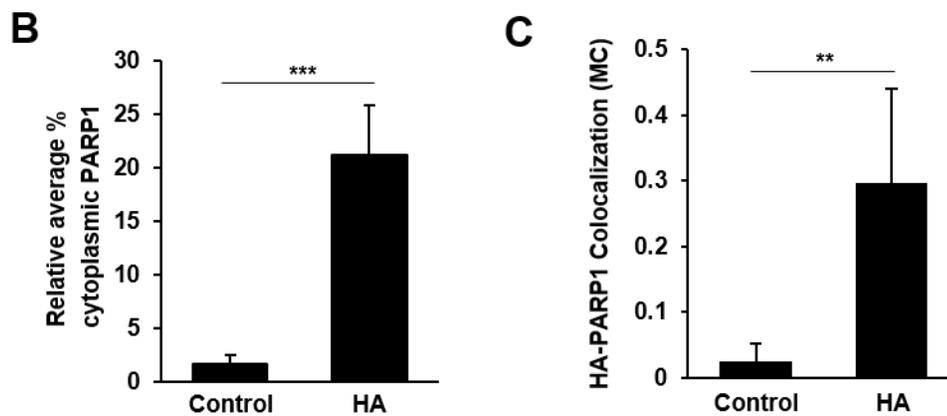
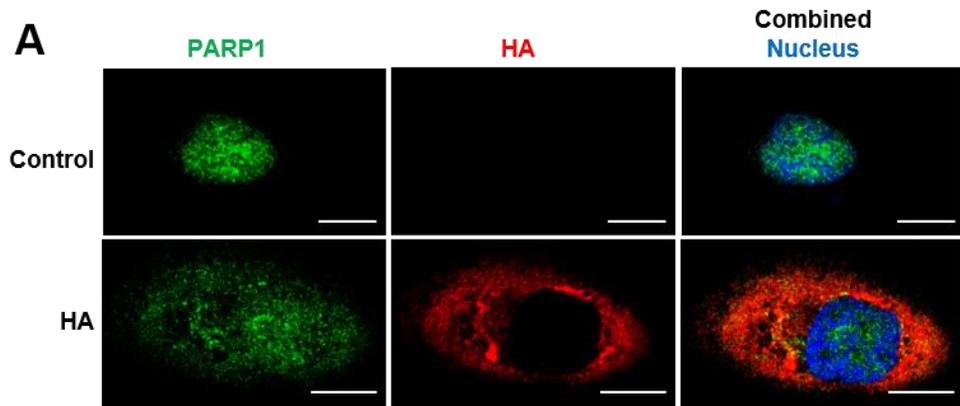


Figure 30: PARP1 localization is altered upon IAV HA expression. (A) A549 cells were transfected with plasmids encoding myc-tagged IAV HA or a control vector. At 24 h posttransfection, cells were fixed and stained using Draq5 for nuclei, as well as anti-PARP1 and anti-myc antibodies. Samples were visualized using confocal microscopy. **(B and C)** PARP1 fluorescence from confocal images was quantified using ImageJ, and the Manders colocalization coefficient (MC) between HA and PARP1 **(B)** and PARP1 cytoplasmic fluorescence **(C)** were determined. Eight to 10 images per condition per experiment were used. Statistical analysis was conducted using a two-tailed unpaired t test. The data represent mean values \pm SD (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

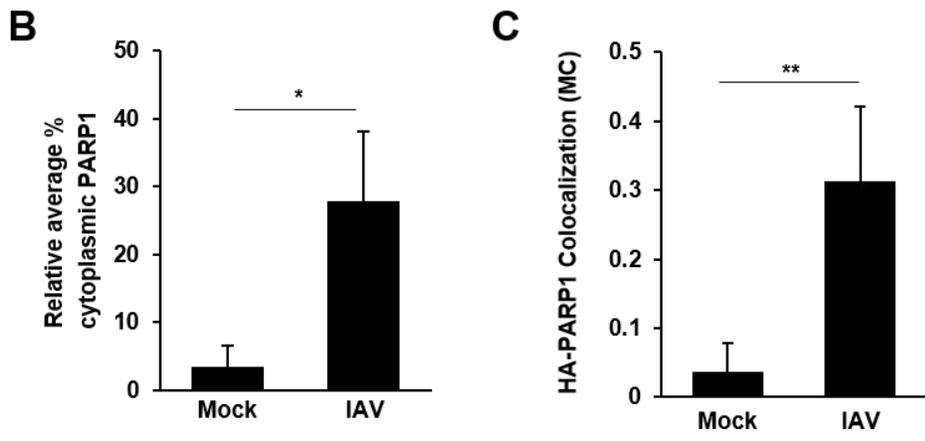
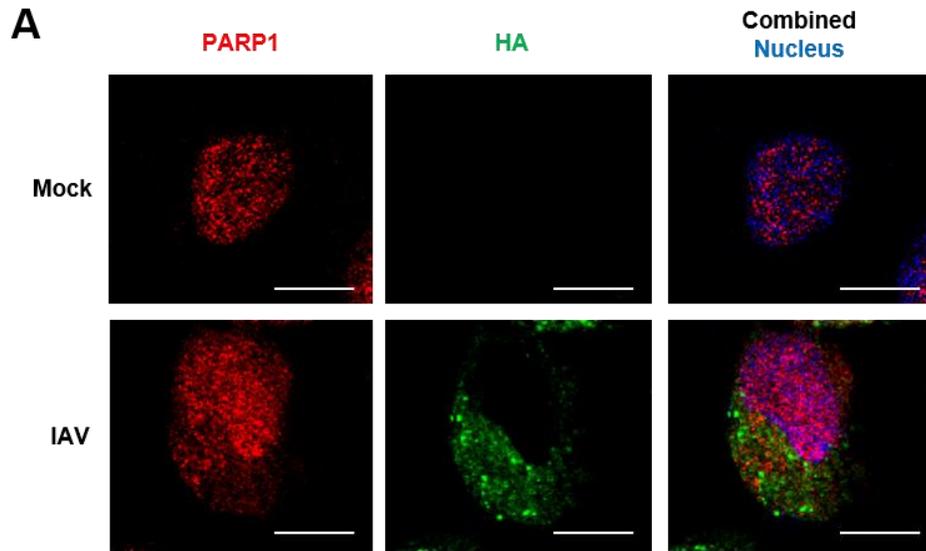


Figure 31: PARP1 localization is altered upon IAV infection. (A) A549 cells were infected with 1 MOI of IAV PR8 and were fixed at 24 hpi. Cells were stained using Draq5 for nuclei, as well as anti-PARP1 and anti-IAV HA antibodies. Samples were visualized using confocal microscopy. (B and C) PARP1 fluorescence from confocal images was quantified using ImageJ, and MC between IAV HA and PARP1 (B) and PARP1 cytoplasmic fluorescence (C) were determined. Scale bars represent 10 μ m. Data are representative of at least two independent experiments. Eight to 10 images per condition per experiment were used. Statistical analysis was conducted using a two-tailed unpaired t test. The data represent mean values \pm SD (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

PARP1 inhibition suppresses IAV propagation which is dependent on IFNAR1

To confirm the regulatory effects of PARP1 on IAV replication, A549 cells were pre-treated with PARP1 inhibitor (ABT-888), followed by IAV infection. The effect of the inhibitor on IAV replication was evaluated by comparing expression levels of viral proteins. IAV HA protein expression decreased at 12, 24, and 36 hpi with the treatment of PARP1 inhibitor (Figure 32A), and as expected, virus-induced downregulation of IFNAR1 was suppressed following PARP1 inhibitor treatment (Figure 32A). To further clarify the role of type I IFN signaling in PARP1-mediated IAV replication, A549 cells were transfected with either scrambled control siRNA (SCR) or IFNAR1-specific siRNA (Figure 32B and 32C). The siRNA transfected cells were then infected with IAV in the presence or absence of PARP1 inhibitor. PARP1 inhibitor treatment reduced the production of infectious IAV from cells transfected with SCR at 1, 2, or 3 days post infection (dpi) (Figure 32B), which is consistent with the suppression of viral protein levels (Figure 32A). However, when endogenous IFNAR1 was knocked down, treatment with PARP1 inhibitor failed to significantly suppress virus propagation in comparison to solvent-treated samples (Figure 32C). Thus, PARP1 inhibition substantially restricts IAV replication, which requires intact IFNAR expression in A549 cells. Altogether, these data suggest the regulatory effect of PARP1 on IAV propagation is closely associated with the type I IFN signaling pathway.

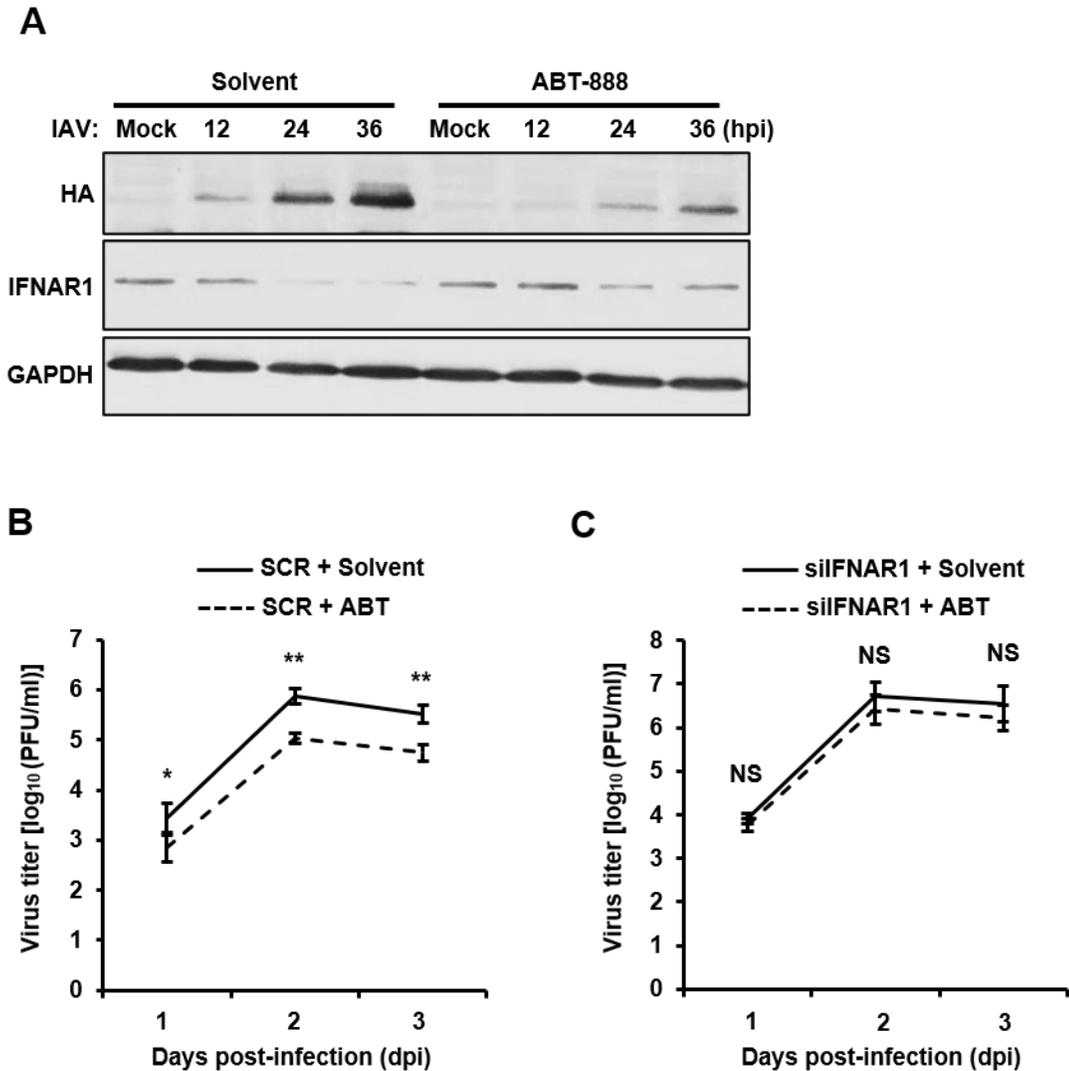


Figure 32: PARP1 inhibition suppresses IAV propagation which is dependent on IFNAR1. (A) A549 cells were infected with pandemic influenza A/CA/04/09 virus (IAV) at an MOI of 1 and treated with DMSO (Solvent) or an ABT-888 (20 μ M). The levels of Viral HA, IFNAR1, and GAPDH were detected at 12, 24, and 36 hours post infection. (B and C) A549 cells were transfected with nonspecific scrambled control siRNA (SCR) (B) or siRNA specific to IFNAR1 (siIFNAR1) (C). At 24 hours post transfection, cells were treated with either DMSO (Solvent) or 20 μ M ABT-888 and infected with IAV at an MOI of 0.001. The titers of infectious virus in the supernatants of the culture were assessed by plaque assays on MDCK cells at 1, 2, or 3 days post infection (dpi). Each data point on the curve represents the mean of three independently obtained samples. Data represent mean values \pm SD (*, $p < 0.05$, **, $p < 0.01$, NS = not significant).

IV-E. Discussion

Successful influenza virus replication relies heavily on the subversion and evasion of host type I IFN signaling (Grandvaux et al., 2002; Pitha and Kunzi, 2007; Seo and Hahm, 2010). Our lab has previously shown that viral HA protein induces the degradation of type I IFN receptor IFNAR1 during IAV infection (Xia et al., 2016). IFNAR1 degradation during infection results in decreased sensitivity to type I IFNs, subsequently facilitating viral propagation. However, the mechanisms behind HA-induced IFNAR1 degradation remain obscure. We demonstrate that PARP1 plays a key role in IAV HA-induced degradation of IFNAR1, which subsequently affects the type I IFN innate immune response to IAV (Figure 33).

Several cellular mechanisms regulate IFNAR1 degradation, such as the ligand (IFN) dependent pathway or the PERK-dependent ER-stress response (also known as unfolded protein response, UPR) (Kumar et al., 2003; Liu et al., 2009b; Suresh Kumar et al., 2004). Our previous study determined that influenza viral HA eliminates IFNAR1 using CK1 α . However, this degradation process is independent on both the ligand dependent pathway and the PERK-dependent ER stress response (Xia et al., 2016). In the UPR-induced IFNAR1 phosphorylation pathway, the primary phosphorylation of IFNAR1 by p38 is crucial to ensure subsequent IFNAR1 phosphorylation and ubiquitination (Bhattacharya et al., 2011). Given that p38 activation is dispensable for IFNAR1 downregulation upon HA expression (Xia et al., 2016), there could be an undiscovered mechanism utilized by viral HA to activate the signaling pathway for IFNAR1 degradation.

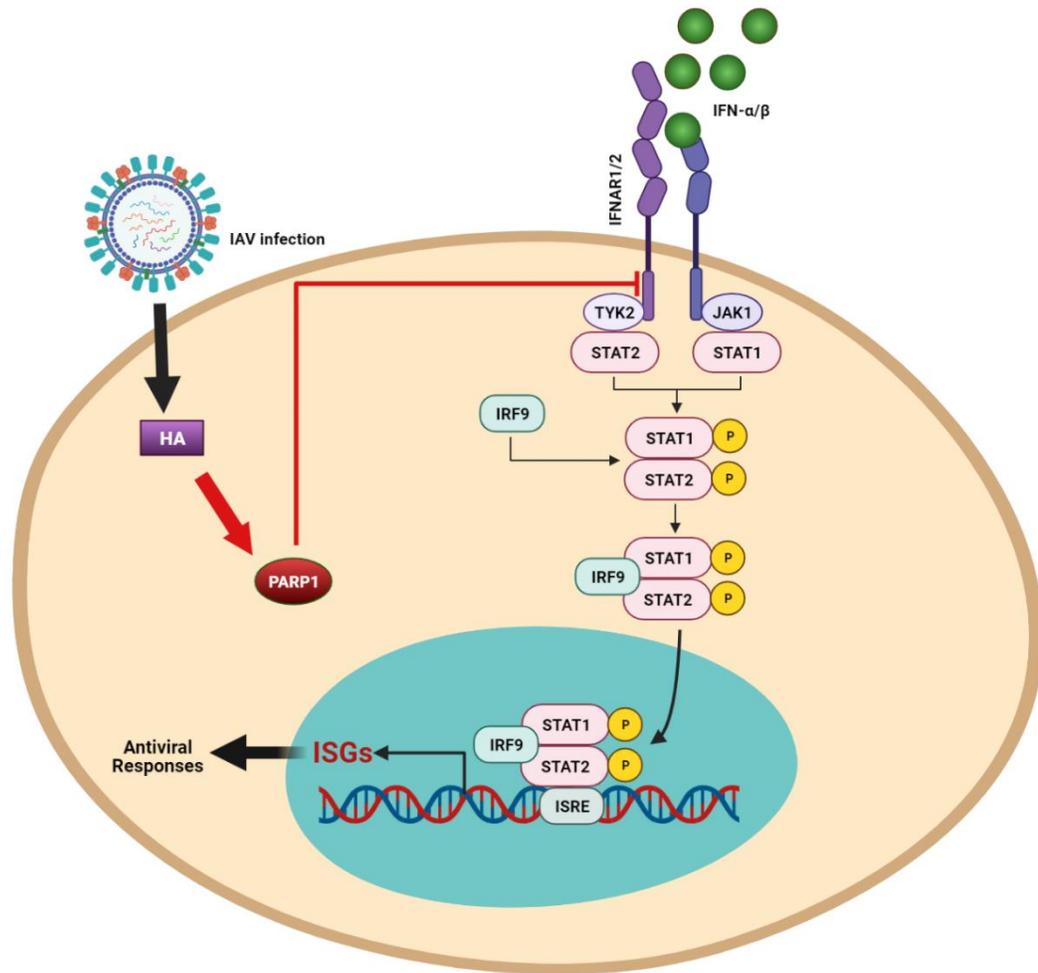


Figure 33: PARP1 enables IAV HA-induced IFNAR1 degradation. IAV infection induces the degradation of type I IFN receptor IFNAR1 using viral HA protein. However, PARP1 is key to IFNAR1 downregulation mediated by IAV HA.

Utilizing viral HA as bait and subsequent mass spec analysis led to the identification of PARP1 as an interacting partner (data not shown). PARP1 is commonly known as a chromatin-associated protein and mainly exists in the nucleus under normal conditions (Liu and Yu, 2015). However, it has long been questioned whether it could also localize in other cellular compartments (Scovassi, 2004). Certain conditions, such as oxidative stress, have been reported to induce the nuclear-to-cytoplasm release of PARP1, which relies on the interaction between PARP1 and ring finger protein 146 (RNF146) (Gerö et al., 2014). PARP1 has also been shown to interact with mitofilin, a mitochondrial protein, and localizes in mitochondria where it plays a role in the maintenance of mitochondrial DNA integrity (Rossi et al., 2009). Nuclear-cytoplasmic expression of PARP1 has also been shown to occur in breast cancer (Donizy et al., 2014) as well as pancreatic cancer in human patients (Xu et al., 2019). Additionally, PARP1 localization is cytoplasmic during HIV-1 Vpr expression in cells (Muthumani et al., 2006), which could indicate that proteins of other viruses also have the potential to affect PARP1 localization. These findings suggest that PARP1 could have several functions in the host cell cytoplasm during different viral infections, which requires further examination.

PARP1 and PARP13.1 have been previously shown to regulate the function of influenza viral polymerases (Bortz et al., 2011; Liu et al., 2015; Westera et al., 2019). However, under our experimental conditions, PARP1 interacted with HA during IAV infection and facilitated IFNAR1 degradation, regulating host antiviral IFN responses. Notably, PARP1 localization became nuclear-cytoplasmic during both IAV HA expression and IAV infection (Figure 30 and 31). Our results as well as the findings of others indicate the possibility that PARP1 is utilized in multiple ways to enhance influenza virus infection. Our results show an approximate two-fold difference in IAV production during the downregulation of IFNAR1 and treatment with PARP1 inhibitor (Figure 32B and 32C).

While these results are not statistically significant under our experimental conditions, they could correspond to the known effects of IAV polymerase interacting with PARP1 (Westera et al., 2019). It is likely that the presence of PARP1 in the nucleus and cytoplasm could also have different but necessary effects for robust influenza virus replication.

Cell surface levels of IFNAR1 are correlated with type I IFN mediated antiviral responses (Fuchs, 2013). Lack of IFNAR1 on the cell surface desensitizes cells to type I IFNs, creating a more favorable environment for viral replication. In this study, we identified host PARP1 as a pro-IAV factor that regulates viral propagation via controlling IFNAR1 degradation. Pharmacologic inhibition or PARP1 knockdown strongly suppressed IAV replication (Figure 28 and Figure 29). Thus, PARP1 may represent a new cellular target for controlling influenza virus propagation. Since known PARP1 inhibitors are currently used to treat cancer patients in the clinic, it would be worthwhile to test the effects of these inhibitors on influenza virus infection.

In conclusion, our work reveals the importance of host protein PARP1 in mediating IAV HA-induced degradation of IFNAR1, therefore regulating IAV propagation. This study expands our knowledge of the interactions between IAV and the host and unveils novel cellular targets that have the potential to be novel therapeutics to control influenza viral replication.

IV-F. Acknowledgements

The work was supported by University of Missouri (B.H.). We thank Dr. Serge Fuchs (UPenn) and the NIH Vaccine Research Center for the kind provision of reagents as described in the materials/methods section. We also thank the University of Missouri Proteomics Core Facility for processing and analyzing the mass spectrometry data. We thank the University of Missouri Molecular Cytology Core Facility and Dr. Michael Baldwin (University of Missouri) for helping with the confocal fluorescence microscopy. Figures and text from this dissertation chapter were published in Journal of Virology (Xia et al., 2020). American Society for Microbiology journals permits authors to use the full published article in dissertations.

IV-F. Data Collection and Analysis

Jennifer J. Wolf: Investigation, Validation, Formal analysis, Writing-Original draft preparation, Writing - Review & Editing, Conceptualization, Visualization. **Chuan Xia:** Investigation, Validation, Formal analysis, Writing-Original draft preparation, Writing - Review & Editing, Conceptualization, Visualization. **Caleb J Studstill:** Investigation, Conceptualization. **Ngo H:** Investigation. **Hahm B:** Funding acquisition, Project administration, Supervision, Writing - Review & Editing, Conceptualization, Visualization.

Chapter V. Discussion

Viral infection is an intricate web of interactions where the host attempts to eliminate the virus while the virus must circumvent host defenses in order to further its replication. There are still many virus-host interactions which remain unknown. In this work, IAV has been shown to interact with the host in various ways to promote its replication. IAV dampens the type I IFN innate immune response by using its NS1 protein to downregulate SPL and by using host PARP1 in an IAV HA-dependent manner to promote the degradation of IFNAR1. Additionally, IAV utilizes host SK2 to further its replication, increasing SK2 expression in a MEK/ERK-dependent manner.

Our study showed that the anti-influenza activity of SPL was applicable in primary human tracheal epithelial cells (Figure 9A and 9B). However, the effect of SPL during infection in mice remains unknown. This is due to SPL KO mice dying when they reach 2-4 weeks of age (Fyrst and Saba, 2008), which makes it difficult to study the role of SPL in antiviral immunity and host innate immune signaling. However, SPL gene (*Sgpl1*) floxed mice have been developed in the lab of Dr. Julie D. Saba, and these conditional SPL KO mice will be useful to study SPL antiviral activity (Degagné et al., 2014; Zamora-Pineda et al., 2016). SPL was shown to have antiviral activity against IAV subtypes H1N1 and H3N2, IBV, as well as measles virus (Figure 7, Figure 10, and Figure 11). However, it is yet to be determined whether SPL has antiviral capabilities against other respiratory RNA viruses such as SARS-CoV-2 or more pathogenic influenza viruses such as IAV H5N1.

Previous immunofluorescence studies in our lab revealed that IKK ϵ and SPL complex and form punctate structures when they are both overexpressed. While not shown with immunofluorescence, MAVS has been shown with co-IP analysis to complex with IKK ϵ and SPL (data not shown). However, the structural basis behind the interaction is unknown. It would be interesting to further characterize and refine the interaction

between IKK ϵ and SPL by using structural methods, such as cryogenic transmission electron microscopy or crystallography, in order to more completely understand the structural basis behind it. It is also unknown where this interaction happens in the cell. Cellular sensing of viral RNAs could possibly induce post-translational modification or re-localization of SPL to specific sites for interaction with IKK ϵ . While SPL was not phosphorylated upon infection (data not shown), further investigation into these possibilities may be required to more fully understand the antiviral function of SPL.

Although we have shown that SPL is degraded by IAV NS1, it is yet to be determined which portion of NS1 is necessary for this downregulation to occur. The domains of NS1 could be tested for their ability to induce the ubiquitination and degradation of endogenous SPL. Conducting further deletion and mutation analyses based on those results could identify the amino acid position of NS1 that is important for SPL degradation during IAV infection. After determining the necessary amino acid of NS1, small molecule inhibitors could be developed based on that, which should have antiviral effects. Likewise, it is also unknown which amino acid residue of SPL is ubiquitinated on during NS1 overexpression or IAV infection, and determining this will help to further clarify the mechanism for SPL ubiquitination.

IAV NS1 was found to promote the ubiquitination and subsequent degradation of SPL. However, the exact mechanism for this is unclear. Preliminary data have indicated that host SYVN1 could mediate NS1-induced SPL ubiquitination and downregulation. However, further research is required to confirm this. In co-IP analyses, SYVN1, but not NS1, was pulled down with FLAG-SPL during transient NS1 overexpression. SYVN1 could interact transiently with NS1, or there could potentially be another host protein mediating this. IAV infection has also been associated with global remodeling of the ER, resulting in IAV vRNPs and Rab11 at the membrane of a modified, tubulated ER that extends

throughout the cell, mediating the transport of vRNPs (de Castro Martin et al., 2017). Therefore, it is a possibility this remodeling could alter the observed SPL degradation over time. Performing co-IP experiments with FLAG-tagged SYVN1 or FLAG-tagged SPL during IAV NS1 expression with subsequent proteomic analysis could provide further insight as to if there are other proteins that could mediate NS1-induced downregulation of SPL.

Further experiments are necessary for determining the mechanism behind NS1-induced SPL degradation. To clarify the role of NS1 in SPL downregulation, NS1 mutants could be utilized to determine which portion of NS1 is essential for SPL ubiquitination and downregulation. Expressing various NS1 mutant proteins in A549 cells and either subsequent denatured IP analyses of FLAG-tagged SPL and measurement of ubiquitination of SPL or measurement of endogenous SPL protein levels could further examine this. Furthermore, it could be useful to assess which amino acid residues of SPL are ubiquitinated using proteomic analysis. FLAG-tagged SPL could be harvested using denatured IP after expression of wild type NS1 and compared to a control harvested without NS1 expression. Proteomics could be performed using the MU proteomics core, and this experiment could be performed either with transfection of IAV NS1-expressing plasmid or infection with either wild type or Δ NS1 IAV. Proteomics analysis could also be used after co-IP of FLAG-SPL during infection or NS1 expression to assess SPL binding partners. Host SYVN1 could be confirmed to have involvement with NS1-mediated SPL ubiquitination by using denatured IP and either knockdown or inhibition of either host SYVN1 or another ubiquitin ligase as a control. A similar experiment could be performed to confirm if host SYVN1 is necessary for NS1-induced SPL degradation. Additionally, co-IP analysis could be performed with a FLAG-tagged NS1 to determine if IAV NS1 interacts with SYVN1 either during NS1 expression or during infection. FLAG-tagged NS1 could

also be used to confirm that IAV NS1 does not interact with host SPL, since the larger size of tagged NS1 will ensure greater separation of NS1 from light chain IgG.

Inhibition of ERK prior to infection was shown to interfere with the IAV-induced increase of SK2 and pSK2 levels throughout infection (Figure 26). This result indicates that ERK activation is important for IAV-induced upregulation of SK2 and pSK2. However, it is yet to be determined whether ERK1 or ERK2 themselves are important for increasing SK2 and pSK2 levels during IAV infection. ERK has been previously shown to be activated in a biphasic manner upon infection (Marjuki et al., 2011), which could mean that the increase in SK2 and pSK2 levels observed during infection could also be biphasic. Indeed, our western blotting data (Figure 26) indicate an increase at 2 hpi and 8 hpi. We also observed an increase in SK2 mRNA at 8 hpi (data not shown), which could correlate with the observed increase in SK2 protein levels. However, the observed increase in SK2 levels at 2 hpi suggests that there may be another mechanism for increased SK2 and pSK2 levels. One possibility is that SK2 may interact with ERK1/2, increasing SK2 stability and activation. ERK has been previously reported to be critical for the export of vRNPs from the nucleus and other pathways, but the underlying mechanism is not fully understood. If ERK's pro-influenza activity is largely dependent on SK2 activation, then ERK inhibition would have little effect on IAV replication in SK2-deficient cells. However, ERK may have multiple downstream targets, and SK2 could have specific functions that regulate IAV replication. Further investigation may be required to fully assess this.

Expression of p21 was impaired by SK2 inhibition specifically during IAV infection (Figure 27), which suggests that SK2 regulates p21 during infection. This has implications for SK2 modulating the expression of genes important for IAV replication, especially since SK2 and S1P have been previously shown to interact with HDAC 1/2 in the nucleus upon PMA stimulation, which subsequently regulated the expression of genes such as p21 (Hait

et al., 2009). Because IAV has been previously shown to induce temporal cell cycle arrest in the G0/G1 phase in a p21-dependent manner (He et al., 2010), the next logical step would be to determine if SK2 is mediating IAV-induced cell cycle arrest. A study has shown that SK2 binds to and inhibits HDAC 1/2 in breast cancer cells during transient overexpression of SK2 (Hait et al., 2009). Thus, investigating if SK2 interacts with HDAC 1/2 during infection is another avenue of interest. SK2 may regulate p21 at the transcriptional level. If this is determined with RT-PCR, it could be worthwhile to research if SK2 could be affecting histone acetylation during infection in DNA regions associated with p21 transcription complex binding. We ultimately anticipate that during infection, SK2 binds to HDAC 1/2 as well as promoters of p21 to regulate histone acetylation and gene expression.

SK2 and SPL are primarily known for their ability to metabolize S1P. SK2 phosphorylates sphingosine to make S1P, and SPL irreversibly catabolizes S1P into hexadecenal and phosphoethanolamine. S1P has been shown to have many functions in the cell and organism. However, exogenously supplied S1P did not alter IAV replication, indicating that extracellular S1P does not influence IAV replication (Marsolais et al., 2009; Seo et al., 2010). Furthermore, an SPL mutant incapable of metabolizing S1P was shown to still have antiviral activity against SPL (Vijayan et al., 2017). While S1P metabolism does not appear to affect the antiviral activity of SPL, it is yet to be determined if the pro-influenza activity of SK2 is dependent upon intracellular S1P metabolism.

IAV HA was shown to induce accumulation of PARP1 in cytoplasm and partially colocalize with PARP1 in the cytoplasm (Figure 30 and 31), suggesting that the association occurs transiently to elicit further signaling events in order to induce IFNAR1 degradation. It is currently unclear how IAV HA manipulates PARP1 to trigger IFNAR1 degradation. PARP1 can regulate various cellular processes (Chaitanya et al., 2010; Du

et al., 2016; Kaneko et al., 2016; Liu and Yu, 2015) and modify other proteins through poly ADP-ribosylation, a posttranslational modification (Liu and Yu, 2015). Upon IAV infection, PARP1 in the cytoplasm could trigger the activation of PARP1 ADP-ribose polymerase, leading to the modification of an unknown protein involved in the IFNAR1 degradation process. Use of inhibitors blocking PARP1 activation indicates that PARP1 activity is crucial for HA-induced IFNAR1 degradation (Figure 29). Poly-ADP ribosylation leads substrates to proteasomal degradation (Levaot et al., 2011; Li et al., 2015) under certain conditions. Since IAV HA-induced IFNAR1 degradation is dependent on both proteasomal and lysosomal pathways (Xia et al., 2016), it would be interesting to investigate the involvement of PARP1 in the IFNAR1 degradation pathways. It is possible that poly-ADP ribosylation of IFNAR1 or its unknown regulatory protein is required prior to the degradation process during IAV infection. A recent study has shown that another PARP family member, PARP11, inhibits the type I interferon-mediated response by catalyzing mono-ADP-ribosylation of E3 ligase β -TrCP and, in turn, promotes the ubiquitination and degradation of IFNAR1 (Guo et al., 2019). It would therefore be of interest to determine whether PARP1 and PARP11 cooperate, leading to IFNAR1 degradation, or if they have distinct functions to regulate IFNAR1, enhancing viral infection. Another possibility is that PARP1 induces the activation of a kinase such as CK1 α by poly ADP-ribosylation. The kinase could then phosphorylate IFNAR1, leading to degradation of the receptor. It is possible that PARP1 could function to regulate phosphorylation, ubiquitination, or degradation of IFNAR1, but further investigation is required to determine this.

Our work reveals novel insight into host-influenza interactions, particularly how IAV evades and manipulates the host system to further its own replication. Antiviral therapeutics to treat IAV infection can have a limited scope because the high mutability of

IAV allows it to quickly develop resistance. Targeting host factors is advantageous because it has the potential to counteract several different subtypes of IAV. Understanding how IAV interacts with the host gives us a more comprehensive view of infection and allows us to determine new targets for potential therapeutics to more effectively control influenza. Thus, identifying host mediators of viral replication is a promising avenue of research that ultimately has potential therapeutic applications.

References

- Aaronson, D.S., Horvath, C.M., 2002. A road map for those who don't know JAK-STAT. *Science* 296, 1653–1655. <https://doi.org/10.1126/science.1071545>
- Adeyemi, R.O., Fuller, M.S., Pintel, D.J., 2014. Efficient Parvovirus Replication Requires CRL4Cdt2-Targeted Depletion of p21 to Prevent Its Inhibitory Interaction with PCNA. *PLoS Pathog.* 10, e1004055. <https://doi.org/10.1371/journal.ppat.1004055>
- Aguilar, A., Saba, J.D., 2012. Truth and consequences of sphingosine-1-phosphate lyase. *Adv. Biol. Regul.* 52, 17–30. <https://doi.org/10.1016/j.advenzreg.2011.09.015>
- Alexis Dinno, 2015. Conover-Iman test of multiple comparisons using rank sums.
- Alshaker, H., Sauer, L., Monteil, D., Ottaviani, S., Srivats, S., Böhler, T., Pchejetski, D., 2013. Therapeutic Potential of Targeting SK1 in Human Cancers, in: *Advances in Cancer Research*. Academic Press Inc., pp. 143–200. <https://doi.org/10.1016/B978-0-12-394274-6.00006-6>
- Alvarez, S.E., Harikumar, K.B., Hait, N.C., Allegood, J., Strub, G.M., Kim, E.Y., MacEyka, M., Jiang, H., Luo, C., Kordula, T., Milstien, S., Spiegel, S., 2010. Sphingosine-1-phosphate is a missing cofactor for the E3 ubiquitin ligase TRAF2. *Nature* 465, 1084–1088. <https://doi.org/10.1038/nature09128>
- Aragón, T., de la Luna, S., Novoa, I., Carrasco, L., Ortín, J., Nieto, A., 2000. Eukaryotic Translation Initiation Factor 4GI Is a Cellular Target for NS1 Protein, a Translational Activator of Influenza Virus. *Mol. Cell. Biol.* 20, 6259–6268. <https://doi.org/10.1128/mcb.20.17.6259-6268.2000>
- Ayllon, J., García-Sastre, A., 2015. The NS1 protein: A multitasking virulence factor.

- Curr. Top. Microbiol. Immunol. 386, 73–107. https://doi.org/10.1007/82_2014_400
- Bamborschke, D., Pergande, M., Becker, K., Koerber, F., Dötsch, J., Vierzig, A., Weber, L.T., Cirak, S., 2018. A novel mutation in sphingosine-1-phosphate lyase causing congenital brain malformation. *Brain Dev.* 40, 480–483.
<https://doi.org/10.1016/j.braindev.2018.02.008>
- Bandhuvula, P., Saba, J.D., 2007. Sphingosine-1-phosphate lyase in immunity and cancer: silencing the siren. *Trends Mol. Med.* 13, 210–217.
<https://doi.org/10.1016/j.molmed.2007.03.005>
- Banerjee, I., Miyake, Y., Philip Nobs, S., Schneider, C., Horvath, P., Kopf, M., Matthias, P., Helenius, A., Yamauchi, Y., 2014. Influenza A virus uses the aggresome processing machinery for host cell entry. *Science* 346, 473–477.
<https://doi.org/10.1126/science.1257037>
- Banerjee, I., Yamauchi, Y., Helenius, A., Horvath, P., 2013. High-Content Analysis of Sequential Events during the Early Phase of Influenza A Virus Infection. *PLoS One* 8, 68450. <https://doi.org/10.1371/journal.pone.0068450>
- Barro, M., Patton, J.T., 2005. Rotavirus nonstructural protein 1 subverts innate immune response by inducing degradation of IFN regulatory factor 3. *Proc. Natl. Acad. Sci. U. S. A.* 102, 4114–4119. <https://doi.org/10.1073/pnas.0408376102>
- Benedetti, M.D., Marangi, A., Bozzetti, S., Gobbin, F., Turatti, M., Pea, M., Gajofatto, A., Mocella, S., 2018. HPV-related papillary squamous cell carcinoma of the tonsil during treatment with fingolimod. *Mult. Scler. Relat. Disord.* 23, 24–26.
<https://doi.org/10.1016/j.msard.2018.04.018>
- Bergmann, M., Garcia-Sastre, A., Carnero, E., Pehamberger, H., Wolff, K., Palese, P.,

- Muster, T., 2000. Influenza Virus NS1 Protein Counteracts PKR-Mediated Inhibition of Replication. *J. Virol.* 74, 6203–6206. <https://doi.org/10.1128/jvi.74.13.6203-6206.2000>
- Bhattacharya, S., Qian, J., Tzimas, C., Baker, D.P., Koumenis, C., Diehl, J.A., Fuchs, S.Y., 2011. Role of p38 protein kinase in the ligand-independent ubiquitination and down-regulation of the IFNAR1 chain of type I interferon receptor. *J. Biol. Chem.* 286, 22069–22076. <https://doi.org/10.1074/jbc.M111.238766>
- Blanton, L., Dugan, V.G., Abd Elal, A.I., Alabi, N., Barnes, J., Brammer, L., Budd, A.P., Burns, E., Cummings, C.N., Garg, S., Garten, R., Gubareva, L., Kniss, K., Kramer, N., O'Halloran, A., Reed, C., Rolfes, M., Sessions, W., Taylor, C., Xu, X., Fry, A.M., Wentworth, D.E., Katz, J., Jernigan, D., 2019. Update: Influenza Activity — United States, September 30, 2018–February 2, 2019. *MMWR. Morb. Mortal. Wkly. Rep.* 68, 125–134. <https://doi.org/10.15585/mmwr.mm6806a1>
- Bolte, S., Cordelières, F.P., 2006. A guided tour into subcellular colocalization analysis in light microscopy. *J. Microsc.* 224, 213–232. <https://doi.org/10.1111/j.1365-2818.2006.01706.x>
- Bornholdt, Z.A., Prasad, B.V.V., 2006. X-ray structure of influenza virus NS1 effector domain. *Nat. Struct. Mol. Biol.* 13, 559–560. <https://doi.org/10.1038/nsmb1099>
- Bortz, E., Westera, L., Maamary, J., Steel, J., Albrecht, R.A., Manicassamy, B., Chase, G., Martínez-Sobrido, L., Schwemmle, M., García-Sastrea, A., 2011. Host- and strain-specific regulation of influenza virus polymerase activity by interacting cellular proteins. *MBio.* 2, e00151–11. <https://doi.org/10.1128/mBio.00151-11>
- Bourquin, F., Riezman, H., Capitani, G., Grütter, M.G., 2010. Structure and function of sphingosine-1-phosphate lyase, a key enzyme of sphingolipid metabolism.

Structure 18, 1054–1065. <https://doi.org/10.1016/j.str.2010.05.011>

Brinkmann, V., Billich, A., Baumruker, T., Heining, P., Schmouder, R., Francis, G., Aradhye, S., Burtin, P., 2010. Fingolimod (FTY720): Discovery and development of an oral drug to treat multiple sclerosis, *Nature Reviews Drug Discovery*. *Nat Rev Drug Discov.* 9, 883–897. <https://doi.org/10.1038/nrd3248>

Brinkmann, V., Davis, M.D., Heise, C.E., Albert, R., Cottens, S., Hof, R., Bruns, C., Prieschl, E., Baumruker, T., Hiestand, P., Foster, C.A., Zollinger, M., Lynch, K.R., 2002. The immune modulator FTY720 targets sphingosine 1-phosphate receptors. *J. Biol. Chem.* 277, 21453–21457. <https://doi.org/10.1074/jbc.C200176200>

Britten, C.D., Garrett-Mayer, E., Chin, S.H., Shirai, K., Ogretmen, B., Bentz, T.A., Brisendine, A., Anderton, K., Cusack, S.L., Maines, L.W., Zhuang, Y., Smith, C.D., Thomas, M.B., 2017. A phase I study of ABC294640, a first-in-class sphingosine kinase-2 inhibitor, in patients with advanced solid tumors. *Clin. Cancer Res.* 23, 4642–4650. <https://doi.org/10.1158/1078-0432.CCR-16-2363>

Brooke, C.B., 2017. Population Diversity and Collective Interactions during Influenza Virus Infection. *J. Virol.* 91. e00164–17. <https://doi.org/10.1128/jvi.01164-17>

Camerer, E., Regard, J.B., Cornelissen, I., Srinivasan, Y., Duong, D.N., Palmer, D., Pham, T.H., Wong, J.S., Pappu, R., Coughlin, S.R., 2009. Sphingosine-1-phosphate in the plasma compartment regulates basal and inflammation-induced vascular leak in mice. *J. Clin. Invest.* 119, 1871–1879. <https://doi.org/10.1172/jci38575>

Carrillo, B., Choi, J.-M., Bornholdt, Z.A., Sankaran, B., Rice, A.P., Prasad, B.V. V., 2014. The Influenza A Virus Protein NS1 Displays Structural Polymorphism. *J. Virol.* 88, 4113–4122. <https://doi.org/10.1128/jvi.03692-13>

- Chaitanya, G.V., Alexander, J.S., Babu, P.P., 2010. PARP-1 cleavage fragments: Signatures of cell-death proteases in neurodegeneration. *Cell Commun. Signal.* <https://doi.org/10.1186/1478-811X-8-31>
- Chakrabarti, A.K., Pasricha, G., 2013. An insight into the PB1F2 protein and its multifunctional role in enhancing the pathogenicity of the influenza A viruses. *Virology.* 440, 97–104. <https://doi.org/10.1016/j.virol.2013.02.025>
- Chen, R.H., Chen, Y.H., Huang, T.Y., 2019. Ubiquitin-mediated regulation of autophagy. *J. Biomed. Sci.* 26, 80. <https://doi.org/10.1186/s12929-019-0569-y>
- Cheng, P.K.C., To, A.P.C., Leung, T.W.C., Leung, P.C.K., Lee, C.W.C., Lim, W.W.L., 2010. Oseltamivir- and amantadine-resistant influenza virus A H1N1. *Emerg. Infect. Dis.* 16, 155–156. <https://doi.org/10.3201/eid1601.091304>
- Chesarino, N.M., McMichael, T.M., Yount, J.S., 2015. E3 Ubiquitin Ligase NEDD4 Promotes Influenza Virus Infection by Decreasing Levels of the Antiviral Protein IFITM3. *PLoS Pathog.* 11, e1005095. <https://doi.org/10.1371/journal.ppat.1005095>
- Chi, H., 2011. Sphingosine-1-phosphate and immune regulation: Trafficking and beyond. *Trends Pharmacol. Sci.* 32, 16–24. <https://doi.org/10.1016/j.tips.2010.11.002>
- Chien, C.Y., Tejero, R., Huang, Y., Zimmerman, D.E., Rios, C.B., Krug, R.M., Montelione, G.T., 1997. A novel RNA-binding motif in influenza A virus non-structural protein 1. *Nat. Struct. Biol.* 4, 891–895. <https://doi.org/10.1038/nsb1197-891>
- Choi, Y.J., Saba, J.D., 2019. Sphingosine phosphate lyase insufficiency syndrome (SPLIS): A novel inborn error of sphingolipid metabolism. *Adv. Biol. Regul.* 71, 128–140. <https://doi.org/10.1016/j.jbior.2018.09.004>

- Clark, C.C., Weitzel, J.N., O'Connor, T.R., 2012. Enhancement of synthetic lethality via combinations of ABT-888, a PARP inhibitor, and carboplatin In Vitro and in Vivo using BRCA1 and BRCA2 isogenic models. *Mol. Cancer Ther.* 11, 1948–1958. <https://doi.org/10.1158/1535-7163.MCT-11-0597>
- Cooper, G.M., 2000. *The Cell: A Molecular Approach. Lysosomes*, 2nd ed. Sinauer Associates, Sunderland (MA).
- Cowling, B.J., Jin, L., Lau, E.H.Y., Liao, Q., Wu, P., Jiang, H., Tsang, T.K., Zheng, J., Fang, V.J., Chang, Z., Ni, M.Y., Zhang, Q., Ip, D.K.M., Yu, J., Li, Y., Wang, L., Tu, W., Meng, L., Wu, J.T., Luo, H., Li, Q., Shu, Y., Li, Z., Feng, Z., Yang, W., Wang, Y., Leung, G.M., Yu, H., 2013. Comparative epidemiology of human infections with avian influenza A H7N9 and H5N1 viruses in China: A population-based study of laboratory-confirmed cases. *Lancet* 382, 129–137. [https://doi.org/10.1016/S0140-6736\(13\)61171-X](https://doi.org/10.1016/S0140-6736(13)61171-X)
- Cuvillier, O., Pirianov, G., Kleuser, B., Vanek, P.G., Cosot, O.A., Gutkind, J.S., Spiegel, S., 1996. Suppression of ceramide-mediated programmed cell death by sphingosine- 1-phosphate. *Nature* 381, 800–803. <https://doi.org/10.1038/381800a0>
- Dai, L., Plaisance-Bonstaff, K., Voelkel-Johnson, C., Smith, C.D., Ogretmen, B., Qin, Z., Parsons, C., 2014. Sphingosine kinase-2 maintains viral latency and survival for KSHV-infected endothelial cells. *PLoS One* 9, e102314. <https://doi.org/10.1371/journal.pone.0102314>
- Davidson, S., 2018. Treating influenza infection, from now and into the future. *Front. Immunol.* 9, 1946. <https://doi.org/10.3389/fimmu.2018.01946>
- de Brito, O.M., Scorrano, L., 2009. Mitofusin-2 regulates mitochondrial and endoplasmic reticulum morphology and tethering: The role of Ras. *Mitochondrion* 9, 222–226.

<https://doi.org/10.1016/j.mito.2009.02.005>

de Castro Martin, I.F., Fournier, G., Sachse, M., Pizarro-Cerda, J., Risco, C., Naffakh, N., 2017. Influenza virus genome reaches the plasma membrane via a modified endoplasmic reticulum and Rab11-dependent vesicles. *Nat. Commun.* 8, 1396. <https://doi.org/10.1038/s41467-017-01557-6>

de la Luna, S., Fortes, P., Beloso, A., Ortín, J., 1995. Influenza virus NS1 protein enhances the rate of translation initiation of viral mRNAs. *J. Virol.* 69, 2427–2433. <https://doi.org/10.1128/jvi.69.4.2427-2433.1995>

Degagné, E., Pandurangan, A., Bandhuvula, P., Kumar, A., Eltanawy, A., Zhang, M., Yoshinaga, Y., Nefedov, M., De Jong, P.J., Fong, L.G., Young, S.G., Bittman, R., Ahmedi, Y., Saba, J.D., 2014. Sphingosine-1-phosphate lyase downregulation promotes colon carcinogenesis through STAT3-activated microRNAs. *J. Clin. Invest.* 124, 5368–5384. <https://doi.org/10.1172/JCI74188>

Dharan, N.J., Gubareva, L. V., Meyer, J.J., Margaret, Okomo-Adhiambo, McClinton, R.C., Marshall, S.A., George, K.S., Epperson, S., Brammer, L., Klimov, A.I., Bresee, J.S., Fry, A.M., 2009. Infections with oseltamivir-resistant influenza A(H1N1) virus in the united states. *JAMA - J. Am. Med. Assoc.* 301, 1034–1041. <https://doi.org/10.1001/jama.2009.294>

Dickinson, J.D., Sweeter, J.M., Warren, K.J., Ahmad, I.M., De Deken, X., Zimmerman, M.C., Brody, S.L., 2018. Autophagy regulates DUOX1 localization and superoxide production in airway epithelial cells during chronic IL-13 stimulation. *Redox Biol.* 14, 272–284. <https://doi.org/10.1016/j.redox.2017.09.013>

Don, A.S., Martinez-Lamenca, C., Webb, W.R., Proia, R.L., Roberts, E., Rosen, H., 2007. Essential requirement for sphingosine kinase 2 in a sphingolipid apoptosis

pathway activated by FTY720 analogues. *J. Biol. Chem.* 282, 15833–15842.

<https://doi.org/10.1074/jbc.M609124200>

Donizy, P., Pietrzyk, G., Halon, A., Kozyra, C., Gansukh, T., Lage, H., Surowiak, P., Matkowski, R., 2014. Nuclear-cytoplasmic PARP-1 expression as an unfavorable prognostic marker in lymph node-negative early breast cancer: 15-year follow-up. *Oncol. Rep.* 31, 1777–1787. <https://doi.org/10.3892/or.2014.3024>

Du, Y., Yamaguchi, H., Wei, Y., Hsu, J.L., Wang, H.L., Hsu, Y.H., Lin, W.C., Yu, W.H., Leonard, P.G., Lee, G.R., Chen, M.K., Nakai, K., Hsu, M.C., Chen, C. Te, Sun, Y., Wu, Y., Chang, W.C., Huang, W.C., Liu, C.L., Chang, Y.C., Chen, C.H., Park, M., Jones, P., Hortobagyi, G.N., Hung, M.C., 2016. Blocking c-Met-mediated PARP1 phosphorylation enhances anti-tumor effects of PARP inhibitors. *Nat. Med.* 22, 194–201. <https://doi.org/10.1038/nm.4032>

Eisfeld, A.J., Neumann, G., Kawaoka, Y., 2014. Influenza A virus isolation, culture and identification. *Nat. Protoc.* 9, 2663–2681. <https://doi.org/10.1038/nprot.2014.180>

Falcón, A.M., Marión, R.M., Zürcher, T., Gómez, P., Portela, A., Nieto, A., Ortín, J., 2004. Defective RNA Replication and Late Gene Expression in Temperature-Sensitive Influenza Viruses Expressing Deleted Forms of the NS1 Protein. *J. Virol.* 78, 3880–3888. <https://doi.org/10.1128/jvi.78.8.3880-3888.2004>

Feng, L., Zhang, J., Zhu, N., Ding, Q., Zhang, X., Yu, J., Qiang, W., Zhang, Z., Ma, Y., Huang, D., Shen, Yujun, Fang, S., Yu, Y., Wang, H., Shen, Yuxian, 2017. Ubiquitin ligase SYVN1/HRD1 facilitates degradation of the SERPINA1 Z variant/ α -1-antitrypsin Z variant via SQSTM1/p62-dependent selective autophagy. *Autophagy* 13, 686–702. <https://doi.org/10.1080/15548627.2017.1280207>

Foot, N., Henshall, T., Kumar, S., 2017. Ubiquitination and the regulation of membrane

proteins. *Physiol. Rev.* 97, 253–281. <https://doi.org/10.1152/physrev.00012.2016>

Fraser, C., Donnelly, C.A., Cauchemez, S., Hanage, W.P., Van Kerkhove, M.D., Hollingsworth, T.D., Griffin, J., Baggaley, R.F., Jenkins, H.E., Lyons, E.J., Jombart, T., Hinsley, W.R., Grassly, N.C., Balloux, F., Ghani, A.C., Ferguson, N.M., Rambaut, A., Pybus, O.G., Lopez-Gatell, H., Alpuche-Aranda, C.M., Chapela, I.B., Zavala, E.P., Ma. Espejo Guevara, D., Checchi, F., Garcia, E., Hugonnet, S., Roth, C., 2009. Pandemic potential of a strain of influenza A (H1N1): Early findings. *Science* 324, 1557–1561. <https://doi.org/10.1126/science.1176062>

French, K.J., Schrecengost, R.S., Lee, B.D., Zhuang, Y., Smith, S.N., Eberly, J.L., Yun, J.K., Smith, C.D., 2003. Discovery and Evaluation of Inhibitors of Human Sphingosine Kinase 1, *Cancer Res.* 63, 5962–5969.

French, M.E., Koehler, C.F., Hunter, T., 2021. Emerging functions of branched ubiquitin chains. *Cell Discov.* <https://doi.org/10.1038/s41421-020-00237-y>

Fuchs, S.Y., 2013. Hope and fear for interferon: The receptor-centric outlook on the future of interferon therapy. *J. Interf. Cytokine Res.* <https://doi.org/10.1089/jir.2012.0117>

Fyrst, H., Saba, J.D., 2010. An update on sphingosine-1-phosphate and other sphingolipid mediators. *Nat. Chem. Biol.* <https://doi.org/10.1038/nchembio.392>

Fyrst, H., Saba, J.D., 2008. Sphingosine-1-phosphate lyase in development and disease: Sphingolipid metabolism takes flight. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids.* <https://doi.org/10.1016/j.bbalip.2008.05.005>

Gack, M.U., Albrecht, R.A., Urano, T., Inn, K.S., Huang, I.C., Carnero, E., Farzan, M., Inoue, S., Jung, J.U., García-Sastre, A., 2009. Influenza A Virus NS1 Targets the

- Ubiquitin Ligase TRIM25 to Evade Recognition by the Host Viral RNA Sensor RIG-I. *Cell Host Microbe* 5, 439–449. <https://doi.org/10.1016/j.chom.2009.04.006>
- Gale, M., Foy, E.M., 2005. Evasion of intracellular host defence by hepatitis C virus. *Nature*. 436, 939–945. <https://doi.org/10.1038/nature04078>
- Gannagé, M., Dormann, D., Albrecht, R., Dengjel, J., Torossi, T., Rämer, P.C., Lee, M., Strowig, T., Arrey, F., Conenello, G., Pypaert, M., Andersen, J., García-Sastre, A., Münz, C., 2009. Matrix Protein 2 of Influenza A Virus Blocks Autophagosome Fusion with Lysosomes. *Cell Host Microbe* 6, 367–380. <https://doi.org/10.1016/j.chom.2009.09.005>
- Gao, S., Song, L., Li, J., Zhang, Z., Peng, H., Jiang, W., Wang, Q., Kang, T., Chen, S., Huang, W., 2012. Influenza A virus-encoded NS1 virulence factor protein inhibits innate immune response by targeting IKK. *Cell. Microbiol.* 14, 1849–1866. <https://doi.org/10.1111/cmi.12005>
- Garaigorta, U., Ortín, J., 2007. Mutation analysis of a recombinant NS replicon shows that influenza virus NS1 protein blocks the splicing and nucleo-cytoplasmic transport of its own viral mRNA. *Nucleic Acids Res.* 35, 4573–4582. <https://doi.org/10.1093/nar/gkm230>
- García-Sastre, A., 2011. Induction and evasion of type I interferon responses by influenza viruses. *Virus Res.* 162, 12. <https://doi.org/10.1016/J.VIRUSRES.2011.10.017>
- George, A.J., Hoffiz, Y.C., Charles, A.J., Zhu, Y., Mabb, A.M., 2018. A comprehensive atlas of E3 ubiquitin ligase mutations in neurological disorders. *Front. Genet.* <https://doi.org/10.3389/fgene.2018.00029>

- Gerö, D., Szoleczky, P., Chatzianastasiou, A., Papapetropoulos, A., Szabo, C., 2014. Modulation of poly(ADP-Ribose) polymerase-1 (PARP-1)-mediated oxidative cell injury by ring finger protein 146 (RNF146) in cardiac myocytes. *Mol. Med.* 20, 313–328. <https://doi.org/10.2119/molmed.2014.00102>
- Ghosh, R., Roy, S., Franco, S., 2018. PARP1 depletion induces RIG-I-dependent signaling in human cancer cells. *PLoS One* 13, e0194611. <https://doi.org/10.1371/journal.pone.0194611>
- Goldstein, G., Scheid, M., Hammerling, U., Schlesinger, D.H., Niall, H.D., Boyse, E.A., 1975. Isolation of a polypeptide that has lymphocyte differentiating properties and is probably represented universally in living cells. *Proc. Natl. Acad. Sci. U. S. A.* 72, 11–15. <https://doi.org/10.1073/pnas.72.1.11>
- Goubau, D., Deddouche, S., Reis e Sousa, C., 2013. Cytosolic Sensing of Viruses. *Immunity*. <https://doi.org/10.1016/j.immuni.2013.05.007>
- Grandvaux, N., TenOever, B.R., Servant, M.J., Hiscott, J., 2002. The interferon antiviral response: From viral invasion to evasion. *Curr. Opin. Infect. Dis.* <https://doi.org/10.1097/00001432-200206000-00008>
- Greenspan, D., Palese, P., Krystal, M., 1988. Two nuclear location signals in the influenza virus NS1 nonstructural protein. *J. Virol.* 62, 3020–3026. <https://doi.org/10.1128/jvi.62.8.3020-3026.1988>
- Grossmann, C., Ganem, D., 2008. Effects of NFκB activation on KSHV latency and lytic reactivation are complex and context-dependent. *Virology* 375, 94–102. <https://doi.org/10.1016/j.virol.2007.12.044>
- Guo, T., Zuo, Y., Qian, L., Liu, J., Yuan, Y., Xu, K., Miao, Y., Feng, Q., Chen, X., Jin, L.,

- Zhang, L., Dong, C., Xiong, S., Zheng, H., 2019. ADP-ribosyltransferase PARP11 modulates the interferon antiviral response by mono-ADP-ribosylating the ubiquitin E3 ligase β -TrCP. *Nat. Microbiol.* 1. <https://doi.org/10.1038/s41564-019-0428-3>
- Hait, N.C., Allegood, J., Maceyka, M., Strub, G.M., Harikumar, K.B., Singh, S.K., Luo, C., Marmorstein, R., Kordula, T., Milstien, S., Spiegel, S., 2009. Regulation of histone acetylation in the nucleus by sphingosine-1-phosphate. *Science* 325, 1254–7. <https://doi.org/10.1126/science.1176709>
- Hait, N.C., Bellamy, A., Milstien, S., Kordula, T., Spiegel, S., 2007. Sphingosine kinase type 2 activation by ERK-mediated phosphorylation. *J. Biol. Chem.* 282, 12058–12065. <https://doi.org/10.1074/jbc.M609559200>
- Hale, B.G., Albrecht, R.A., García-Sastre, A., 2010. Innate immune evasion strategies of influenza viruses. *Future Microbiol.* <https://doi.org/10.2217/fmb.09.108>
- Hale, B.G., Barclay, W.S., Randall, R.E., Russell, R.J., 2008a. Structure of an avian influenza A virus NS1 protein effector domain. *Virology* 378, 1–5. <https://doi.org/10.1016/j.virol.2008.05.026>
- Hale, B.G., Randall, R.E., Ortin, J., Jackson, D., 2008b. The multifunctional NS1 protein of influenza A viruses. *J. Gen. Virol.* <https://doi.org/10.1099/vir.0.2008/004606-0>
- Hao, R., Nanduri, P., Rao, Y., Panichelli, R.S., Ito, A., Yoshida, M., Yao, T.P., 2013. Proteasomes Activate Aggresome Disassembly and Clearance by Producing Unanchored Ubiquitin Chains. *Mol. Cell* 51, 819–828. <https://doi.org/10.1016/j.molcel.2013.08.016>
- Hasanifard, L., Sheervalilou, R., Majidinia, M., Yousefi, B., 2019. New insights into the roles and regulation of SphK2 as a therapeutic target in cancer chemoresistance. *J.*

Cell. Physiol. <https://doi.org/10.1002/jcp.27612>

- He, Y., Xu, K., Keiner, B., Zhou, J., Czudai, V., Li, T., Chen, Z., Liu, J., Klenk, H.-D., Shu, Y.L., Sun, B., 2010. Influenza A Virus Replication Induces Cell Cycle Arrest in G0/G1 Phase. *J. Virol.* 84, 12832–12840. <https://doi.org/10.1128/jvi.01216-10>
- Herr, D.R., Fyrst, H., Phan, V., Heinecke, K., Georges, R., Harris, G.L., Saba, J.D., 2003. Sply regulation of sphingolipid signaling molecules is essential for Drosophila development. *Development.* <https://doi.org/10.1242/dev.00456>
- Hobbelen, P.H.F., Elbers, A.R.W., Werkman, M., Koch, G., Velkers, F.C., Stegeman, A., Hagenaars, T.J., 2020. Estimating the introduction time of highly pathogenic avian influenza into poultry flocks. *Sci. Rep.* 10, 1–14. <https://doi.org/10.1038/s41598-020-68623-w>
- Hobson, J.P., Rosenfeldt, H.M., Barak, L.S., Olivera, A., Poulton, S., Caron, M.G., Milstien, S., Spiegel, S., 2001. Role of the sphingosine-1-phosphate receptor EDG-1 in PDGF-induced cell motility. *Science* 291, 1800–1803. <https://doi.org/10.1126/science.1057559>
- Hoffmann, H.H., Schneider, W.M., Rice, C.M., 2015. Interferons and viruses: An evolutionary arms race of molecular interactions. *Trends Immunol.* <https://doi.org/10.1016/j.it.2015.01.004>
- Hu, S., Yin, L., Mei, S., Li, J., Xu, F., Sun, H., Liu, X., Cen, S., Liang, C., Li, A., Guo, F., 2017. BST-2 restricts IAV release and is countered by the viral M2 protein. *Biochem. J.* 474, 715–730. <https://doi.org/10.1042/BCJ20160861>
- Huang, H., Chen, Y., Ye, J., 2007. Inhibition of hepatitis C virus replication by peroxidation of arachidonate and restoration by vitamin E. *Proc. Natl. Acad. Sci. U.*

S. A. 104, 18666–70. <https://doi.org/10.1073/pnas.0708423104>

Hussain, M., Galvin, H.D., Haw, T.Y., Nutsford, A.N., Husain, M., 2017. Drug resistance in influenza a virus: The epidemiology and management. *Infect. Drug Resist.*

<https://doi.org/10.2147/IDR.S105473>

Igarashi, N., Okada, T., Hayashi, S., Fujita, T., Jahangeer, S., Nakamura, S.I., 2003.

Sphingosine Kinase 2 Is a Nuclear Protein and Inhibits DNA Synthesis. *J. Biol.*

Chem. 278, 46832–46839. <https://doi.org/10.1074/jbc.M306577200>

Irwin, K.K., Renzette, N., Kowalik, T.F., Jensen, J.D., 2016. Antiviral drug resistance as

an adaptive process. *Virus Evol.* 2. <https://doi.org/10.1093/ve/vew014>

Isaacs, A., Lindenmann, J., 1957. Virus interference. I. The interferon. *Proc. R. Soc.*

London. Ser. B - Biol. Sci. 147, 258–267. <https://doi.org/10.1098/rspb.1957.0048>

Iuliano, A.D., Roguski, K.M., Chang, H.H., Muscatello, D.J., Palekar, R., Tempia, S.,

Cohen, C., Gran, J.M., Schanzer, D., Cowling, B.J., Wu, P., Kyncl, J., Ang, L.W.,

Park, M., Redlberger-Fritz, M., Yu, H., Espenhain, L., Krishnan, A., Emukule, G.,

van Asten, L., Pereira da Silva, S., Aungkulanon, S., Buchholz, U., Widdowson,

M.A., Bresee, J.S., Network, G.S.I.M.C., 2018. Estimates of global seasonal

influenza-associated respiratory mortality: a modelling study. *Lancet* 391, 1285–

1300. [https://doi.org/10.1016/S0140-6736\(17\)33293-2](https://doi.org/10.1016/S0140-6736(17)33293-2)

Jahan, A.S., Biquand, E., Muñoz-Moreno, R., Le Quang, A., Mok, C.K.P., Wong, H.H.,

Teo, Q.W., Valkenburg, S.A., Chin, A.W.H., Man Poon, L.L., te Velthuis, A., García-

Sastre, A., Demeret, C., Sanyal, S., 2020. OTUB1 Is a Key Regulator of RIG-I-

Dependent Immune Signaling and Is Targeted for Proteasomal Degradation by

Influenza A NS1. *Cell Rep.* 30, 1570-1584.e6.

<https://doi.org/10.1016/j.celrep.2020.01.015>

JASP Team, 2020. JASP (Version 0.14.1).

Jones, J.C., Kumar, G., Barman, S., Najera, I., White, S.W., Webby, R.J., Govorkova, E.A., 2018. Identification of the I38T PA substitution as a resistance marker for next-generation influenza virus endonuclease inhibitors. *MBio.* 9.
<https://doi.org/10.1128/mBio.00430-18>

Jureka, A.S., Kleinpeter, A.B., Tipper, J.L., Harrod, K.S., Petit, C.M., 2020. The influenza NS1 protein modulates RIG-I activation via a strain-specific direct interaction with the second CARD of RIG-I. *J. Biol. Chem.* 295, 1153–1164.
[https://doi.org/10.1016/s0021-9258\(17\)49923-6](https://doi.org/10.1016/s0021-9258(17)49923-6)

Kaneko, M., Ishiguro, M., Niinuma, Y., Uesugi, M., Nomura, Y., 2002. Human HRD1 protects against ER stress-induced apoptosis through ER-associated degradation. *FEBS Lett.* 532, 147–152. [https://doi.org/10.1016/S0014-5793\(02\)03660-8](https://doi.org/10.1016/S0014-5793(02)03660-8)

Kaneko, M., Iwase, I., Yamasaki, Y., Takai, T., Wu, Y., Kanemoto, S., Matsuhisa, K., Asada, R., Okuma, Y., Watanabe, T., Imaizumi, K., Nomura, Y., 2016. Genome-wide identification and gene expression profiling of ubiquitin ligases for endoplasmic reticulum protein degradation. *Sci. Rep.* 6, 1–10. <https://doi.org/10.1038/srep30955>

Kapadia, S.B., Chisari, F. V., 2005. Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. *Proc. Natl. Acad. Sci. U. S. A.* 102, 2561–2566.
<https://doi.org/10.1073/pnas.0409834102>

Kharel, Y., Lee, S., Snyder, A.H., Sheasley-O'Neill, S.L., Morris, M.A., Setiady, Y., Zhu, R., Zigler, M.A., Burcin, T.L., Ley, K., Tung, K.S.K., Engelhard, V.H., Macdonald, T.L., Pearson-White, S., Lynch, K.R., 2005. Sphingosine kinase 2 is required for modulation of lymphocyte traffic by FTY720. *J. Biol. Chem.* 280, 36865–36872.
<https://doi.org/10.1074/jbc.M506293200>

- Killip, M.J., Fodor, E., Randall, R.E., 2015. Influenza virus activation of the interferon system. *Virus Res.* 209, 11–22. <https://doi.org/10.1016/j.virusres.2015.02.003>
- Kirui, J., Mondal, A., Mehle, A., 2016. Ubiquitination Upregulates Influenza Virus Polymerase Function. *J. Virol.* 90, 10906–10914. <https://doi.org/10.1128/jvi.01829-16>
- Kleuser, B., 2018. The enigma of sphingolipids in health and disease. *Int. J. Mol. Sci.* <https://doi.org/10.3390/ijms19103126>
- Kochs, G., García-Sastre, A., Martínez-Sobrido, L., 2007. Multiple Anti-Interferon Actions of the Influenza A Virus NS1 Protein. *J. Virol.* 81, 7011–7021. <https://doi.org/10.1128/jvi.02581-06>
- Krammer, F., García-Sastre, A., Palese, P., 2018. Is it possible to develop a “universal” influenza virus vaccine?: Potential target antigens and critical aspects for a universal influenza vaccine. *Cold Spring Harb. Perspect. Biol.* 10, a028845. <https://doi.org/10.1101/cshperspect.a028845>
- Krug, R.M., 2015. Functions of the influenza A virus NS1 protein in antiviral defense. *Curr. Opin. Virol.* <https://doi.org/10.1016/j.coviro.2015.01.007>
- Kumar, A., Wessels, D., Daniels, K.J., Alexander, H., Alexander, S., Soll, D.R., 2004. Sphingosine-1-phosphate plays a role in the suppression of lateral pseudopod formation during *Dictyostelium discoideum* cell migration and chemotaxis. *Cell Motil. Cytoskeleton* 59, 227–241. <https://doi.org/10.1002/cm.20035>
- Kumar, K.G.S., Tang, W., Ravindranath, A.K., Clark, W.A., Croze, E., Fuchs, S.Y., 2003. SCFHOS ubiquitin ligase mediates the ligand-induced down-regulation of the interferon- α receptor. *EMBO J.* 22, 5480–5490.

<https://doi.org/10.1093/emboj/cdg524>

- Kuo, R.L., Li, Z.H., Li, L.H., Lee, K.M., Tam, E.H., Liu, H.M., Liu, H.P., Shih, S.R., Wu, C.C., 2016. Interactome analysis of the NS1 protein encoded by influenza A H1N1 virus reveals a positive regulatory role of host protein PRP19 in viral replication. *J. Proteome Res.* 15, 1639–1648. <https://doi.org/10.1021/acs.jproteome.6b00103>
- Lai, S., Qin, Y., Cowling, B.J., Ren, X., Wardrop, N.A., Gilbert, M., Tsang, T.K., Wu, P., Feng, L., Jiang, H., Peng, Z., Zheng, J., Liao, Q., Li, S., Horby, P.W., Farrar, J.J., Gao, G.F., Tatem, A.J., Yu, H., 2016. Global epidemiology of avian influenza A H5N1 virus infection in humans, 1997-2015: A systematic review of individual case data. *Lancet Infect. Dis.* [https://doi.org/10.1016/S1473-3099\(16\)00153-5](https://doi.org/10.1016/S1473-3099(16)00153-5)
- Lam, T.T., Tang, J.W., Lai, F.Y., Zaraket, H., Dbaibo, G., Bialasiewicz, S., Tozer, S., Heraud, J.M., Drews, S.J., Hachette, T., Chan, P.K., Koay, E.S., Lee, H.K., Tee, K.K., Liu, Y., Fraaij, P. LA, Jennings, L., Waris, M., Kraiden, M., Corriveau, A., Jalal, H., Nishimura, H., Nymadawa, P., Badarch, D., Watanabe, A., Kabanda, A., Sloots, T., Kok, J., Dwyer, D.E., Koopmans, M., 2019. Comparative global epidemiology of influenza, respiratory syncytial and parainfluenza viruses, 2010–2015. *J. Infect.* 79, 373–382. <https://doi.org/10.1016/j.jinf.2019.07.008>
- Lecker, S.H., Goldberg, A.L., Mitch, W.E., 2006. Protein degradation by the ubiquitin-proteasome pathway in normal and disease states. *J. Am. Soc. Nephrol.* <https://doi.org/10.1681/ASN.2006010083>
- Lee, J., Yu, H., Li, Yonghai, Ma, J., Lang, Y., Duff, M., Henningson, J., Liu, Q., Li, Yuhao, Nagy, A., Bawa, B., Li, Z., Tong, G., Richt, J.A., Ma, W., 2017. Impacts of different expressions of PA-X protein on 2009 pandemic H1N1 virus replication, pathogenicity and host immune responses. *Virology* 504, 25–35.

<https://doi.org/10.1016/j.virol.2017.01.015>

Lee, M.J., Thangada, S., Claffey, K.P., Ancellin, N., Liu, C.H., Kluk, M., Volpi, M., Sha'afi, R.I., Hla, T., 1999. Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. *Cell* 99, 301–312.

[https://doi.org/10.1016/S0092-8674\(00\)81661-X](https://doi.org/10.1016/S0092-8674(00)81661-X)

Levaot, N., Voytyuk, O., Dimitriou, I., Sircoulomb, F., Chandrakumar, A., Deckert, M., Krzyzanowski, P.M., Scotter, A., Gu, S., Janmohamed, S., Cong, F., Simonic, P.D., Ueki, Y., La Rose, J., Rottapel, R., 2011. Loss of Tankyrase-mediated destruction of 3BP2 is the underlying pathogenic mechanism of cherubism. *Cell* 147, 1324–1339. <https://doi.org/10.1016/j.cell.2011.10.045>

Lewis, C.S., Voelkel-Johnson, C., Smith, C.D., 2018. Targeting Sphingosine Kinases for the Treatment of Cancer. *Adv. Cancer Res.* 140, 295–325.

<https://doi.org/10.1016/bs.acr.2018.04.015>

Li, G., Alexander, H., Schneider, N., Alexander, S., 2000. Molecular basis for resistance to the anticancer drug cisplatin in *Dictyostelium*. *Microbiology* 146, 2219–2227.

<https://doi.org/10.1099/00221287-146-9-2219>

Li, N., Zhang, Y., Han, X., Liang, K., Wang, J., Feng, L., Wang, W., Songyang, Z., Lin, C., Yang, L., Yu, Y., Chen, J., 2015. Poly-ADP ribosylation of PTEN by tankyrases promotes PTEN degradation and tumor growth. *Genes Dev.* 29, 157–170.

<https://doi.org/10.1101/gad.251785.114>

Li, S.F., Gong, M.J., Zhao, F.R., Shao, J.J., Xie, Y.L., Zhang, Y.G., Chang, H.Y., 2018.

Type I interferons: Distinct biological activities and current applications for viral infection. *Cell. Physiol. Biochem.* <https://doi.org/10.1159/000495897>

- Li, Y., Xie, P., Lu, L., Wang, J., Diao, L., Liu, Z., Guo, F., He, Y., Liu, Y., Huang, Q., Liang, H., Li, D., He, F., 2017. An integrated bioinformatics platform for investigating the human E3 ubiquitin ligase-substrate interaction network. *Nat. Commun.* 8, 1–9. <https://doi.org/10.1038/s41467-017-00299-9>
- Li, Y., Yamakita, Y., Krug, R.M., 1998. Regulation of a nuclear export signal by an adjacent inhibitory sequence: The effector domain of the influenza virus NS1 protein. *Proc. Natl. Acad. Sci. U. S. A.* 95, 4864–4869. <https://doi.org/10.1073/pnas.95.9.4864>
- Liedmann, S., Hrinčius, E.R., Guy, C., Anhlan, D., Dierkes, R., Carter, R., Wu, G., Staeheli, P., Green, D.R., Wolff, T., McCullers, J.A., Ludwig, S., Ehrhardt, C., 2014. Viral suppressors of the RIG-I-mediated interferon response are pre-packaged in influenza virions. *Nat. Commun.* 5. <https://doi.org/10.1038/ncomms6645>
- Liu, C., Yu, X., 2015. ADP-Ribosyltransferases and Poly ADP-Ribosylation. *Curr. Protein Pept. Sci.* 16, 491–501.
- Liu, C.H., Zhou, L., Chen, G., Krug, R.M., 2015. Battle between influenza A virus and a newly identified antiviral activity of the PARP-containing ZAPL protein. *Proc. Natl. Acad. Sci. U. S. A.* 112, 14048–14053. <https://doi.org/10.1073/pnas.1509745112>
- Liu, J., Carvalho, L.P., Bhattacharya, S., Carbone, C.J., Kumar, K.G.S., Leu, N.A., Yau, P.M., Donald, R.G.K., Weiss, M.J., Baker, D.P., McLaughlin, K.J., Scott, P., Fuchs, S.Y., 2009a. Mammalian Casein Kinase 1 α and Its Leishmanial Ortholog Regulate Stability of IFNAR1 and Type I Interferon Signaling. *Mol. Cell. Biol.* 29, 6401–6412. <https://doi.org/10.1128/mcb.00478-09>
- Liu, J., HuangFu, W.C., Kumar, K.G.S., Qian, J., Casey, J.P., Hamanaka, R.B., Grigoriadou, C., Aldabe, R., Diehl, J.A., Fuchs, S.Y., 2009b. Virus-Induced

Unfolded Protein Response Attenuates Antiviral Defenses via Phosphorylation-Dependent Degradation of the Type I Interferon Receptor. *Cell Host Microbe* 5, 72–83. <https://doi.org/10.1016/j.chom.2008.11.008>

Liu, J., Lynch, P.A., Chien, C.Y., Montelione, G.T., Krug, R.M., Berman, H.M., 1997.

Crystal structure of the unique RNA-binding domain of the influenza virus NS1 protein. *Nat. Struct. Biol.* 4, 896–899. <https://doi.org/10.1038/nsb1197-896>

Liu, Y., Wada, R., Yamashita, T., Mi, Y., Deng, C.X., Hobson, J.P., Rosenfeldt, H.M.,

Nava, V.E., Chae, S.S., Lee, M.J., Liu, C.H., Hla, T., Spiegel, S., Proia, R.L., 2000. Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. *J. Clin. Invest.* 106, 951–961. <https://doi.org/10.1172/JCI10905>

Lohrmann, F., Dijkman, R., Stertz, S., Thiel, V., Haller, O., Staeheli, P., Kochs, G., 2013.

Emergence of a C-Terminal Seven-Amino-Acid Elongation of NS1 in Around 1950 Conferred a Minor Growth Advantage to Former Seasonal Influenza A Viruses. *J. Virol.* 87, 11300–11303. <https://doi.org/10.1128/jvi.01271-13>

Loo, Y.-M., Fornek, J., Crochet, N., Bajwa, G., Perwitasari, O., Martinez-Sobrido, L.,

Akira, S., Gill, M.A., García-Sastre, A., Katze, M.G., Gale, M., 2008. Distinct RIG-I and MDA5 Signaling by RNA Viruses in Innate Immunity. *J. Virol.* 82, 335–345. <https://doi.org/10.1128/jvi.01080-07>

Lovric, S., Goncalves, S., Gee, H.Y., Oskouian, B., Srinivas, H., Choi, W.-I. II, Shril, S.,

Ashraf, S., Tan, W., Rao, J., Airik, M., Schapiro, D., Braun, D.A., Sadowski, C.E., Widmeier, E., Jobst-Schwan, T., Schmidt, J.M., Girik, V., Capitani, G., Suh, J.H., Lachaussée, N., Arrondel, C., Patat, J., Gribouval, O., Furlano, M., Boyer, O., Schmitt, A., Vuiblet, V., Hashmi, S., Wilcken, R., Bernier, F.P., Innes, A.M., Parboosingh, J.S., Lamont, R.E., Midgley, J.P., Wright, N., Majewski, J., Zenker,

M., Schaefer, F., Kuss, N., Greil, J., Giese, T., Schwarz, K., Catheline, V., Schanze, D., Franke, I., Sznajder, Y., Truant, A.S., Adams, B., Désir, J., Biemann, R., Pei, Y., Ars, E., Lloberas, N., Madrid, A., Dharnidharka, V.R., Connolly, A.M., Willing, M.C., Cooper, M.A., Lifton, R.P., Simons, M., Riezman, H., Antignac, C., Saba, J.D., Hildebrandt, F., 2017. Mutations in sphingosine-1-phosphate lyase cause nephrosis with ichthyosis and adrenal insufficiency. *J. Clin. Invest.* 127, 912–928.
<https://doi.org/10.1172/JCI89626>

Lu, Y., Qian, X.Y., Krug, R.M., 1994. The influenza virus NS1 protein: A novel inhibitor of pre-mRNA splicing. *Genes Dev.* 8, 1817–1828.
<https://doi.org/10.1101/gad.8.15.1817>

Luo, Z., Liu, L.F., Jiang, Y.N., Tang, L.P., Li, W., Ouyang, S.H., Tu, L.F., Wu, Y.P., Gong, H.B., Yan, C.Y., Jiang, S., Lu, Y.H., Liu, T., Jiang, Z., Kurihara, H., Yu, Y., Yao, X.S., Li, Y.F., He, R.R., 2020. Novel insights into stress-induced susceptibility to influenza: corticosterone impacts interferon- β responses by Mfn2-mediated ubiquitin degradation of MAVS. *Signal Transduct. Target. Ther.* 5, 1–12.
<https://doi.org/10.1038/s41392-020-00238-z>

Ma, W., Belisle, S.E., Mosier, D., Li, X., Stigger-Rosser, E., Liu, Q., Qiao, C., Elder, J., Webby, R., Katze, M.G., Richt, J.A., 2011. 2009 Pandemic H1N1 Influenza Virus Causes Disease and Upregulation of Genes Related to Inflammatory and Immune Responses, Cell Death, and Lipid Metabolism in Pigs. *J. Virol.* 85, 11626–11637.
<https://doi.org/10.1128/jvi.05705-11>

Maceyka, M., Rohrbach, T., Milstien, S., Spiegel, S., 2020. Role of sphingosine kinase 1 and sphingosine-1-phosphate axis in hepatocellular carcinoma, in: *Handbook of Experimental Pharmacology*. Springer Science and Business Media Deutschland

GmbH, pp. 3–17. https://doi.org/10.1007/164_2019_217

Maceyka, M., Sankala, H., Hait, N.C., Le Stunff, H., Liu, H., Toman, R., Collier, C., Zhang, M., Satin, L.S., Merrill, A.H., Milstien, S., Spiegel, S., 2005. SphK1 and SphK2, sphingosine kinase isoenzymes with opposing functions in sphingolipid metabolism. *J. Biol. Chem.* 280, 37118–29. <https://doi.org/10.1074/jbc.M502207200>

Machesky, N.J., Zhang, G., Raghavan, B., Zimmerman, P., Kelly, S.L., Merrill, A.H., Waldman, W.J., Van Brocklyn, J.R., Trgovcich, J., 2008. Human cytomegalovirus regulates bioactive sphingolipids. *J. Biol. Chem.* 283, 26148–26160. <https://doi.org/10.1074/jbc.M710181200>

Mandala, S., Hajdu, R., Bergstrom, J., Quackenbush, E., Xie, J., Milligan, J., Thornton, R., Shei, G.J., Card, D., Keohane, C.A., Rosenbach, M., Hale, J., Lynch, C.L., Rupprecht, K., Parsons, W., Rosen, H., 2002. Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science* 296, 346–349. <https://doi.org/10.1126/science.1070238>

Marc, D., 2014. Influenza virus non-structural protein NS1: Interferon antagonism and beyond. *J. Gen. Virol.* <https://doi.org/10.1099/vir.0.069542-0>

Marjuki, H., Alam, M.I., Ehrhardt, C., Wagner, R., Planz, O., Klenk, H.D., Ludwig, S., Pleschka, S., 2006. Membrane accumulation of influenza A virus hemagglutinin triggers nuclear export of the viral genome via protein kinase C α -mediated activation of ERK signaling. *J. Biol. Chem.* 281, 16707–16715. <https://doi.org/10.1074/jbc.M510233200>

Marjuki, H., Gornitzky, A., Marathe, B.M., Ilyushina, N.A., Aldridge, J.R., Desai, G., Webby, R.J., Webster, R.G., 2011. Influenza A virus-induced early activation of ERK and PI3K mediates V-ATPase-dependent intracellular pH change required for

fusion. *Cell. Microbiol.* 13, 587–601. <https://doi.org/10.1111/j.1462-5822.2010.01556.x>

Marjuki, H., Mishin, V.P., Chesnokov, A.P., Jones, J., De La Cruz, J.A., Sleeman, K., Tamura, D., Nguyen, H.T., Wu, H.S., Chang, F.Y., Liu, M.T., Fry, A.M., Cox, N.J., Villanueva, J.M., Davis, C.T., Gubareva, L. V., 2015. Characterization of drug-resistant influenza a(H7N9) variants isolated from an oseltamivir-treated patient in Taiwan. *J. Infect. Dis.* 211, 249–257. <https://doi.org/10.1093/infdis/jiu447>

Marsolais, D., Hahm, B., Walsh, K.B., Edelmann, K.H., McGavern, D., Hatta, Y., Kawaoka, Y., Rosen, H., Oldstone, M.B.A., 2009. A critical role for the sphingosine analog AAL-R in dampening the cytokine response during influenza virus infection. *Proc. Natl. Acad. Sci. U. S. A.* 106, 1560–5. <https://doi.org/10.1073/pnas.0812689106>

Mattie, M., Brooker, G., Spiegel, S., 1994. Sphingosine-1-phosphate, a Putative Second Messenger, Mobilizes Calcium from Internal Stores via an Inositol Trisphosphate-independent Pathway*. *J. Biol. Chem.* 269, 3181–3188. [https://doi.org/10.1016/S0021-9258\(17\)41846-1](https://doi.org/10.1016/S0021-9258(17)41846-1)

McAuley, J.L., Gilbertson, B.P., Trifkovic, S., Brown, L.E., McKimm-Breschkin, J.L., 2019. Influenza virus neuraminidase structure and functions. *Front. Microbiol.* <https://doi.org/10.3389/fmicb.2019.00039>

McGowan, E.M., Haddadi, N., Nassif, N.T., Lin, Y., 2020. Targeting the SPHK-S1P-SIPR pathway as a potential therapeutic approach for COVID-19. *Int. J. Mol. Sci.* <https://doi.org/10.3390/ijms21197189>

Meerbrey, K.L., Hu, G., Kessler, J.D., Roarty, K., Li, M.Z., Fang, J.E., Herschkowitz, J.I., Burrows, A.E., Ciccia, A., Sun, T., Schmitt, E.M., Bernardi, R.J., Fu, X., Bland, C.S.,

- Cooper, T.A., Schiff, R., Rosen, J.M., Westbrook, T.F., Elledge, S.J., 2011. The pINDUCER lentiviral toolkit for inducible RNA interference in vitro and in vivo. *Proc. Natl. Acad. Sci. U. S. A.* 108, 3665–3670. <https://doi.org/10.1073/pnas.1019736108>
- Melén, K., Kinnunen, L., Fagerlund, R., Ikonen, N., Twu, K.Y., Krug, R.M., Julkunen, I., 2007. Nuclear and Nucleolar Targeting of Influenza A Virus NS1 Protein: Striking Differences between Different Virus Subtypes. *J. Virol.* 81, 5995–6006. <https://doi.org/10.1128/jvi.01714-06>
- Melén, K., Tynell, J., Fagerlund, R., Roussel, P., Hernandez-Verdun, D., Julkunen, I., 2012. Influenza A H3N2 subtype virus NS1 protein targets into the nucleus and binds primarily via its C-terminal NLS2/NoLS to nucleolin and fibrillarin. *Viol. J.* 9, 1–13. <https://doi.org/10.1186/1743-422X-9-167>
- Mibayashi, M., Martínez-Sobrido, L., Loo, Y.-M., Cárdenas, W.B., Gale, M., García-Sastre, A., 2007. Inhibition of Retinoic Acid-Inducible Gene I-Mediated Induction of Beta Interferon by the NS1 Protein of Influenza A Virus. *J. Virol.* 81, 514–524. <https://doi.org/10.1128/jvi.01265-06>
- Min, J., Mesika, A., Sivaguru, M., Van Veldhoven, P.P., Alexander, H., Futerman, A.H., Alexander, S., 2007. (Dihydro)ceramide synthase 1-regulated sensitivity to cisplatin is associated with the activation of p38 mitogen-activated protein kinase and is abrogated by sphingosine kinase 1. *Mol. Cancer Res.* 5, 801–812. <https://doi.org/10.1158/1541-7786.MCR-07-0100>
- Min, J., Traynor, D., Stegner, A.L., Zhang, L., Hanigan, M.H., Alexander, H., Alexander, S., 2005. Sphingosine kinase regulates the sensitivity of Dictyostelium discoideum cells to the anticancer drug cisplatin. *Eukaryot. Cell* 4, 178–189. <https://doi.org/10.1128/EC.4.1.178-189.2005>

- Min, J.Y., Krug, R.M., 2006. The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: Inhibiting the 2'-5' oligo (A) synthetase/RNase L pathway. *Proc. Natl. Acad. Sci. U. S. A.* 103, 7100–7105.
<https://doi.org/10.1073/pnas.0602184103>
- Min, J.Y., Li, S., Sen, G.C., Krug, R.M., 2007. A site on the influenza A virus NS1 protein mediates both inhibition of PKR activation and temporal regulation of viral RNA synthesis. *Virology* 363, 236–243. <https://doi.org/10.1016/j.virol.2007.01.038>
- Monick, M.M., Cameron, K., Powers, L.S., Butler, N.S., McCoy, D., Mallampalli, R.K., Hunninghake, G.W., 2004. Sphingosine kinase mediates activation of extracellular signal-related kinase and Akt by respiratory syncytial virus. *Am. J. Respir. Cell Mol. Biol.* 30, 844–852. <https://doi.org/10.1165/rcmb.2003-0424OC>
- Morens, D.M., Fauci, A.S., 2007. The 1918 Influenza Pandemic: Insights for the 21st Century. *J. Infect. Dis.* 195, 1018–1028. <https://doi.org/10.1086/511989>
- Muñoz-Moreno, R., Martínez-Romero, C., García-Sastre, A., 2020. Induction and Evasion of Type-I Interferon Responses during Influenza A Virus Infection. *Cold Spring Harb. Perspect. Med.* a038414.
<https://doi.org/10.1101/cshperspect.a038414>
- Muthumani, K., Choo, A.Y., Zong, W.X., Madesh, M., Hwang, D.S., Premkumar, A., Thieu, K.P., Emmanuel, J., Kumar, S., Thompson, C.B., Weiner, D.B., 2006. The HIV-1 Vpr and glucocorticoid receptor complex is a gain-of-function interaction that prevents the nuclear localization of PARP-1. *Nat. Cell Biol.* 8, 170–179.
<https://doi.org/10.1038/ncb1352>
- Nemeroff, M.E., Qian, X.Y., Krug, R.M., 1995. The Influenza Virus NS1 Protein Forms Multimers in Vitro and in Vivo. *Virology* 212, 422–428.

<https://doi.org/10.1006/viro.1995.1499>

Neubauer, H.A., Pham, D.H., Zebol, J.R., Moretti, P.A.B., Peterson, A.L., Leclercq, T.M., Chan, H., Powell, J.A., Pitman, M.R., Samuel, M.S., Bonder, C.S., Creek, D.J., Gliddon, B.L., Pitson, S.M., 2016. An oncogenic role for sphingosine kinase 2. *Oncotarget* 7, 64886–64899. <https://doi.org/10.18632/oncotarget.11714>

Neubauer, H.A., Pitson, S.M., 2013. Roles, regulation and inhibitors of sphingosine kinase 2, in: *FEBS Journal*. pp. 5317–5336. <https://doi.org/10.1111/febs.12314>

Neumann, G., Watanabe, T., Ito, H., Watanabe, S., Goto, H., Gao, P., Hughes, M., Perez, D.R., Donis, R., Hoffmann, E., Hobom, G., Kawaoka, Y., 1999. Generation of influenza A viruses entirely from cloned cDNAs. *Proc. Natl. Acad. Sci. U. S. A.* 96, 9345–9350. <https://doi.org/10.1073/pnas.96.16.9345>

Nogales, A., Martinez-Sobrido, L., Topham, D.J., Dediego, M.L., 2018. Modulation of innate immune responses by the influenza A NS1 and PA-X proteins. *Viruses*. <https://doi.org/10.3390/v10120708>

O'Connor, P., Comi, G., Montalban, X., Antel, J., Radue, E.W., de Vera, A., Pohlmann, H., Kappos, L., O'Connor, P., Comi, G., Montalban, X., Antel, J., Radue, E.W., de Vera, A., Pohlmann, H., Kappos, L., FTY720 D2201 Study Group, 2009. Oral fingolimod (FTY720) in multiple sclerosis: Two-year results of a phase II extension study. *Neurology* 72, 73–79. <https://doi.org/10.1212/01.wnl.0000338569.32367.3d>

Oh, E., Akopian, D., Rape, M., 2018. Principles of Ubiquitin- Dependent Signaling. *Annu. Rev. Cell Dev. Biol.* <https://doi.org/10.1146/annurev-cellbio-100617-062802>

Ohtake, F., Tsuchiya, H., Saeki, Y., Tanaka, K., 2018. K63 ubiquitylation triggers proteasomal degradation by seeding branched ubiquitin chains. *Proc. Natl. Acad.*

Sci. U. S. A. 115, E1401–E1408. <https://doi.org/10.1073/pnas.1716673115>

Olivera, A., Spiegel, S., 1993. Sphingosine-1-phosphate as second messenger in cell proliferation induced by PDGF and FCS mitogens. *Nature* 365, 557–560.

<https://doi.org/10.1038/365557a0>

Orr Gandy, K.A., Obeid, L.M., 2013. Targeting the sphingosine kinase/sphingosine 1-phosphate pathway in disease: Review of sphingosine kinase inhibitors. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 1831, 157–166.

<https://doi.org/10.1016/j.bbalip.2012.07.002>

Oskouian, B., Saba, J.D., 2010. Cancer treatment strategies targeting sphingolipid metabolism. *Adv. Exp. Med. Biol.* 688, 185–205. https://doi.org/10.1007/978-1-4419-6741-1_13

Patel, D., Schultz, L.W., Umland, T.C., 2013. Influenza A polymerase subunit PB2 possesses overlapping binding sites for polymerase subunit PB1 and human MAVS proteins. *Virus Res.* 172, 75–80. <https://doi.org/10.1016/j.virusres.2012.12.003>

Paz, S., Vilasco, M., Arguello, M., Sun, Q., Lacoste, J., Nguyen, T.L.-A., Zhao, T., Shestakova, E.A., Zaari, S., Bibeau-Poirier, A., Servant, M.J., Lin, R., Meurs, E.F., Hiscott, J., 2009. Ubiquitin-Regulated Recruitment of I κ B Kinase ϵ to the MAVS Interferon Signaling Adapter. *Mol. Cell. Biol.* 29, 3401–3412.

<https://doi.org/10.1128/mcb.00880-08>

Peiris, J.S.M., De Jong, M.D., Guan, Y., 2007. Avian influenza virus (H5N1): A threat to human health. *Clin. Microbiol. Rev.* <https://doi.org/10.1128/CMR.00037-06>

Perot, B.P., Boussier, J., Yatim, N., Rossman, J.S., Ingersoll, M.A., Albert, M.L., 2018. Autophagy diminishes the early interferon- β response to influenza A virus resulting

- in differential expression of interferon-stimulated genes. *Cell Death Dis.* 9.
<https://doi.org/10.1038/s41419-018-0546-5>
- Pestka, S., Krause, C.D., Walter, M.R., 2004. Interferons, interferon-like cytokines, and their receptors. *Immunol. Rev.* 202, 8–32. <https://doi.org/10.1111/j.0105-2896.2004.00204.x>
- Pichlmair, A., Schulz, O., Tan, C.P., Näslund, T.I., Liljeström, P., Weber, F., Reis E Sousa, C., 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* 314, 997–1001.
<https://doi.org/10.1126/science.1132998>
- Pinto, L.H., Lamb, R.A., 2006. The M2 proton channels of influenza A and B viruses. *J. Biol. Chem.* <https://doi.org/10.1074/jbc.R500020200>
- Piroth, L., Cottenet, J., Mariet, A.S., Bonniaud, P., Blot, M., Tubert-Bitter, P., Quantin, C., 2021. Comparison of the characteristics, morbidity, and mortality of COVID-19 and seasonal influenza: a nationwide, population-based retrospective cohort study. *Lancet Respir. Med.* 9, 251–259. [https://doi.org/10.1016/S2213-2600\(20\)30527-0](https://doi.org/10.1016/S2213-2600(20)30527-0)
- Pitha, P.M., Kunzi, M.S., 2007. Type I interferon: The ever unfolding story. *Curr. Top. Microbiol. Immunol.* https://doi.org/10.1007/978-3-540-71329-6_4
- Pitson, Stuart M, 2011. Regulation of sphingosine kinase and sphingolipid signaling. *Trends Biochem. Sci.* 36, 97–107. <https://doi.org/10.1016/j.tibs.2010.08.001>
- Pitson, Stuart M., 2011. Regulation of sphingosine kinase and sphingolipid signaling. *Trends Biochem. Sci.* <https://doi.org/10.1016/j.tibs.2010.08.001>
- Pizzorno, A., Dubois, J., Machado, D., Cartet, G., Traversier, A., Julien, T., Lina, B., Bourdon, J.C., Rosa-Calatrava, M., Terrier, O., 2018. Influenza A viruses alter the

- stability and antiviral contribution of host E3-ubiquitin ligase Mdm2 during the time-course of infection. *Sci. Rep.* 8. <https://doi.org/10.1038/s41598-018-22139-6>
- Platanias, L.C., 2005. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat. Rev. Immunol.* <https://doi.org/10.1038/nri1604>
- Pleschka, S., Wolff, T., Ehrhardt, C., Hobom, G., Planz, O., Rapp, U.R., Ludwig, S., 2001. Influenza virus propagation is impaired by inhibition of the Raf/MEK/ERK signalling cascade. *Nat. Cell Biol.* 3, 301–305. <https://doi.org/10.1038/35060098>
- Plotch, S.J., Krug, R.M., 1986. In vitro splicing of influenza viral NS1 mRNA and NS1- β -globin chimeras: Possible mechanisms for the control of viral mRNA splicing. *Proc. Natl. Acad. Sci. U. S. A.* 83, 5444–5448. <https://doi.org/10.1073/pnas.83.15.5444>
- Pohl, C., Dikic, I., 2019. Cellular quality control by the ubiquitin-proteasome system and autophagy. *Science* 366, 818–822. <https://doi.org/10.1126/science.aax3769>
- Poland, G.A., Jacobson, R.M., Ovsyannikova, I.G., 2009. Influenza virus resistance to antiviral agents: A plea for rational use. *Clin. Infect. Dis.* <https://doi.org/10.1086/598989>
- Prasad, R., Hadjidemetriou, I., Maharaj, A., Meimaridou, E., Buonocore, F., Saleem, M., Hurcombe, J., Bierzynska, A., Barbagelata, E., Bergadá, I., Cassinelli, H., Das, U., Krone, R., Hacıhamdioglu, B., Sari, E., Yesilkaya, E., Storr, H.L., Clemente, M., Fernandez-Cancio, M., Camats, N., Ram, N., Achermann, J.C., Van Veldhoven, P.P., Guasti, L., Braslavsky, D., Guran, T., Metherell, L.A., Sgene, G., Krone, R., Hacıhamdioglu, B., Sari, E., Yesilkaya, E., Storr, H.L., Clemente, M., Fernandez-Cancio, M., Camats, N., Ram, N., Achermann, J.C., Van Veldhoven, P.P., Guasti, L., Braslavsky, D., Guran, T., Metherell, L.A., 2017. Sphingosine-1-phosphate lyase mutations cause primary adrenal insufficiency and steroid-resistant nephrotic

- syndrome. *J. Clin. Invest.* 127, 942–953. <https://doi.org/10.1172/JCI90171>
- Pritzl, C.J., Seo, Y.-J., Xia, C., Vijayan, M., Stokes, Z.D., Hahm, B., 2015. A Ceramide Analogue Stimulates Dendritic Cells To Promote T Cell Responses upon Virus Infections. *J. Immunol.* 194, 4339–4349. <https://doi.org/10.4049/jimmunol.1402672>
- Pyne, N.J., Pyne, S., 2010. Sphingosine 1-phosphate and cancer. *Nat. Rev. Cancer.* <https://doi.org/10.1038/nrc2875>
- Qin, Z., Dai, L., Trillo-Tinoco, J., Senkal, C., Wang, W., Reske, T., Bonstaff, K., Del Valle, L., Rodriguez, P., Flemington, E., Voelkel-Johnson, C., Smith, C.D., Ogretmen, B., Parsons, C., 2014. Targeting sphingosine kinase induces apoptosis and tumor regression for KSHV-associated primary effusion lymphoma. *Mol. Cancer Ther.* 13, 154–164. <https://doi.org/10.1158/1535-7163.MCT-13-0466>
- R Core Team, 2020. R: A language and environment for statistical computing.
- Rajsbaum, R., Albrecht, R.A., Wang, M.K., Maharaj, N.P., Versteeg, G.A., Nistal-Villán, E., García-Sastre, A., Gack, M.U., 2012. Species-Specific Inhibition of RIG-I Ubiquitination and IFN Induction by the Influenza A Virus NS1 Protein. *PLoS Pathog.* 8, e1003059. <https://doi.org/10.1371/journal.ppat.1003059>
- Rape, M., 2018. Post-Translational Modifications: Ubiquitylation at the crossroads of development and disease. *Nat. Rev. Mol. Cell Biol.* <https://doi.org/10.1038/nrm.2017.83>
- Reid, S.P. atric., Tritsch, S.R., Kota, K., Chiang, C.Y., Dong, L., Kenny, T., Brueggemann, E.E., Ward, M.D., Cazares, L.H., Bavari, S., 2015. Sphingosine kinase 2 is a chikungunya virus host factor co-localized with the viral replication complex. *Emerg. Microbes Infect.* 4, e61. <https://doi.org/10.1038/emi.2015.61>

- Robertson, J.S., Schubert, M., Lazzarini, R.A., 1981. Polyadenylation sites for influenza virus mRNA. *J. Virol.* 38, 157–163. <https://doi.org/10.1128/jvi.38.1.157-163.1981>
- Rock, K.L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., Goldberg, A.L., 1994. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* 78, 761–771. [https://doi.org/10.1016/S0092-8674\(94\)90462-6](https://doi.org/10.1016/S0092-8674(94)90462-6)
- Rolando, M., Escoll, P., Buchrieser, C., 2016. *Legionella pneumophila* restrains autophagy by modulating the host's sphingolipid metabolism. *Autophagy*. <https://doi.org/10.1080/15548627.2016.1166325>
- Rosário-Ferreira, N., Preto, A.J., Melo, R., Moreira, I.S., Brito, R.M.M., 2020. The central role of non-structural protein 1 (NS1) in influenza biology and infection. *Int. J. Mol. Sci.* 21. <https://doi.org/10.3390/ijms21041511>
- Rossi, M.N., Carbone, M., Mostocotto, C., Mancone, C., Tripodi, M., Malone, R., Amati, P., 2009. Mitochondrial localization of PARP-1 requires interaction with mitofilin and is involved in the maintenance of mitochondrial DNA integrity. *J. Biol. Chem.* 284, 31616–31624. <https://doi.org/10.1074/jbc.M109.025882>
- Rueden, C.T., Schindelin, J., Hiner, M.C., DeZonia, B.E., Walter, A.E., Arena, E.T., Eliceiri, K.W., 2017. ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinformatics* 18. <https://doi.org/10.1186/s12859-017-1934-z>
- Saba, J.D., Hla, T., 2004. Point-Counterpoint of Sphingosine 1-Phosphate Metabolism. *Circ. Res.* <https://doi.org/10.1161/01.RES.0000122383.60368.24>
- Salvador, R., Tanquilut, N., Macmac, R., Na Lampang, K., Chaisowwong, W., Pfeiffer, D., Punyapornwithaya, V., 2020. Evaluation of strategies using simulation model to

control a potential outbreak of highly pathogenic avian influenza among poultry farms in Central Luzon, Philippines. *PLoS One* 15, e0238815.

<https://doi.org/10.1371/journal.pone.0238815>

Saric, T., Graef, C.I., Goldberg, A.L., 2004. Pathway for degradation of peptides generated by proteasomes: A key role for thimet oligopeptidase and other metallopeptidases. *J. Biol. Chem.* 279, 46723–46732.

<https://doi.org/10.1074/jbc.M406537200>

Sato, M., Suemori, H., Hata, N., Asagiri, M., Ogasawara, K., Nakao, K., Nakaya, T., Katsuki, M., Noguchi, S., Tanaka, N., Taniguchi, T., 2000. Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN- α/β gene induction. *Immunity* 13, 539–548. [https://doi.org/10.1016/S1074-7613\(00\)00053-4](https://doi.org/10.1016/S1074-7613(00)00053-4)

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: An open-source platform for biological-image analysis. *Nat. Methods*.

<https://doi.org/10.1038/nmeth.2019>

Schoggins, J.W., 2014. Interferon-stimulated genes: Roles in viral pathogenesis. *Curr. Opin. Virol.* <https://doi.org/10.1016/j.coviro.2014.03.006>

Schröder, B., Wrocklage, C., Pan, C., Jäger, R., Kösters, B., Schäfer, H., Elsässer, H.P., Mann, M., Hasilik, A., 2007. Integral and associated lysosomal membrane proteins. *Traffic* 8, 1676–1686. <https://doi.org/10.1111/j.1600-0854.2007.00643.x>

Schwab, S.R., Pereira, J.P., Matloubian, M., Xu, Y., Huang, Y., Cyster, J.G., 2005.

Immunology: Lymphocyte sequestration through S1P lyase inhibition and disruption

of S1P gradients. *Science* 309, 1735–1739.

<https://doi.org/10.1126/science.1113640>

Schwertman, P., Bekker-Jensen, S., Mailand, N., 2016. Regulation of DNA double-strand break repair by ubiquitin and ubiquitin-like modifiers. *Nat. Rev. Mol. Cell Biol.*

<https://doi.org/10.1038/nrm.2016.58>

Scovassi, A.I., 2004. Mitochondrial poly(ADP-ribosylation): from old data to new perspectives. *FASEB J.* 18, 1487–1488. <https://doi.org/10.1096/fj.04-1841rev>

Seo, Y.-J., Blake, C., Alexander, S., Hahm, B., 2010. Sphingosine 1-Phosphate-Metabolizing Enzymes Control Influenza Virus Propagation and Viral Cytopathogenicity. *J. Virol.* 84, 8124–8131. <https://doi.org/10.1128/jvi.00510-10>

Seo, Y.-J., Pritzl, C.J., Vijayan, M., Blake, C.R., McClain, M.E., Hahm, B., 2012. Sphingosine analogue AAL-R increases TLR7-mediated dendritic cell responses via p38 and type I IFN signaling pathways. *J. Immunol.* 188, 4759–68.

<https://doi.org/10.4049/jimmunol.1102754>

Seo, Y.-J.J., Pritzl, C.J., Vijayan, M., Bomb, K., McClain, M.E., Alexander, S., Hahm, B., 2013. Sphingosine Kinase 1 Serves as a Pro-Viral Factor by Regulating Viral RNA Synthesis and Nuclear Export of Viral Ribonucleoprotein Complex upon Influenza Virus Infection. *PLoS One* 8, e75005. <https://doi.org/10.1371/journal.pone.0075005>

Seo, Y.J., Alexander, S., Hahm, B., 2011. Does cytokine signaling link sphingolipid metabolism to host defense and immunity against virus infections?, *Cytokine and Growth Factor Reviews*. Pergamon. <https://doi.org/10.1016/j.cytogfr.2010.12.001>

Seo, Y.J., Hahm, B., 2010. Type I interferon modulates the battle of host immune system against viruses, in: *Advances in Applied Microbiology*. Academic Press Inc., pp. 83–

101. [https://doi.org/10.1016/S0065-2164\(10\)73004-5](https://doi.org/10.1016/S0065-2164(10)73004-5)

Seo, Y.J., Pritzl, C.J., Vijayan, M., Bomb, K., McClain, M.E., Alexander, S., Hahm, B.,
2013. Sphingosine Kinase 1 Serves as a Pro-Viral Factor by Regulating Viral RNA
Synthesis and Nuclear Export of Viral Ribonucleoprotein Complex upon Influenza
Virus Infection. *PLoS One* 8. <https://doi.org/10.1371/journal.pone.0075005>

Serra, M., Saba, J.D., 2010. Sphingosine 1-phosphate lyase, a key regulator of
sphingosine 1-phosphate signaling and function. *Adv. Enzyme Regul.* 50, 349–362.
<https://doi.org/10.1016/j.advenzreg.2009.10.024>

Settas, N., Persky, R., Faucz, F.R., Sheanon, N., Voutetakis, A., Lodish, M., Metherell,
L.A., Stratakis, C.A., 2019. SGPL1 deficiency: A rare cause of primary adrenal
insufficiency. *J. Clin. Endocrinol. Metab.* 104, 1484–1490.
<https://doi.org/10.1210/jc.2018-02238>

Shapira, S.D., Gat-Viks, I., Shum, B.O.V., Dricot, A., de Grace, M.M., Wu, L., Gupta,
P.B., Hao, T., Silver, S.J., Root, D.E., Hill, D.E., Regev, A., Hacohen, N., 2009. A
Physical and Regulatory Map of Host-Influenza Interactions Reveals Pathways in
H1N1 Infection. *Cell* 139, 1255–1267. <https://doi.org/10.1016/j.cell.2009.12.018>

Sharma, S., TenOever, B.R., Grandvaux, N., Zhou, G.P., Lin, R., Hiscott, J., 2003.
Triggering the interferon antiviral response through an IKK-related pathway.
Science 300, 1148–1151. <https://doi.org/10.1126/science.1081315>

Shimizu, T., Takizawa, N., Watanabe, K., Nagata, K., Kobayashi, N., 2011. Crucial role
of the influenza virus NS2 (NEP) C-terminal domain in M1 binding and nuclear
export of vRNP. *FEBS Lett.* 585, 41–46.
<https://doi.org/10.1016/j.febslet.2010.11.017>

- Shu, X., Wu, W., Mosteller, R.D., Broek, D., 2002. Sphingosine Kinase Mediates Vascular Endothelial Growth Factor-Induced Activation of Ras and Mitogen-Activated Protein Kinases. *Mol. Cell. Biol.* 22, 7758–7768.
<https://doi.org/10.1128/mcb.22.22.7758-7768.2002>
- Singhania, A., Graham, C.M., Gabryšová, L., Moreira-Teixeira, L., Stavropoulos, E., Pitt, J.M., Chakravarty, P., Warnatsch, A., Branchett, W.J., Conejero, L., Lin, J.W., Davidson, S., Wilson, M.S., Bancroft, G., Langhorne, J., Frickel, E., Sesay, A.K., Priestnall, S.L., Herbert, E., Ioannou, M., Wang, Q., Humphreys, I.R., Dodd, J., Openshaw, P.J.M., Mayer-Barber, K.D., Jankovic, D., Sher, A., Lloyd, C.M., Baldwin, N., Chaussabel, D., Papayannopoulos, V., Wack, A., Banchereau, J.F., Pascual, V.M., O'Garra, A., 2019. Transcriptional profiling unveils type I and II interferon networks in blood and tissues across diseases. *Nat. Commun.* 10, 1–21.
<https://doi.org/10.1038/s41467-019-10601-6>
- Slade, D., 2020. PARP and PARG inhibitors in cancer treatment. *Genes Dev.*
<https://doi.org/10.1101/gad.334516.119>
- Spiegel, S., Milstien, S., 2011. The outs and the ins of sphingosine-1-phosphate in immunity. *Nat. Rev. Immunol.* <https://doi.org/10.1038/nri2974>
- Spiegel, S., Milstien, S., 2003. Sphingosine-1-phosphate: An enigmatic signalling lipid. *Nat. Rev. Mol. Cell Biol.* <https://doi.org/10.1038/nrm1103>
- Su, W.-C., Yu, W.-Y., Huang, S.-H., Lai, M.M.C., 2017. Ubiquitination of the Cytoplasmic Domain of Influenza A Virus M2 Protein is Crucial for Production of Infectious Virus Particles. *J. Virol.* 92, JVI.01972-17. <https://doi.org/10.1128/jvi.01972-17>
- Su, W.C., Chen, Y.C., Tseng, C.H., Hsu, P.W.C., Tung, K.F., Jeng, K.S., Lai, M.M.C., 2013. Pooled RNAi screen identifies ubiquitin ligase Itch as crucial for influenza A

- virus release from the endosome during virus entry. *Proc. Natl. Acad. Sci. U. S. A.* 110, 17516–17521. <https://doi.org/10.1073/pnas.1312374110>
- Suarez, D.L., Perdue, M.L., 1998. Multiple alignment comparison of the non-structural genes of influenza A viruses. *Virus Res.* 54, 59–69. [https://doi.org/10.1016/S0168-1702\(98\)00011-2](https://doi.org/10.1016/S0168-1702(98)00011-2)
- Suresh Kumar, K.G., Krolewski, J.J., Fuchs, S.Y., 2004. Phosphorylation and specific ubiquitin acceptor sites are required for ubiquitination and degradation of the IFNAR1 subunit of type I interferon receptor. *J. Biol. Chem.* 279, 46614–46620. <https://doi.org/10.1074/jbc.M407082200>
- Swiecki, M., Colonna, M., 2011. Type I interferons: Diversity of sources, production pathways and effects on immune responses. *Curr. Opin. Virol.* <https://doi.org/10.1016/j.coviro.2011.10.026>
- Tagawa, A., Ogawa, T., Tetsuka, S., Otsuka, M., Hashimoto, R., Kato, H., Ando, K., Tanabe, H., 2016. Hepatitis C virus (HCV) reactivation during fingolimod treatment for relapsing and remitting multiple sclerosis. *Mult. Scler. Relat. Disord.* 9, 155–157. <https://doi.org/10.1016/j.msard.2016.08.003>
- Taha, T.A., Hannun, Y.A., Obeid, L.M., 2006. Sphingosine kinase: Biochemical and cellular regulation and role in disease. *J. Biochem. Mol. Biol.* <https://doi.org/10.5483/bmbrep.2006.39.2.113>
- Tan, J.M.M., Wong, E.S.P., Kirkpatrick, D.S., Pletnikova, O., Ko, H.S., Tay, S.P., Ho, M.W.L., Troncoso, J., Gygi, S.P., Lee, M.K., Dawson, V.L., Dawson, T.M., Lim, K.L., 2008. Lysine 63-linked ubiquitination promotes the formation and autophagic clearance of protein inclusions associated with neurodegenerative diseases. *Hum. Mol. Genet.* 17, 431–439. <https://doi.org/10.1093/hmg/ddm320>

- Tenover, B.R., Ng, S.-L., Chua, M.A., McWhirter, S.M., García-Sastre, A., Maniatis, T., 2007. Multiple functions of the IKK-related kinase IKKepsilon in interferon-mediated antiviral immunity. *Science* 315, 1274–1278.
<https://doi.org/10.1126/science.1136567>
- Thudichum, J.L.W., 1964. *A Treatise on the Chemical Constitution of the Brain*. Archon Books, North Haven, CT, USA. <https://doi.org/10.1086/349859>
- Tiper, I. V., East, J.E., Subrahmanyam, P.B., Webb, T.J., 2016. *Sphingosine 1-phosphate signaling impacts lymphocyte migration, inflammation and infection, Pathogens and Disease*. Oxford University Press.
<https://doi.org/10.1093/femspd/ftw063>
- Trigueiro-Louro, J.M., Correia, V., Santos, L.A., Guedes, R.C., Brito, R.M.M., Rebelo-de-Andrade, H., 2019. To hit or not to hit: Large-scale sequence analysis and structure characterization of influenza A NS1 unlocks new antiviral target potential. *Virology* 535, 297–307. <https://doi.org/10.1016/j.virol.2019.04.009>
- Tu, J., Guo, J., Zhang, A., Zhang, W., Zhao, Z., Zhou, H., Liu, C., Chen, H., Jin, M., 2011. Effects of the C-terminal truncation in NS1 protein of the 2009 pandemic H1N1 influenza virus on host gene expression. *PLoS One* 6, 26175.
<https://doi.org/10.1371/journal.pone.0026175>
- Varble, A., Albrecht, R.A., Backes, S., Crumiller, M., Bouvier, N.M., Sachs, D., García-Sastre, A., Tenover, B.R., 2014. Influenza a virus transmission bottlenecks are defined by infection route and recipient host. *Cell Host Microbe* 16, 691–700.
<https://doi.org/10.1016/j.chom.2014.09.020>
- Varga, Z.T., Grant, A., Manicassamy, B., Palese, P., 2012. Influenza Virus Protein PB1-F2 Inhibits the Induction of Type I Interferon by Binding to MAVS and Decreasing

- Mitochondrial Membrane Potential. *J. Virol.* 86, 8359–8366.
<https://doi.org/10.1128/jvi.01122-12>
- Vazquez, C., Horner, S.M., 2015. MAVS Coordination of Antiviral Innate Immunity. *J. Virol.* 89, 6974–6977. <https://doi.org/10.1128/jvi.01918-14>
- Vijayan, M., Seo, Y.J., Pritzl, C.J., Squires, S.A., Alexander, S., Hahm, B., 2014. Sphingosine kinase 1 regulates measles virus replication. *Virology* 450–451, 55–63.
<https://doi.org/10.1016/j.virol.2013.11.039>
- Vijayan, M., Xia, C., Song, Y.E., Ngo, H., Studstill, C.J., Drews, K., Fox, T.E., Johnson, M.C., Hiscott, J., Kester, M., Alexander, S., Hahm, B., 2017. Sphingosine 1-Phosphate Lyase Enhances the Activation of IKK ϵ To Promote Type I IFN–Mediated Innate Immune Responses to Influenza A Virus Infection. *J. Immunol.* 199, 677–687. <https://doi.org/10.4049/jimmunol.1601959>
- Voges, D., Zwickl, P., Baumeister, W., 1999. The 26S Proteasome: A Molecular Machine Designed for Controlled Proteolysis. *Annu. Rev. Biochem.* 68, 1015–1068.
<https://doi.org/10.1146/annurev.biochem.68.1.1015>
- Voronkov, A., Holsworth, D.D., Waaler, J., Wilson, S.R., Ekblad, B., Perdreau-Dahl, H., Dinh, H., Drewes, G., Hopf, C., Morth, J.P., Krauss, S., 2013. Structural basis and SAR for G007-LK, a lead stage 1,2,4-triazole based specific tankyrase 1/2 inhibitor. *J. Med. Chem.* 56, 3012–3023. <https://doi.org/10.1021/jm4000566>
- Vyas, S., Chesarone-Cataldo, M., Todorova, T., Huang, Y.H., Chang, P., 2013. A systematic analysis of the PARP protein family identifies new functions critical for cell physiology. *Nat. Commun.* 4. <https://doi.org/10.1038/ncomms3240>
- Walker, S., Brew, B., 2016. Kaposi sarcoma in a fingolimod-treated patient with multiple

- sclerosis. *J. Clin. Neurosci.* 31, 217–218. <https://doi.org/10.1016/j.jocn.2016.03.001>
- Wan, Y., Hopper, A.K., 2018. From powerhouse to processing plant: Conserved roles of mitochondrial outer membrane proteins in tRNA splicing. *Genes Dev.* 32, 1309–1314. <https://doi.org/10.1101/gad.316257.118>
- Wang, H., Li, Q., Shen, Yujun, Sun, A., Zhu, X., Fang, S., Shen, Yuxian, 2011. The ubiquitin ligase Hrd1 promotes degradation of the Z variant alpha 1-antitrypsin and increases its solubility. *Mol. Cell. Biochem.* 346, 137–145. <https://doi.org/10.1007/s11010-010-0600-9>
- Wang, S., Chi, X., Wei, H., Chen, Y., Chen, Z., Huang, S., Chen, J.-L., 2014a. Influenza A Virus-Induced Degradation of Eukaryotic Translation Initiation Factor 4B Contributes to Viral Replication by Suppressing IFITM3 Protein Expression. *J. Virol.* 88, 8375–8385. <https://doi.org/10.1128/jvi.00126-14>
- Wang, S., Chi, X., Wei, H., Chen, Y., Chen, Z., Huang, S., Chen, J.-L., 2014b. Influenza A Virus-Induced Degradation of Eukaryotic Translation Initiation Factor 4B Contributes to Viral Replication by Suppressing IFITM3 Protein Expression. *J. Virol.* 88, 8375–8385. <https://doi.org/10.1128/jvi.00126-14>
- Wang, T.T., Palese, P., 2009. Unraveling the Mystery of Swine Influenza Virus. *Cell.* <https://doi.org/10.1016/j.cell.2009.05.032>
- Wang, W., Krug, R.M., 1998. U6atac snRNA, the highly divergent counterpart of U6 snRNA, is the specific target that mediates inhibition of AT-AC splicing by the influenza virus NS1 protein. *RNA* 4, 55–64. <https://doi.org/10.1017/S1355838298980992>
- Wang, W., Riedel, K., Lynch, P., Chien, C.Y., Montelione, G.T., Krug, R.M., 1999. RNA

binding by the novel helical domain of the influenza virus NS1 protein requires its dimer structure and a small number of specific basic amino acids. *RNA* 5, 195–205. <https://doi.org/10.1017/S1355838299981621>

Wang, X., Li, M., Zheng, H., Muster, T., Palese, P., Beg, A.A., García-Sastre, A., 2000. Influenza A Virus NS1 Protein Prevents Activation of NF- κ B and Induction of Alpha/Beta Interferon. *J. Virol.* 74, 11566–11573. <https://doi.org/10.1128/jvi.74.24.11566-11573.2000>

Wang, X., Robbins, J., 2014. Proteasomal and lysosomal protein degradation and heart disease. *J. Mol. Cell. Cardiol.* <https://doi.org/10.1016/j.yjmcc.2013.11.006>

Wang, Z., Robb, N.C., Lenz, E., Wolff, T., Fodor, E., Pleschka, S., 2010. NS Reassortment of an H7-Type Highly Pathogenic Avian Influenza Virus Affects Its Propagation by Altering the Regulation of Viral RNA Production and Antiviral Host Response. *J. Virol.* 84, 11323–11335. <https://doi.org/10.1128/jvi.01034-10>

Wati, S., Rawlinson, S.M., Ivanov, R.A., Dorstyn, L., Beard, M.R., Jans, D.A., Pitson, S.M., Burrell, C.J., Li, P., Carr, J.M., 2011. Tumour necrosis factor alpha (TNF-alpha) stimulation of cells with established dengue virus type 2 infection induces cell death that is accompanied by a reduced ability of TNF-alpha to activate nuclear factor kappaB and reduced sphingosine kinase-1 activity. *J. Gen. Virol.* 92, 807–18. <https://doi.org/10.1099/vir.0.028159-0>

Wei, C.-J., Xu, L., Kong, W.-P., Shi, W., Canis, K., Stevens, J., Yang, Z.-Y., Dell, A., Haslam, S.M., Wilson, I.A., Nabel, G.J., 2008. Comparative Efficacy of Neutralizing Antibodies Elicited by Recombinant Hemagglutinin Proteins from Avian H5N1 Influenza Virus. *J. Virol.* 82, 6200–6208. <https://doi.org/10.1128/jvi.00187-08>

Westera, L., Jennings, A.M., Maamary, J., Schwemmler, M., García-Sastre, A., Bortz, E.,

2019. Poly-ADP Ribosyl Polymerase 1 (PARP1) Regulates Influenza A Virus Polymerase. *Adv. Virol.* 2019. <https://doi.org/10.1155/2019/8512363>
- Wickham, H., Francois, R., Henry, L., Muller, K., RStudio, 2021. *A Grammar of Data Manipulation* R package version 1.0.3.
- Widjaja, I., de Vries, E., Tscherne, D.M., García-Sastre, A., Rottier, P.J.M., de Haan, C.A.M., 2010. Inhibition of the Ubiquitin-Proteasome System Affects Influenza A Virus Infection at a Postfusion Step. *J. Virol.* 84, 9625–9631. <https://doi.org/10.1128/jvi.01048-10>
- Wolf, J.J., Studstill, C.J., Hahm, B., 2019. Emerging Connections of S1P-Metabolizing Enzymes with Host Defense and Immunity During Virus Infections. *Viruses* 2019, Vol. 11, Page 1097 11, 1097. <https://doi.org/10.3390/V11121097>
- Wolf, J.J., Xia, C., Studstill, C.J., Ngo, H., Brody, S.L., Anderson, P.E., Hahm, B., 2021. Influenza A virus NS1 induces degradation of sphingosine 1-phosphate lyase to obstruct the host innate immune response. *Virology* 558, 67–75. <https://doi.org/10.1016/j.virol.2021.02.006>
- Xia, C., Anderson, P., Hahm, B., 2018a. Viral dedication to vigorous destruction of interferon receptors. *Virology*. <https://doi.org/10.1016/j.virol.2018.06.017>
- Xia, C., Seo, Y.J., Studstill, C.J., Vijayan, M., Wolf, J.J., Hahm, B., 2018b. Transient inhibition of sphingosine kinases confers protection to influenza A virus infected mice. *Antiviral Res.* 158, 171–177. <https://doi.org/10.1016/j.antiviral.2018.08.010>
- Xia, C., Vijayan, M., Pritzl, C.J., Fuchs, S.Y., McDermott, A.B., Hahm, B., 2016. Hemagglutinin of Influenza A Virus Antagonizes Type I Interferon (IFN) Responses by Inducing Degradation of Type I IFN Receptor 1. *J. Virol.* 90, 2403–2417.

- Xia, C., Wolf, J.J., Sun, C., Xu, M., Studstill, C.J., Chen, J., Ngo, H., Zhu, H., Hahm, B., 2020. PARP1 Enhances Influenza A Virus Propagation by Facilitating Degradation of Host Type I Interferon Receptor. *J. Virol.* 94. <https://doi.org/10.1128/jvi.01572-19>
- Xia, C., Wolf, J.J., Vijayan, M., Studstill, C.J., Ma, W., Hahm, B., 2018c. Casein Kinase 1 α Mediates the Degradation of Receptors for Type I and Type II Interferons Caused by Hemagglutinin of Influenza A Virus. *J. Virol.* 92. <https://doi.org/10.1128/jvi.00006-18>
- Xia, C., Wolf, J.J., Vijayan, M., Studstill, C.J., Ma, W., Hahm, B., 2018d. Casein Kinase 1 α Mediates the Degradation of Receptors for Type I and Type II Interferons Caused by Hemagglutinin of Influenza A Virus. *J. Virol.* 92, 6–18. <https://doi.org/10.1128/jvi.00006-18>
- Xu, F., Sun, Y., Yang, S., Zhou, T., Jhala, N., McDonald, J., Chen, Y., 2019. Cytoplasmic PARP-1 promotes pancreatic cancer tumorigenesis and resistance. *Int. J. Cancer* 145, 474–483. <https://doi.org/10.1002/ijc.32108>
- Yamane, D., McGivern, D.R., Wauthier, E., Yi, M., Madden, V.J., Welsch, C., Antes, I., Wen, Y., Chugh, P.E., McGee, C.E., Widman, D.G., Misumi, I., Bandyopadhyay, S., Kim, S., Shimakami, T., Oikawa, T., Whitmire, J.K., Heise, M.T., Dittmer, D.P., Kao, C.C., Pitson, S.M., Merrill, A.H., Reid, L.M., Lemon, S.M., 2014. Regulation of the hepatitis C virus RNA replicase by endogenous lipid peroxidation. *Nat. Med.* 20, 927–35. <https://doi.org/10.1038/nm.3610>
- Yamane, D., Zahoor, M.A., Mohamed, Y.M., Azab, W., Kato, K., Tohya, Y., Akashi, H., 2009. Inhibition of sphingosine kinase by bovine viral diarrhea virus NS3 is crucial for efficient viral replication and cytopathogenesis. *J. Biol. Chem.* 284, 13648–13659. <https://doi.org/10.1074/jbc.M807498200>

- Yin, C., Khan, J.A., Swapna, G.V.T., Ertekin, A., Krug, R.M., Tong, L., Montelione, G.T., 2007. Conserved surface features form the double-stranded RNA binding site of non-structural protein 1 (NS1) from influenza A and B viruses. *J. Biol. Chem.* 282, 20584–20592. <https://doi.org/10.1074/jbc.M611619200>
- Zamora-Pineda, J., Kumar, A., Suh, J.H., Zhang, M., Saba, J.D., 2016. Dendritic cell sphingosine-1-phosphate lyase regulates thymic egress. *J. Exp. Med.* 213, 2773–2791. <https://doi.org/10.1084/jem.20160287>
- Zemann, B., Kinzel, B., Müller, M., Reuschel, R., Mechtcheriakova, D., Urtz, N., Bornancin, F., Baumruker, T., Billich, A., 2006. Sphingosine kinase type 2 is essential for lymphopenia induced by the immunomodulatory drug FTY720. *Blood* 107, 1454–1458. <https://doi.org/10.1182/blood-2005-07-2628>
- Zhang, H., Desai, N.N., Olivera, A., Seki, T., Brooker, G., Spiegel, S., 1991. Sphingosine-1-phosphate, a novel lipid, involved in cellular proliferation. *J. Cell Biol.* 114, 155–167. <https://doi.org/10.1083/jcb.114.1.155>
- Zhang, L., Xu, M., Scotti, E., Chen, Z.J., Tontonoz, P., 2013. Both K63 and K48 ubiquitin linkages signal lysosomal degradation of the LDL receptor. *J. Lipid Res.* 54, 1410–1420. <https://doi.org/10.1194/jlr.M035774>
- Zheng, Hui, Qian, J., Baker, D.P., Fuchs, S.Y., 2011. Tyrosine phosphorylation of protein kinase D2 mediates ligand-inducible elimination of the type 1 interferon receptor. *J. Biol. Chem.* 286, 35733–35741. <https://doi.org/10.1074/jbc.M111.263608>
- Zheng, H., Qian, J., Varghese, B., Baker, D.P., Fuchs, S., 2011. Ligand-Stimulated Downregulation of the Alpha Interferon Receptor: Role of Protein Kinase D2. *Mol. Cell. Biol.* 31, 710–720. <https://doi.org/10.1128/mcb.01154-10>

- Zhirnov, O.P., Klenk, H.D., 2013. Influenza A Virus Proteins NS1 and Hemagglutinin Along with M2 Are Involved in Stimulation of Autophagy in Infected Cells. *J. Virol.* 87, 13107–13114. <https://doi.org/10.1128/jvi.02148-13>
- Zhong, X., Lee, H., Kim, S.H., Park, S., Kim, W., Cha, Y., Surh, Y., 2018. Myc-nick promotes efferocytosis through M2 macrophage polarization during resolution of inflammation. *FASEB J.* 32, 5312–5325. <https://doi.org/10.1096/fj.201800223R>
- Zhu, X., Zhou, F., Qin, D., Zeng, Y., Lv, Z., Yao, S., Lu, C., 2011. Human immunodeficiency virus type 1 induces lytic cycle replication of Kaposi's-sarcoma-associated herpesvirus: role of Ras/c-Raf/MEK1/2, PI3K/AKT, and NF-κB signaling pathways. *J. Mol. Biol.* 410, 1035–51. <https://doi.org/10.1016/j.jmb.2011.03.055>
- Ziemssen, T., Medin, J., Couto, C.A.-M.M., Mitchell, C.R., 2017. Multiple sclerosis in the real world: A systematic review of fingolimod as a case study, *Autoimmunity Reviews*. Elsevier B.V. <https://doi.org/10.1016/j.autrev.2017.02.007>

Vita

Jennifer J. Wolf was born in College Station, TX and grew up in Ardmore, OK. She earned her Bachelor of Science degree in microbiology at University of Oklahoma in Norman, OK. Jennifer is completing her Doctor of Philosophy degree in molecular microbiology and immunology in Dr. Bumsuk Hahm's lab at University of Missouri-Columbia.