Role of Fibronectin-interacting cellular proteins in Influenza A virus infection in human lung epithelial cells

BY

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ABSTRACT

Influenza A virus (IAV) alters the expression of many host cellular proteins in the infected cells; many of these proteins interact with fibronectin. Proteasome subunit alpha type-2 (PSMA2), Chloride intracellular channel protein 1(CLIC1) and Heat Shock Protein Family A (Hsp70) member 5 (HSPA5) are three fibronectin interacting proteins that were highly expressed in IAV-infected cells. PSMA2 is part of the 20S proteasome complex, involved in proteolytic modification and recycling of cellular proteins. CLIC1 is a chloride channel that regulates intracellular pH, cell volume and trans-epithelial ion transport. HSPA5 helps to translocate nascent proteins to endoplasmic reticulum and subsequent folding. In this study, I have investigated the importance of PSMA2, CLIC1 and HSPA5 in IAV replication cycle. The knockdown (KD) of PSMA2, CLIC1 and HSPA5 in A549 cells, caused a significant reduction in extracellular progeny IAV. Although CLIC1 KD did not affect viral protein translation, IAV-nucleoprotein (NP) was accumulated in HSPA5 KD and PSMA2 KD cells. Viral RNAs were significantly higher in CLIC1 KD cells after IAV infection, but transcription was unaffected in PSMA2 KD and HSPA5 KD cells. The results indicate that PSMA2, HSPA5 and CLIC1 are critical host factors for IAV and are possibly involve in the terminal stages of viral replication. Further proteomic analysis to understand the role of PSMA2 KD in IAV infection at the proteomic level revealed that NRF2-mediated oxidative stress response signaling, was inhibited by IAV infection but was significantly activated by PSMA2 KD. Reactive oxygen species (ROS) level was significantly inhibited in wild-type cells but significantly increased in PSMA2 KD cells after IAV infection. However, IAV infection also caused significantly higher nuclear translocation of NFR2, which was inhibited in the PSMA2 KD cells. Treatment with ROS scavenger was able to reduce the inhibitory impact of PSMA2 KD. This indicates that PSMA2 is required for NRF2-mediated ROS neutralization and that IAV uses PSMA2 to escape viral clearance via NRF2-mediated oxidative response. This study has extended our understanding of the significance of Fibronectin-interacting proteins in the IAV replication cycle; nevertheless, further research is needed to fully comprehend the mechanism and develop antiviral drugs targeting the proteins.

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DEDICATION

I would like to dedicate this achievement to

my mother, Momotaz Begum, and father, Md Abdur Rashid,

without whom I would not have achieved such a remarkable accomplishment.

Publication earned during PhD training.

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1. **Rashid, M. U**., Gao, A., & Coombs, K. M. (2022). Influenza A virus uses PSMA2 for downregulation of NRF2-mediated oxidative stress response. *Journal of virology*, jvi-01990.

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Figures and tables adapted from this article listed below

Figure 8. Impact of siRNA knockdown of fibronectin-interacting proteins on IAV replication in A549 cells.

Figure 17. PSMA2 is required for replication of IAV virus.

Figure 18. PSMA2 KD does not impact translation viral proteins and transcription of vRNAs but impacts maturation.

Figure 19. Proteomic analysis to understand the impact of PSMA2 KD on IAV replication.

Figure 20. Influenza A Virus Utilizes PSMA2 for Downregulation of NRF2-mediated Oxidative Stress Response.

Figure 21. PSMA2 KD reduces proteasome activity but does not affect IAV replication in presence of NAC.

Figure 22. PSMA2 is required for nuclear translocation of NRF2.

Table 1. Numbers of significantly dysregulated by PSMA2 KD, PR8 infection and PR8 infection in PSMA2 KD cells

Table 2. List of significantly dysregulated by PSMA2 KD, PR8 infection and PR8 infection in PSMA2 KD cells

2. Rashid, M. U., & Coombs, K. M. (2019). Serum-reduced media impacts on cell viability and protein expression in human lung epithelial cells. Journal of cellular physiology, 234(6), 7718-7724.

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Figures and tables adapted from this article listed below

Figure 11. Impact of Opti-MEM on A549 cells viability, morphology and protein expression.

Figure 12. CLIC1 knockdown efficacy in DMEM media compared to Opti-MEM.

Figure 13. PSMA2 knockdown efficacy in DMEM media compared to Opti-MEM.

Figure 7: Host factors and their proposed functions in the influenza virus replication. Reprinted form the article "Watanabe, T., Kawakami, E., Shoemaker, J.E., Lopes, T.J., Matsuoka, Y., Tomita, Y., Kozuka-Hata, H., Gorai, T., Kuwahara, T., Takeda, E. and Nagata, A., 2014. Influenza virus-host interactome screen as a platform for antiviral drug development. *Cell host & microbe*, *16*(6), pp.795-805." Permission was obtained from Copyright Clearance Center to reprint in this thesis. Order License ID1207651-1.

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

Abbreviations	Descriptions
µM	Micro Molar
µm	Micrometre
ATCC	American Type Culture Collection
BiP	Binding Immunoglobulin protein
BST1	Bone Marrow Stromal Cell Antigen 1
Cat	Catalogue
CCNA2	Cyclin A2
CD81	Cluster of Differentiation 81
CDK2	Cyclin Dependent Kinase 2
CDKN1B	Cyclin Dependent Kinase Inhibitor 1B
cDNA	Complementary Deoxyribonucleic Acid
CLF1	Cytokine receptor-like factor 1
CLIC1	Chloride intracellular channel protein
CRM1	Chromosomal Maintenance 1
cRNA	Complementary Ribonucleic acid
CST3	Cystatin C
Ct	Cycle threshold
CTSB	Cathepsin B
CUL3	Cullin 3
DMEM	Dulbecco's Modified Eagle Medium
dpi	Days post infection
ECL	Enhanced chemiluminescence
ECM	extracellular matrix
EGCG	Epigallocatechin-3-gallate
EIF4A3	Eukaryotic Translation Initiation Factor 4A3
EMEM	Eagle's minimum essential medium
ER	Endoplasmic reticulum
ErbB4	Erb-B2 Receptor Tyrosine Kinase 4
FAS	Fas Cell Surface Death Receptor
FBS	Fetal Bovine Serum
FN-1	Fibronectin
FUBP1	Far Upstream Element Binding Protein 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GISRS	Global Influenza Surveillance and Response System
GRB2	Growth Factor Receptor Bound Protein 2
GRP78	Glucose-regulated protein

h	Hours
HA	Hemagglutinin
hpi	Hours post-infection
hpt	Hours post transfection
HRP	horseradish peroxidase
HSPA5	Heat shock 70kDa protein 5
IAV	Influenza A virus
IPA	Ingenuity Pathway Analysis
KD	Knockdown
KEAP1	Kelch-like ECH-associated protein 1
LCN2	Lipocalin 2
M1	Matrix protein 1
M2	Matrix protein 2
MCM7	Minichromosome Maintenance Complex Component 7
MDCK	Madin-Darby canine kidney
MOI	Multiplicity of infection
mpi	Minutes post infection
MRC-5	Medical Research Council cell strain 5
mRNA	Messenger Ribonucleic acid
NA	Neuraminidase
NAC	N-Acetyl-L-cysteine m
NBD	Nucleotide-binding domain
NEAA	Non-essential amino acids
NEP	Nuclear export protein
NGF	Nerve Growth Factor
NLS	Nuclear localization signal
nM	Nanomolar
NPPB	5-nitro-2-(3-phenylpropyl-amino) benzoic acid
NRF2	Nuclear factor-erythroid factor 2-related factor 2
NS/ns	Not significant
NS1	Non-structural proteins 1
NS3	Non-structural proteins 3
NSC	Non silencing control/ Scrambled control
Opti-MEM	Optimized-Minimal Essential Medium
ORF	Open reading frame
OT	On-target
PA	Polymerase acidic protein
PAK	P21 (RAC1) Activated Kinase
PB1	Polymerase basic protein 1
PB2	Polymerase basic protein 2

PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
PFU	Plaque-forming units
PSMA2	Proteasome subunit alpha type-2
PSMA6	Proteasome 20S Subunit Alpha 6
PSMA7	Proteasome subunit alpha type-7
PSMB1	Proteasome subunit beta type-1
PSMB7	Proteasome subunit beta type-7
qRT-PCR	Real-Time Quantitative Reverse Transcription PCR
RdRp	RNA-dependent RNA polymerase
RFU	Relative fluorescent units
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
SBD	Substrate-binding domain
siRNA	Small interfering RNA
SP	Smart-pool
STAT3	Signal Transducer And Activator Of Transcription 3
TFPI	Tissue Factor Pathway Inhibitor
TGFBI	Transforming Growth Factor Beta Induced
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome system
UTR	Untranslated regions
vRNA	Viral Ribonucleic acid
WHO	World Health Organization

TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION

1.1 Influenza virus

In 1933, the *Influenza A virus* (IAV) was first isolated from human patients (Smith et al., 1933). It is a negative-sense, single-stranded RNA virus that belongs to the *Orthomyxoviridae* family. *Influenza B virus*, *Influenza C virus*, *Influenza D virus*, *Thogotovirus*, *Quaranfilvirus* and *Infectious salmon anemia virus* (Figure 1) are other members of this virus family (Blümel et al., 2009; Lycett et al., 2019; Wolff & Veit, 2021). Only the influenza A and B viruses cause disease in humans among these seven genera. IAV is subdivided into 18 HA (hemagglutinin) and 11 NA (neuraminidase) serotypes based on antigenic polymorphisms (Schild et al., 1980; Tong et al., 2013; Wu et al., 2014). Human infections are caused by eight HA serotypes (H1, H2, H3, H5, H6, H7, H9, and H10) and two NA serotypes. However, the most prevalent seasonal flu strains are H1N1 and H3N2 strains (Zhang et al., 2019). However, *Influenza B virus* has two antigenically different subtypes known as Victoria and Yamagata (Biere et al., 2010). Wild ducks and shorebirds are the most common natural hosts of influenza viruses (Taubenberger & Kash, 2010). However, ducks, bats, birds, horses, poultry, turkeys, dogs, and pigs are also susceptible to IAV infection (Wahlgren, 2011; Webster et al., 1992). The natural infection of IAV to their host is primarily asymptomatic but may cause mild to severe disease symptoms when transmitted to other animals (Webster et al., 1992). During the past century, IAV was responsible for several devastating pandemics. Thus, it has received a lot of attention in the past few decades and concerns were mounting about potential pandemic outbreaks in the future.

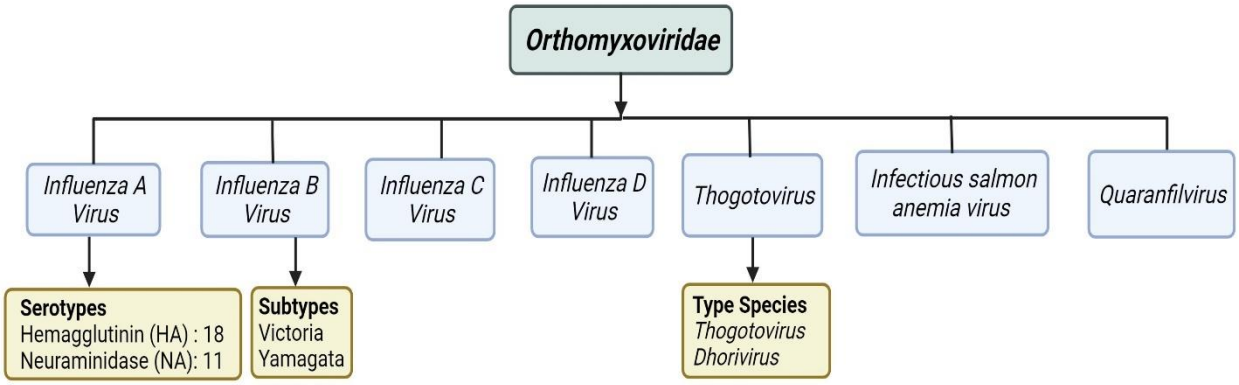


Figure 1: Classification of *Orthomyxoviridae* family. The *Orthomyxoviridae* virus family includes seven genera. The *Influenza A virus* is further divided into 18 HA (hemagglutinin) and 11 NA (neuraminidase) serotypes. The *Influenza B virus* has two subtypes; Victoria and Yamagata. The genus *Thogotovirus* has two type species known as *Thogotovirus* and *Dhorivirus*.

1.2 Influenza Outbreaks and Pandemics

IAV has been associated with seasonal outbreaks and sporadic pandemics. Every year, the influenza virus causes around 1 billion infections, 3-5 million severe incidents, and 0.5 million fatalities globally (WHO, 2019). Children, the elderly, and immunocompromised individuals are most susceptible to IAV infection (Krammer et al., 2018; Lampejo, 2020). Devastating IAV pandemics have caused millions of deaths in the past century (Wikramaratna & Gupta, 2009).

1.2.1 **The Spanish Flu Pandemic (1918-1919)**

In 1918, after the end of World War I, an influenza pandemic broke out worldwide known as the Spanish flu. It was one of the deadliest pandemics to strike humankind, caused by the IAV H1N1 strain (Taubenberger & Kash, 2010). One-third of the world's population suffered the IAV infection, and it killed over 50 million people, making it the world's second-deadliest pandemic in human history (Frost, 1920; Taubenberger & Morens, 2006) after The Black Death of 1346-1353 (DeWitte, 2010; Feehan & Apostolopoulos, 2021). Although the virus could infect individuals of all ages, the majority of fatalities were documented in those aged 20 to 40, resulting in a significant decline in life expectancy (Johnson & Mueller, 2002; Simonsen et al., 1998). The high fatality rate was primarily linked to bacterial pneumonia followed by the viral infection; however, the exact pathogenic mechanism of such lethal infections remains unclear (Morens et al., 2008).

1.2.2 Asian Flu Pandemic (1957-1958)

The Asian pandemic flu first appeared in 1957. The causative agent of the pandemic was IAV H2N2 serotype, which was first identified in China. It subsequently traveled to Hong Kong, Singapore, and the United States. It was able to infect people of all age groups, causing lethal diseases in both children and adults. However, bacterial co-infection was not commonly linked H2N2 infections (Rogers et al., 1958). The H2N2 influenza virus originated from the Spanish flu H1N1 strain. The HA and NA encoding gene segments and the polymerase basic protein 1 (PB1) were obtained from the avian influenza strain. The remaining genomic segments were identical to the Spanish flu strain (Kawaoka et al., 1989; Scholtissek et al., 1978).

1.2.3 The Hong Kong Flu Pandemic (1968)

The IAV H3N2 serotype was responsible for the Hong Kong pandemic in 1968. This virus evolved by reassortment of the genome of avian influenza and a previously circulating H2N2 strain. The H3N2 virus acquired avian-like HA (H3 subtype) and PB1 gene segments from avian species (Kawaoka et al., 1989; Scholtissek et al., 1978), while, the NA segment was carried over from the previous H2N2 Asian Pandemic influenza virus. It was suggested that the severity of the pandemic was reduced by cross-reacting antibodies from previously circulating strains as the newly evolved strain was carrying the same NA (Taubenberger & Kash, 2010).

1.2.4 The Swine flu Pandemic (2009)

The pandemic in 2009 was associated with a newly emerged H1N1 strain, which caused self-limiting but severe pneumonia-related disease. It was first discovered in an outbreak in Mexico (Jain et al., 2009; Perez-Padilla et al., 2009). In Mexico, 854 incidents of pneumonia were documented, with 59 death (Jain et al., 2009). However, during the first year of the 2009 swine flu pandemic, the estimated mortality ranged from 151,700 to 575,400 individuals globally (CDC, 2012). This pandemic strain originated by combining two influenza viruses from the Eurasian and North American swine origins that had been actively causing disease in humans for several years. This strain emerged from the assortment of the IAV genome from bird, swine, and human origin (Garten et al., 2009; Jain et al., 2009).

1.3 Influenza A virus (IAV) structure

IAV has a circular filamentous structure with a diameter of 100-120 nm. It has an envelope made of host-derived lipid membranes (Figure 2). The HA and NA are surface glycoproteins inserted in the lipid membrane at roughly a four-to-one ratio (Wasilewski et al., 2012). This membrane also contains a small number of matrix proteins (M1 and M2), found beneath the envelope. IAV structure also includes the NEP/NS2 (nuclear export protein) (Richardson & Akkina, 1991). The viral core is surrounded by the envelope membrane, consisting of HA, NA, M2 and M1. The segmented viral RNAs are wrapped with nucleoproteins (NP) and polymerase proteins (PA, PB1, and PB2) that reside inside the core (Bouvier & Palese, 2008).

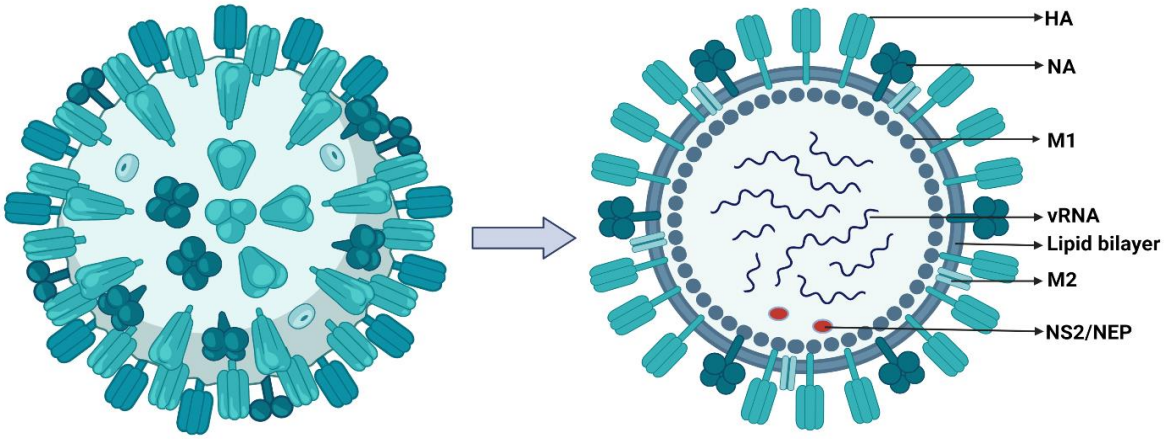


Figure 2: Structure of Influenza A virus. Viral genomic segments (vRNA) reside inside the core surrounded by M1 proteins. The outer envelope is acquired from host cells and contains HA, NA and M2 viral proteins. NS2/NEP is also found inside the core structure. (The Figure was generated using https://app.biorender.com/)

1.3.1 Hemagglutinin (HA)

In the IAV particle, HA is the major antigen located on the surface. HA is the first viral antigen to be recognized by the host adaptive immune system and produces neutralizing antibodies after IAV infection (Stanekov & Varekov, 2010). In the viral envelope, the carboxy terminus of HA is embedded, and the hydrophilic end extends outside the virus particle. There are currently 18 HA subtypes known to exist in nature (Tong et al., 2013; Wu et al., 2014). The mature form of HA is proteolytically cleaved into HA1 and HA2 subunits (Gamblin & Skehel, 2010). HA determines the host specificity by binding to the specific receptors on the cell surface (Abed et al., 2011; Tumpey et al., 2007; B. Zheng et al., 2010). In birds, the IAV HA antigen binds to the 2,3-linked sialic acid receptors, whereas in humans, it recognizes the 2,6-linked sialic acid receptors (Byrd-Leotis et al., 2017). Following attachment, the HA plays a key role in the internalization of virus particles into the cells. The acidic pH of the endosome causes a conformational change in HA, creating a fusion pore in the cytoplasmic membrane with the HA2 peptides. The viral ribonucleoproteins (vRNP) enter the cytoplasm via these fusion pores (Gamblin & Skehel, 2010; Stegmann, 2000).

1.3.2 Neuraminidase (NA)

The second most abundant IAV surface antigen and glycoprotein is NA. After budding from the infected cells, the HA on the progeny virus binds with the sialic acid receptors on the host cell. The cleavage of the HA-sialic acid bond by NA is critical for viral release (Gamblin & Skehel, 2010). There are currently 11 NA subtypes known to exist in nature (Tong et al., 2013; Wu et al., 2014).

1.3.3 Matrix Protein 1 (M1)

The M1 is the most prevalent viral protein in IAV virion. The M1 proteins create a layer under the lipid envelope that encircles the nucleocapsids, and it connects the inner core of virus particles to the surface glycoproteins (Schmitt & Lamb, 2005). In the IAV infected cells, M1 was detected in the nucleus and cytoplasm (Webster et al., 1992). M1 is involved in virus assembly and the nuclear export of viral RNPs to progeny virus particles (Cros & Palese, 2003).

1.3.4 Matrix Protein 2 (M2)

One other membrane integrated protein in IAV particles is M2. During IAV infection, M2 can associate with the proton ion channel on the cell membrane and regulates the entry of vRNPs in the infected cell cytoplasm by acidification of the core of IAV (Rossman & Lamb, 2009). However, the membrane integrated domain of M2 connects with M1, and aids vRNP assembly with viral proteins and the budding of the progeny virions (Chen et al., 2008).

1.3.5 Polymerase proteins (PA, PB1, and PB2)

The IAV RNA-dependent RNA polymerase complex, which is a virulence factor for IAV, is made up of Polymerase acidic protein (PA), Polymerase basic protein 1 (PB1), and 2 (PB2). The RNA polymerase complex is one of the key players in the virulence of the 1918 Spanish flu (Watanabe et al., 2009).

Small pieces of host RNA are used to activate viral RNA transcription, a process known as cap snatching, a critical step in IAV replication. At the beginning of IAV RNA transcription, the PB2 protein binds to the host mRNA at 5' cap (Guilligay et al., 2008) and produces mRNA cap for viral transcription with the help of the endonuclease activity of PA protein (Dias et al., 2009; Yuan et al., 2009). The catalytic activity and structural integrity of influenza RNA polymerase

depend on PB1 (Chu et al., 2012). PB1 also plays a role in elongating viral mRNA by accelerating the successive insertion of nucleotides after cap snatching. Furthermore, PB1 binds to both (-) sense vRNA and complementary (**+)** sense RNA to start the transcription process (González & Ortín, 1999; Noda & Kawaoka, 2010). PB1 mRNA translates two shorter forms of PB1 proteins, PB1-N40 and PB1-F2, via the alternative open reading frame (ORF) (Chen et al., 2001; Wise et al., 2009). PB1-F2 can induce apoptosis in the IAV-infected cells (Zamarin et al., 2005). Both the H5N1 and 1918 H1N1 viruses gained significant virulence from a single amino acid mutation (N66S) in the PB1-F2 protein (Conenello et al., 2007).

1.3.6 Nucleoprotein (NP)

In the IAV virion, NP is the second most prominent protein and is also produced in large quantities inside the infected cells. It coats the entire vRNA and serves as a structural component of viral RNP. The vRNPs must be carried inside the nucleus to initiate transcription. The nuclear localization signal (NLS) on the NP protein enables the transportation of vRNPs from the cytoplasm to the nucleus (O'Neill et al., 1995).

1.3.7 Non-structural protein 1 (NS1)

NS1 is a 202–237 aa long multifunctional protein, abundantly expressed in the infected cells (Krug & Etkind, 1973) and found in all IAV strains (Dundon, 2012). Two distinct categories of IAV NS1 proteins have been identified based on the sequence difference: allele A and allele B. The allele B refers to the NS1 solely for avian origin, whereas allele A includes NS1 proteins from equine, equine, swine, avian, and human IAVs (Ludwig et al., 1991; Treanor et al., 1989). The majority of IAV antigenic alterations occur in HA and NA surface antigens with little or no changes in NS1 antigenic characteristics. Thus there are no significant associations between NS1 and HA or NA subtypes (Brown et al., 1983). NS1 is involved in viral gene splicing (Garaigorta & Ortín, 2007; Robb et al., 2011) and can interfere with the host immune response by inhibiting interferon expression. However, NS1 can also interact with RNA and a large number of host proteins (Rahim et al., 2018) and modulate host cellular functions and signaling pathways in favor of viral replication (Bergmann et al., 2000; Egorov et al., 1998; Hale et al., 2008; Jia et al., 2010; Kochs et al., 2007; Talon et al., 2000; Wang et al., 2000).

1.3.8 Nuclear export protein (NEP/NS2)

The Nuclear export protein (NEP), also known as NS2, plays multiple roles in the influenza virus replication cycle. CRM1 (chromosome region maintenance 1) is a critical nuclear export receptor (Liu et al., 2021; Paterson & Fodor, 2012). Influenza NEP functions as an adapter with CRM1 to facilitate the transfer of vRNPs from infected cells' nucleus to the progeny virion (Boulo et al., 2007; Paterson & Fodor, 2012). Nuclear export signal (NES), a methionine/leucine-rich region at the N-terminus of NEP/NS2, has been shown to play a major role in RNP export (Boulo et al., 2007). NEP has the ability to recruit the F1Fo ATPase, which is essential for the budding of progeny virions (Gorai et al., 2012). Furthermore, NEP plays important role in vRNA transcription and replication, which has been linked to the ability of some avian H5N1 influenza viruses to replicate efficiently in mammalian cells (Mänz et al., 2012).

1.3.9 Non-structural protein 3 (NS3)

NS3, is a novel viral protein synthesized by alternative splicing of the NS1 gene, caused by a D125G (GAT to GGT) mutation (Selman et al., 2012). NS3 is a shortened version of the NS1 protein that lacks the three-antiparallel-strand motif found in the original, which spans only 126 to 168 codons. The NS1-125G (GGT) codon has been detected from 33 IAV strains and linked to the transition from avian to mammalian hosts. The NS3 protein plays a critical role in viral adaptation in new hosts (Hao et al., 2020; Selman et al., 2012).

1.4 Influenza A virus genome

The IAV genome consists of eight single-stranded, segmented negative-sense RNAs. Each of these segments encode for one or more proteins. The PB2, PA, HA, and NP proteins are encoded by viral RNA segments 1, 3, 4, and 5, respectively (Bouvier & Palese, 2008). The polymerase complex subunit PB1 protein is encoded by IAV segment 2. In some IAVs, vRNA segment 2 codes for the PB1-F2 protein, which has pro-apoptotic properties (Bouvier & Palese, 2008; W. Chen et al., 2001). NA and M1 are encoded by segment 6 and segment 7, respectively. However, the M2 protein is produced by alternative splicing (mRNA) of segment 2 (Lamb & Choppin, 1981). The non-structural NS1 protein is encoded by segment 8, while the NEP/NS2 protein is encoded by the alternative splicing of the same segment (Briedis & Lamb, 1982; Lamb et al.,

1980). Although non-coding sections exist in each viral RNA segment, the sequences at the 5' and 3'ends are mostly conserved (Figure 3A).

The viral Ribonucleoproteins (vRNP) complex (Figure 3B) is composed of vRNAs wrapped in NP and polymerase proteins (PB1, PB2, and PA) (Noda & Kawaoka, 2010). A helical hairpin is found at the ends of all vRNAs, which binds to polymerase complexes.

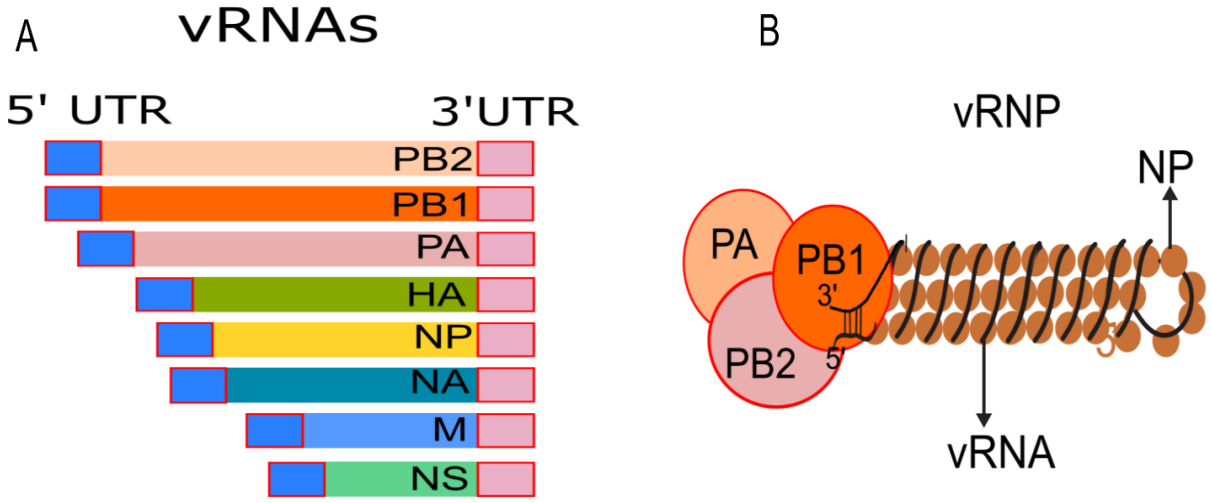


Figure 3: Influenza A virus genomic RNAs. A. Schematic diagram of the eight vRNA genomic segments of IAV. The viral promoters in the 5′ and 3′ untranslated regions (UTRs), whereas the middle portion represents the coding region within each vRNA. B. A helical hairpin is formed when a vRNA segment coils around multiple nucleoproteins (NP), bound by a single tetrameric viral RNA-dependent RNA polymerase with homologous promoter sequences in the 5′ and 3′ UTR regions (PB1, PB2, and PA). (Figure generated by https://app.biorender.com/ and concept adapted from (Dou et al., 2018)

The IAV genome is highly prone to mutation as its RNA polymerase lacks the functional competency of exonuclease proofreading. Thus, wrong nucleotides are easily incorporated into the genome (roughly one error per genome) during replication (Steinhauer & Holland, 1987). Because avian influenza viruses are well adapted in birds, the mutation rate is relatively low. However, the mutation rate in mammalian influenza viruses is much higher than the bird-adapted influenza viruses (Webster et al., 1992). The host innate immune system first encounters the viral surface antigens HA and NA during a viral infection. Thus, under the selective pressure of the host immune system, mutations occur in the antigenic motifs of HA and NA (Chen & Holmes, 2006). HA or NA mutations frequently allow viruses to bypass the host immune system or avoid detection

by neutralizing antibodies and the innate immune system. Antigenic drift is a mutation that changes the viral antigenicity of surface glycoproteins, such as HA or NA (Carrat & Flahault, 2007).

The term "antigenic shift" refers to a substantial genetic alteration that happens in IAV by exchanging genome segments. When two or more strains of IAV infect the same host cell, an exchange of genetic components occurs during viral replication, resulting in the emergence of a new virus strain (Figure 4).

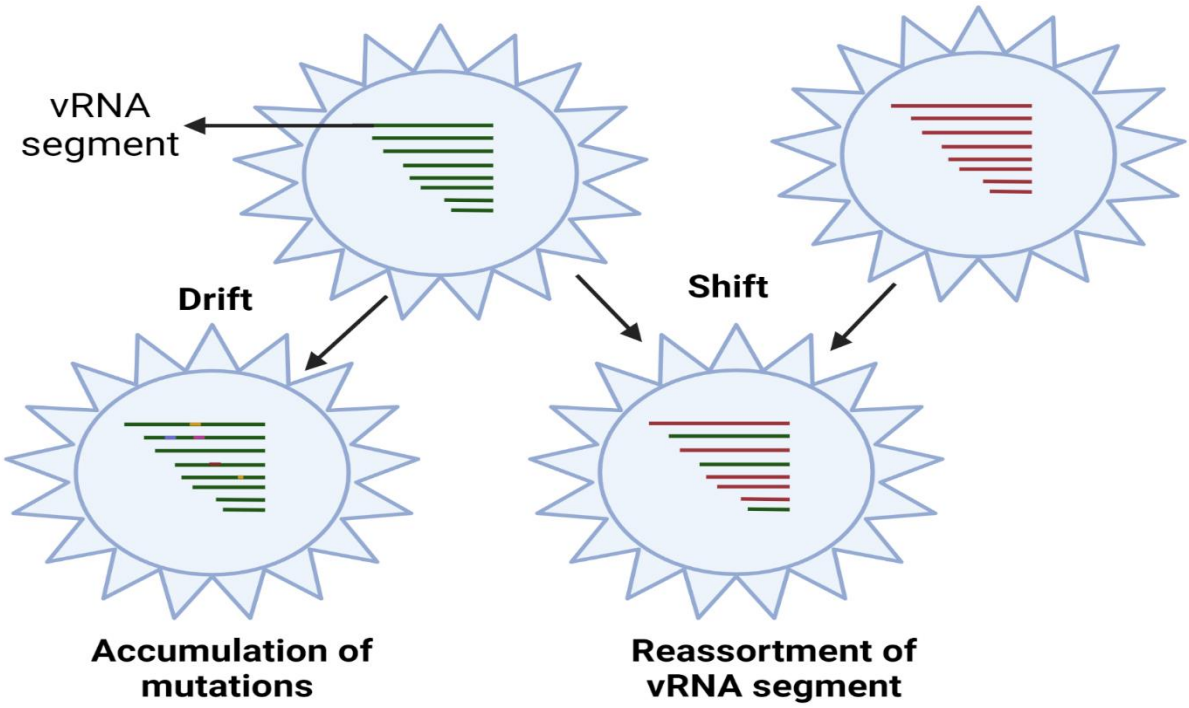


Figure 4: Emergence of new strains of Influenza viruses by genetic shift and drift. The segmented IAV genome is illustrated in the figure. The segments of the genome are coloured differently to reflect the mutations that occur in the genome. RNA segment interchange causes genomic shift, indicated by different colored segments in the same virus.

The newly formed virus will have a different set of genomic segments compared to the parental strains, which will result in a new antigenic type. As a result, human populations usually lack prior immunity against the mutant virus and possess the potential to spread severe outbreaks or pandemics of IAV infection (Ahmed et al., 2007; Bouvier & Palese, 2008). The influenza H1N1 strain of 2009 pandemic, Spanish flu (1918), H2N2 of Asian Flu" Pandemic 1957, and H3N2 of Hong Kong Pandemic 1968 emerged by genomic reassortment or antigenic shifts (Klenk et al., 2011; Schäffr et al., 1993; Sun et al., 2021; Tscherne & García-Sastre, 2011).

1.5 Life cycle of Influenza A virus

1.5.1 Virus attachment

IAV HA protein attaches to the sialic acid (N-acetylneuraminic acid) receptor on the host cells. The HA1 subunit of HA has a binding domain that recognizes the sialic acid receptor via galactose α2,6 linkage in humans and via galactose α2,3 linkage in bird flu viruses (Skehel & Wiley, 2000). In humans, the sialic acid receptors are located on the tracheal or respiratory tract epithelial cells, whereas it is present on the gut epithelium in ducks (Matrosovich et al., 2004; G. N. Rogers & Paulson, 1983). Interestingly the IAV from Swine origin can bind the sialic acid receptors both from human and bird origin. It is suggested that swine is an important intermediate host where human and avian influenza genomes mix, giving rise to recombinant strains with pandemic potential (Sieczkarski & Whittaker, 2005; Skehel & Wiley, 2000).

1.5.2 Virus entry, fusion, and uncoating

The attachment of HA to the cell receptor initiates the clathrin-dependent endocytosis or micropinocytosis of the virion (de Vries et al., 2011; Rust et al., 2004; Sieczkarski & Whittaker, 2002). The virus enters the cell and creates a viral endosome. The low pH of the endosome activates M2 ion channel (Carrat & Flahault, 2007; Lakadamyali et al., 2003; Rust et al., 2004) and causes the conformational change in HA, exposing the HA2 (fusion peptide) (Bullough et al., 1994). The membrane of the endosome and the viral envelope are fused with the help of the HA2 protein and forms a pore to release vRNPs into the cytoplasm (Skehel & Wiley, 2000; Stegmann, 2000). The M2 ion channel protein also facilitates the uncoating process. H+ ions from the. endosome pass into the viral particle through the M2 channel, allowing vRNPs to separate from the M1 protein matrix and be released (Rossman & Lamb, 2009).

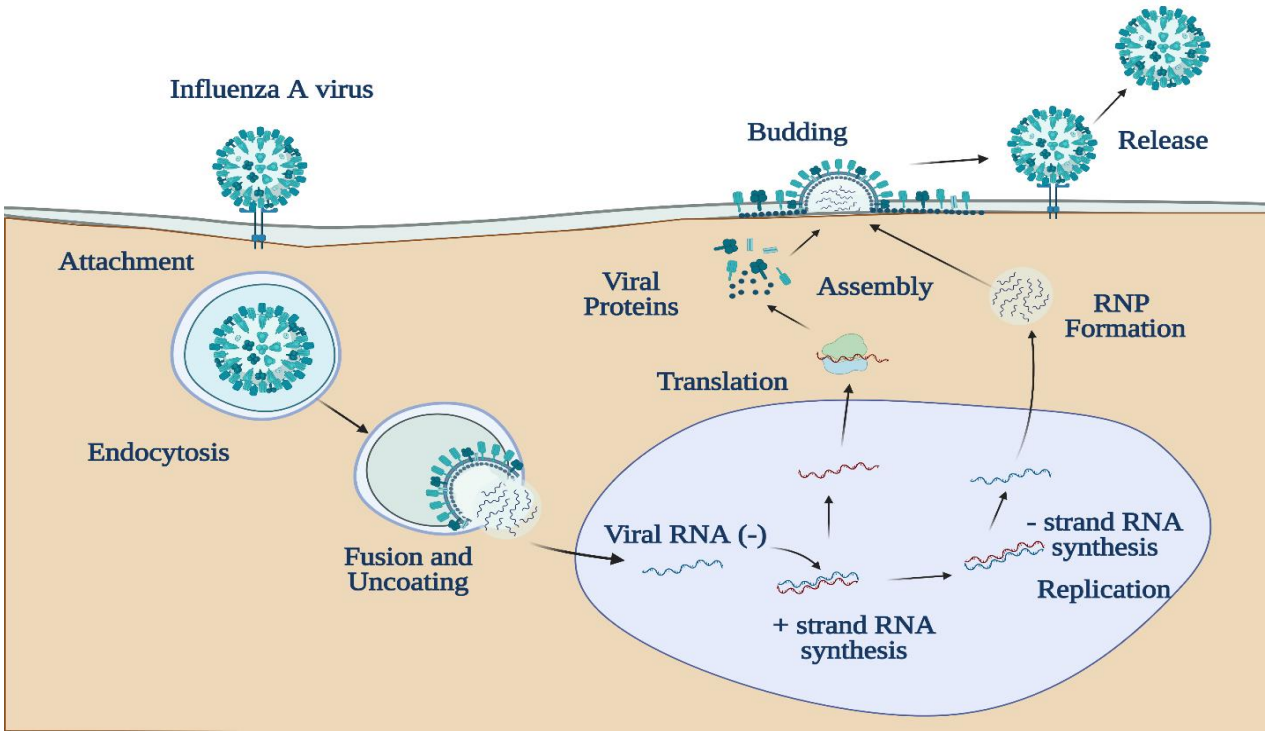


Figure 5: Influenza A virus life cycle. The IAV binds to the host cell membrane via sialic acid receptors and enters inside the cell via receptor-guided endocytosis. The viral RNPs are released from the viral particle after fusing the endosomal membrane and the viral envelope. The viral RNPs are then transported inside the nucleus. Transcription of viral RNAs into viral mRNAs occurs in the nucleus and translocate to the cytoplasm. In the cytoplasm, the viral proteins are translated from the viral mRNAs. Positive sense complementary RNAs generated from the vRNA serve as a template for replicating the viral RNA segments inside the nucleus. The vRNPs are generated by assembling the polymerase complexes and NP protein. The viral structural proteins and vRNPs are transported and assembled in the host cytoplasm. The progeny virus particle exit the cell by budding and is released via neuraminidase activity. (This Figure was generated by https://app.biorender.com/).

1.5.3 Viral ribonucleoproteins (vRNPs) trafficking to the nucleus

For viral RNA to be translated and replicated, influenza vRNPs should translocate inside the nucleus. The cellular import tools recognize the nuclear localization signals (NLSs) in the vRNPs and transport them inside the nucleus through the nuclear pore (Boulo et al., 2007).

1.5.5 Transcription and translation

Viral RNA transcription and replication begin inside the nucleus. Using vRNAs as a template, IAV mRNA transcription happens in a primer-dependent approach. The mature

messenger RNAs (mRNA) from human cells have a methylation cap in the 5' ends and a poly-A tail in the 3', whereas the influenza viral mRNA carries a poly-A tail but lacks the 5'cap. Thus, IAV hijacks the host pre-mRNA transcripts to begin viral mRNA transcription, called cap-snatching (Mikulásová & Varecková, 2000). The PB2 protein identifies the 5' cap of pre-mRNA, and PA's endonuclease activity cleaves the cap (Dias et al., 2009; Guilligay et al., 2008; Yuan et al., 2009). PB1 facilitates the successive addition of nucleotides to viral mRNA utilizing vRNA as a template. Poly-A tail addition in influenza virus transcription differs from cellular polyadenylation. In IAV, Poly-A tail is formed when vRNA transcribes 5-7 uracil units that are translated into (+sense) adenosines (Bouvier & Palese, 2008). After that, viral mRNAs are delivered to the cytoplasm and translated similarly to host mRNAs. Each viral mRNA segment transcribes into a single viral protein except segments 7 and 8, which generate two proteins from each by alternative splicing using host machinery (Engelhardt & Fodor, 2006; Mikulásová A & Varecková E, 2000). vRNAs are also generated in the host nucleus. vRNA replication is a two-step procedure. First, an entire complementary positive-sense RNA (cRNA) is synthesized using negative sense RNA as a template with the help of the RNA-dependent RNA polymerase (RdRp) complex. The starting of cRNA synthesis does not require a capped primer. It has been suggested that NP is essential for complete cRNA transcription (Mikulásová & Varecková, 2000). Then, utilizing cRNA as a template, viral RdRp synthesizes negative-sense genomic vRNAs, which are later coated with NP (Mikulásová & Varecková, 2000).

1.5.6 Translocation vRNP from the nucleus to cytoplasm.

The Crm1 pathway is known to be involved in the export of vRNPs from the nucleus to the cytoplasm (Boulo et al., 2007; Samji, 2009). In the nucleus, NEP/NS2, M1, and viral RNPs interact to create a complex that is essential for vRNP export to the cytoplasm. M1 interacts with the vRNPs and NEP/NS2 proteins and subsequently combines with Crm1 (the export receptor) and multiple nucleoporins, allowing vRNPs to enter the cytoplasm (Boulo et al., 2007; Samji, 2009).

1.5.7 Assembly of virions and budding

All the vRNA segments of IAV must be integrated into the offspring virions to form an infectious virus. There are two proposed models for viral RNA packing. According to the first

model, vRNA segments are packed into the progeny virions in an arbitrary manner (Bancroft & Parslow, 2002; Enami et al., 1991). The second concept suggests that vRNA segments include unique signals that control the vRNAs packaging into progeny virions. The vRNAs carry the packaging signals in their 5' or 3' untranslated (UTR) regions (Fujii et al., 2005; Fujii et al., 2003; Liang et al., 2005; Muramoto et al., 2006; Samji, 2009). All vRNPs and the structural proteins must be delivered into the cytoplasmic membrane before the assembly of the final virion. The exocytic pathway is used by M2, NA, and HA glycoproteins, which are delivered to the host cell membrane via the trans-Golgi network (Nayak et al., 2004). The NA and HA are integrated into the lipid rafts on the cytoplasmic membrane, whereas M2 proteins remain attached to it. M1 transports vRNP to the viral assembly site via two different processes. M1 binds with vRNP and is trafficked to the cytoplasmic membrane via the cytoskeleton called piggybacking or by passive diffusion (Nayak et al., 2004).

The development of plasma membrane curvature is essential for virion budding, and M1 plays a key role in this process. NA and HA trigger budding by deforming the cell membrane. M1initiates polymerization and builds the core structure of the progeny virion (Rossman & Lamb, 2011). It is suggested that M1 could facilitate the recruitment of M2 to the budding sites. M2 with the help of host proteins promotes membrane scission and the separation of encapsulated virions by changing the membrane curvature at the buds' necks (Rossman & Lamb, 2011). After detaching encapsulated virions from the plasma membrane, the new offspring virions' HA attaches to host cell sialic acid receptors (Stegmann, 2000). NA sialidase activity facilitates the release of progeny virus from the host cell by breaking the sialic acid HA bonds (Gamblin & Skehel, 2010).

1.6 Treatment with antiviral drugs

Currently, there are two types of clinically approved drugs for flu treatment: IAV-M2 inhibitors and IAV-NA inhibitors. M2 is a critical viral protein for efficient uncoating of vRNPs during viral entry. Rimantadine and Amantadine are two M2 inhibitors used against IAV (Das et al., 2010). On the other hand, neither M2 inhibitors exhibit any antiviral effect on *Influenza B viruses*. However, the prevalence of Rimantadine and Amantadine-resistant influenza viruses has been increased, limiting the usefulness of M2 inhibitors for influenza treatment (Hayden & Hay, 1992; Pinto & Lamb, 2006). On top of that, the H5N1 strain of IAV detected in birds and humans also demonstrated resistance to the M2 inhibitors (De Clercq & Neyts, 2007). NA inhibitors

zanamivir and oseltamivir have been used as effective drugs against influenza A and B viruses. At the end of IAV replication cycle, the progeny virus particles remain bound on the cell membrane, and the sialidase function of the NA protein is required to free the infectious virus particle. Thus, NA inhibitor prevents the release of the virus particles, limiting further transmission of the viruses to the neighboring cells. NA inhibitors have been an effective drug for managing a recent epidemics event, notably the 2009 H1N1 pandemic (Muthuri et al., 2013), although resistance to NA inhibitors is growing (Lackenby et al., 2008; N. Lee & Hurt, 2018).

1.7 Vaccines against Influenza viruses

Influenza vaccines (commonly known as "flu shots") are designed to provide protection against the most common circulating influenza strains that are predicted to cause disease during the following season (CDC, 2022b). The live attenuated vaccines and inactivated vaccines are the two major forms of influenza vaccines currently available. In most countries, both types of these vaccinations were designed to prevent infection against three different seasonal strains, including influenza A(H3N2), pandemic A(H1N1), and one of two influenza B lineage viruses. However, in recent years, some countries had tetravalent immunizations by including both of the influenza B lineage viruses (CDC, 2022b; WHO, 2022). WHO updates the composition of influenza vaccinations every year using data from the Global Influenza Surveillance and Response System (GISRS). The flu vaccines are made in cell culture or embryonated eggs, and the process takes around six months. As a result, each year, the influenza vaccine is created under significant time constraints, and the upcoming strain that may trigger the next seasonal outbreak is anticipated ahead of time using GISRS data (WHO, 2022). Studies have shown that hospitalization, ICU admission, and death after influenza infection were significantly reduced after flu vaccines (Ferdinands et al., 2021; Thompson et al., 2018). Flu vaccines also can protect pregnant women during and after pregnancy and reduce child death (CDC, 2017; Thompson et al., 2019). Most flu vaccines are safe to use, and it is advised that anyone six months of age or older should get the vaccine every year before flu season (CDC, 2022b). However, due to the wide range of natural reservoirs and antigenic subtypes of influenza virus, determining which strain will cause an outbreak in the future is challenging (Parrish et al., 2015). Therefore, often the flu vaccine ends up with low effectiveness (CDC, 2022a).

1.8 Host and virus interactions

As intracellular parasites, viruses exploit host proteins to facilitate their replication and escape recognition from the immune system. Infection with the influenza virus also causes significant changes in the expression of various cellular proteins. The alteration in protein expression might be due to a pathogenic consequence of the viral infection or a host defensive mechanism, or the protein could be required for viral replication and evasion of the host immune system. Many host proteins were expressed differently in influenza virus-infected cells than in non-infected cells. These proteins are linked to various cellular processes and signaling pathways associated with host signal transduction, transcription, cell adhesion, and cell immunity (Coombs et al., 2010; Kroeker et al., 2013; Kroeker et al., 2012; Vester et al., 2009).

Several studies have used genome-wide RNAi screens to identify host proteins required for IAV replication (Brass et al., 2009; Karlas et al., 2010; König et al., 2009). Various host proteins play critical roles throughout the replication cycle of the influenza virus. So far, hundreds of proteins involved at each stage of the IAV replication cycle have been discovered (Figure 7) (Brass et al., 2009; Watanabe et al., 2010, 2014). It is believed that various viral proteins interact with diverse host proteins to complete different steps of replication. Several host proteins have been discovered to interact with NP, NS1, and the RNA-dependent RNA polymerase complex, with some of them critical for influenza virus replication (Deng et al., 2006; Engelhardt et al., 2005; Huarte et al., 2001; Jorba et al., 2008; Liu et al., 2014; Rahim et al., 2018; Tafforeau et al., 2011). However, antiviral drug targeting a host of cellular proteins has not been discovered yet. So, we need to have a clear understanding of the role of the host cellular proteins in IAV replication.

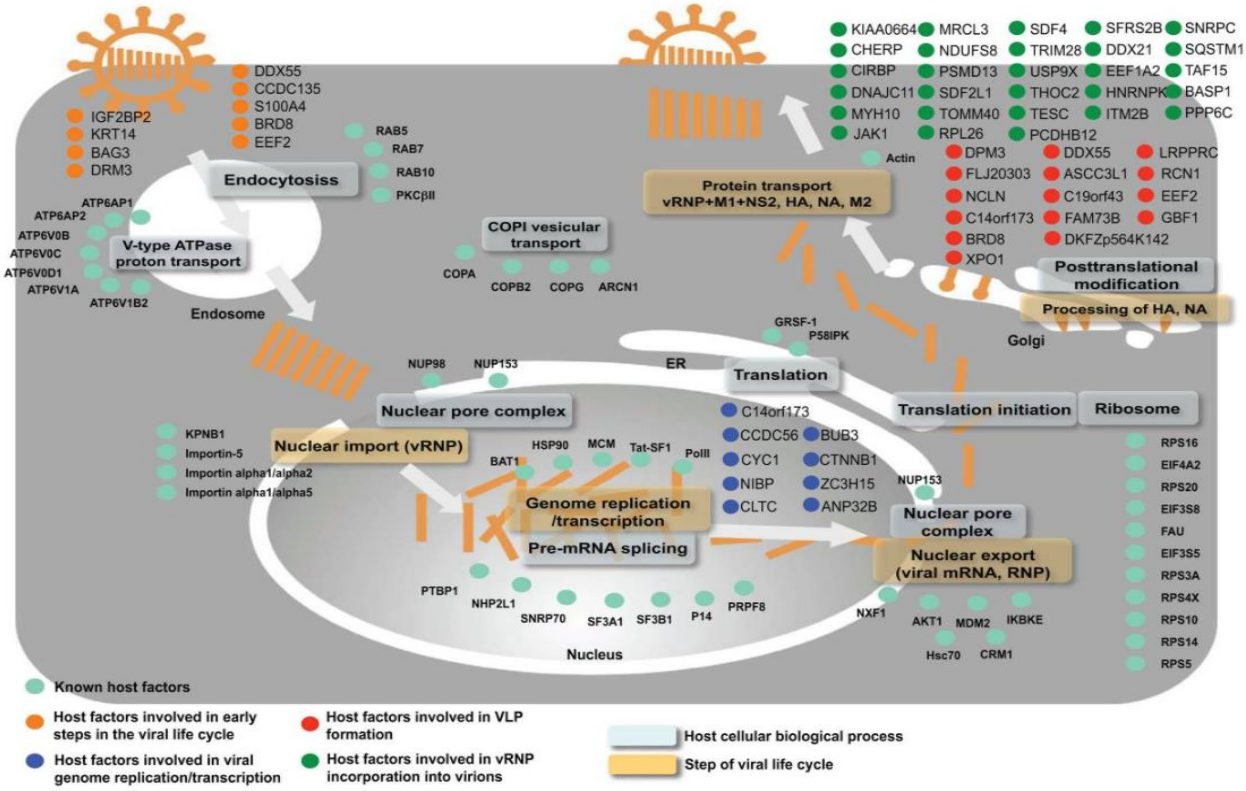


Figure 7: Host factors and their proposed functions in the Influenza virus replication. The bright orange boxes represent specific stages in the IAV replication cycle, whereas the grey boxes represent host cellular activities that are most likely involved. The various host cellular proteins found by Watanabe et al., are categorized by the viral life cycle stages they affected; light green circles relate to host factors identified in previous investigations. The figure was reprinted from Watanabe et al. (Watanabe et al., 2014). Permission was obtained from Copyright Clearance Center to reprint in this thesis. Order License ID1207651-1.

1.9 Fibronectin and fibronectin interacting protein in IAV

Fibronectin (FN-1) is a high molecular weight extracellular matrix (ECM) glycoprotein consisting of two nearly identical subunits (250kDa). Fibronectin is one of the few matrix components that can be found in both soluble and insoluble forms (George et al., 1993; McDonald, 1988). A large quantity of soluble FN-1 is present in plasma (300g/ml) and other bodily fluids (Pankov & Yamada, 2002). The cellular insoluble FN-1 fibrils create linear and branching meshwork around the cells to connect to the nearby cells. The FN-1 facilitates many cellular processes, such as cell proliferation, migration, membrane receptors internalization, inflammation, and is involved in several cell signaling pathways (Mosher, 1989; Pankov & Yamada, 2002; Singh et al., 2010). The structure of FN-1 consists of three types of (type I, II, and III) repeating units

that form a multidomain configuration, which permits the binding of several membrane receptors at the same time, including collagen, integrins, proteoglycans, and others (Singh & Schwarzbauer, 2012). However, several bacteria, fungi, and protozoa have surface proteins that bind with the FN-1 present on the cell membrane (Alderete et al., 2002; Henderson et al., 2011; Hérard et al., 1996), and some (e.g., Staphylococcus aureus) exploit cellular FN-1 to facilitate the infection process (Menzies, 2003). The expression of FN-1 was significantly downregulated in the cells after IAV infection (Simon et al., 2015). IAV infection causes alteration of several cellular proteins in the cell, and many of these proteins were found to interact with fibronectin (Simon et al., 2015). Knockdown (KD) of the expression of several FN-1 interacting proteins significantly impacted the replication of IAV (Figure 8).

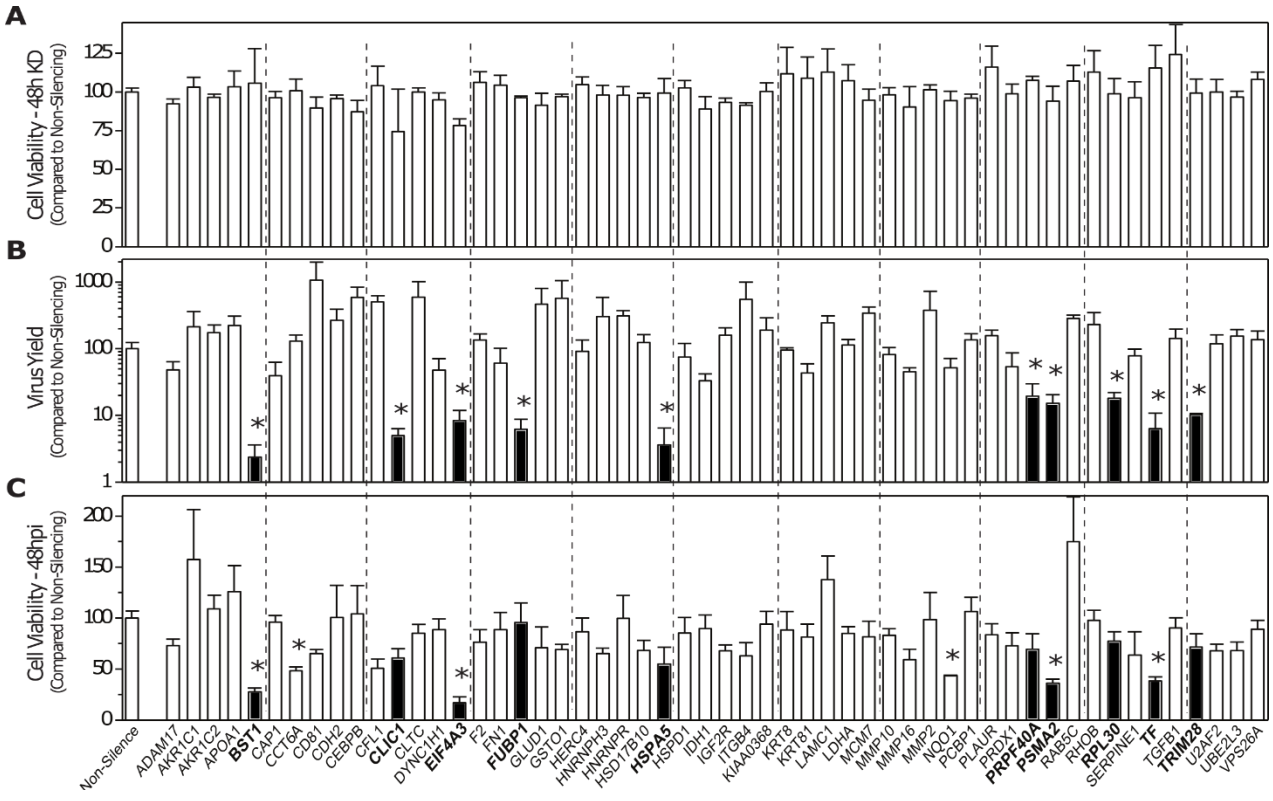


Figure 8. Impact of siRNA knockdown of fibronectin-interacting proteins on IAV replication in A549 cells. A. viability of cells transfected with a 100 nM concentration of the indicated siRNA was determined at 48 hours post-transfection (hpt) by WST-1 assay. B. After 48h-KD cells were infected with PR8 at an MOI of 0.02, supernatants were harvested at 42 hours post-infection (hpi), and viral progeny replication was determined by plaque assay on MDCK cells. C. Viability of the 48-h-transfected/48-h-infected cells determined by WST-1 assay. The data was generated by Dr. Coombs before I joined his lab. (Copyright permission was granted for reprinting from CCC marketplace and the editor of the Journal of Virology) (Rashid et al., 2022).

1.10 Proteasome subunit alpha type 2 (PSMA2)

PSMA2 is encoded by the HC3 gene on human chromosome 7, a 234 amino acid protein with a molecular weight of 25.9 kDa (Tamura et al., 1994) (https://www.ncbi.nlm.nih.gov/gene/5683). PSMA2 is among the 14 fundamental structural subunits identified in the 20S Proteasome. More precisely, PSMA2 creates the ring structure along with the six other alpha subunits that facilitate the substrate entry inside the proteasome (DeMartino et al., 1991; Tamura et al., 1991). The 20S Proteasome is a multi-catalytic proteinase with a compact barrel-shaped structure made from four stacked rings. Each of the two end rings is made up of seven alpha subunits (PSMA1-PSMA7), while the two center rings are made up of seven beta subunits (PSMB1-PSMB7) (Marques et al., 2009). The 20S Proteasome controls various physiological functions, including cell proliferation, oxidative stress response regulation, post-translational modification, and gene transcription. Additionally, extracellular 20S proteasomes are associated with a wide range of biological functions and pathways (Deshmukh et al., 2019; Dianzani et al., 2017, 2019; S. U. Sixt et al., 2012; Sixt et al., 2009).

The core component of the 26S proteasome complex is the 20S proteasome, which is attached to two 19S regulatory particles. In eukaryotic cells, the ubiquitin-proteasome system (UPS) is comprised of the 26S Proteasome, which is a crucial component of the ATP-dependent proteolytic pathway (Thrower et al., 2000). It is responsible for the overall management of protein recycling and quality control, identifying, unfolding, and destroying abnormal proteins. Therefore, the UPS has an impact on the majority of cellular functions (Coux et al., 1996; Finley, 2009; Ravid & Hochstrasser, 2008; Thrower et al., 2000; Tomko & Hochstrasser, 2013). However, many modified versions of the Proteasome, including thymoproteasome, immunoproteasome, the constitutive proteasome, and testis-specific proteasomes, are engaged in specialized activities, such as the immunoproteasome's processing of class I MHC peptides (Rousseau & Bertolotti, 2018).

Metabolic derangement of UPS activity is linked with many diseases, including Parkinson's disease (Le, 2014), Amyotrophic Lateral Sclerosis (Ikeda et al., 2002; Mishto et al., 2010), Huntington's disease (Ortega & Lucas, 2014), Alzheimer's disease (Mishto et al., 2006; Orre et al., 2013), epilepsy (Mishto et al., 2011), Transmissible Spongiform Encephalopathies (Leigh et al., 1991; Neumann et al., 2006) and Creutzfeldt-Jakob disease (Manaka et al., 1992; Zhu et al., 2014).

The UPS is also important in the maintenance of cardiac ventricular hypertrophy, ischemia damage, and heart failure (Calise & Powell, 2013; Powell, 2006; Predmore et al., 2010). Moreover, instability of proteasome expression and activity has been linked to several malignancies, including skeletal muscle cancer (Khal et al., 2005; Kitajima et al., 2020), breast cancer (Chen & Madura, 2005), colon cancer (Arlt et al., 2009), thyroid cancer (Okamura et al., 2003; Qiang et al., 2017) and renal cancer (Corn, 2007).

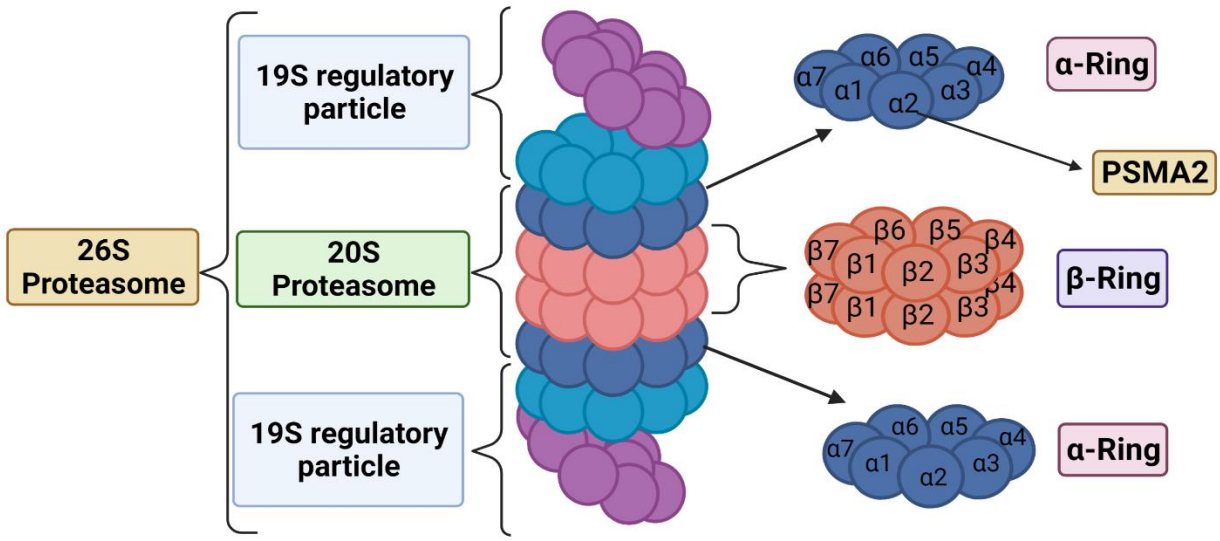


Figure 9: Molecular organization of proteasome subunits in 26S proteasome. 26S proteasome is made with core 20S particle flanked by two 19S regulatory particles. The 20S proteasome has two Alpha and two beta rings, made from seven subunits. One of the seven alpha subunits is called Proteasome subunit alpha type 2 or PSMA2. (The Figure was generated using https://app.biorender.com/ and the concept was adapted from (Murata et al., 2009).

In the UPS, the proteasomes associate with ubiquitin. This complex is primarily responsible for unfolding and degrading faulty proteins in cells, hence regulating a variety of physiological activities such as cell growth and differentiation, post-translational modifications, gene transcription, and oxidative stress response (Coux et al., 1996; Finley, 2009; Ravid & Hochstrasser, 2008; Tomko & Hochstrasser, 2013).

The functional activity of UPS is required for the entrance of mouse minute virus (Ros et al., 2002; Ros & Kempf, 2004) and murine coronavirus (Yu & Lai, 2005) and as well as the release of HIV from the cell membrane (Patnaik et al., 2000; Schubert et al., 2000; Strack et al., 2000). Inhibition of the proteasome negatively impacts viral RNA and protein synthesis of

coxsackievirus (Luo et al., 2003). PSMA2 also plays an important role in West Nile virus genome replication (Gilfoy et al., 2009). In addition, PSMA2 activity was increased in lung epithelial cells after IAV infection (Shahiduzzaman et al., 2014). However, many viral infections also can cause dysregulation of the proteasome activity in host cells (Gao & Luo, 2006).

1.11 Chloride intracellular channel protein (CLIC1)

The chloride intracellular channel proteins (CLICs) are widely distributed in the endoplasmic reticulum, mitochondria, and nuclear membranes. Different CLICs may localize in various sites of the cells or tissue, and they exhibit similar functional properties due to the extensive homologies in their amino acid sequences (Peretti et al., 2015; E. Rousseau et al., 1996). Among the other CLIC family members, CLIC1 was first discovered in humans (Peretti et al., 2015; P. Wang et al., 2012). CLIC1 is a 27-kDa monomer protein, is found in both soluble and integral membrane states in the nucleus and cytoplasm. The tetrameric form of CLICs serve as an intracellular chloride channel (Valenzuela et al., 1997). The roles of this protein include maintaining cell membrane potential and cell volume, transport of molecules, intracellular pH regulation etc. The tetrameric configuration of the subunits in CLIC1 is sufficient to create a functional ion channel (Chang et al., 2009; Wang et al., 2011).

1.12 Heat shock 70kDa protein 5 (HSPA5)

HSPA5, well known as BiP (Binding immunoglobulin protein) and also called glucose-regulated protein (GRP78), is encoded by the HSPA5 gene, which is found on Chromosome 9 of the human genome (Hendershot et al., 1994). HSPA5 is conserved in most eukaryotes and is expressed virtually in all types of human tissues (Brocchieri et al., 2008). There are two functional domains present in HSPA5: substrate-binding domain (SBD) and nucleotide-binding domain (NBD). The SBD has attachment sites for polypeptides, while the NBD interacts with ATP and hydrolyzes it (Jiao Yang et al., 2015). HSPA5 is a molecular chaperone found in the endoplasmic reticulum (ER) lumen of cells that helps peptide folding and reorganization into the appropriate protein structure (Simons et al., 1995). It is also involved in polypeptide transportation to the ER membrane or lumen in an ATP-dependent mechanism (Nguyen et al., 1991; Vogel et al., 1990). HSPA5 regulates the ER stress response via the unfolded protein response (UPR) pathway (Chapman et al., 1998; Korennykh & Walter, 2012; Okamura et al., 2000).

1.13 Rationale

IAV causes an alteration of expression of a wide range of host proteins and signaling pathways after infection (König et al., 2009; Simon et al., 2015; Watanabe et al., 2014). Previous proteomic studies from our lab (Coombs et al., 2010; Kroeker et al., 2012; Simon et al., 2015) and others (Dove et al., 2012; Vester et al., 2009) have found, 100s of host proteins were dysregulated in IAV infected cells. Many of these interact with fibronectin (Mouw et al., 2014; Simon et al., 2015; Sugiyama et al., 2016). However, FN-1 facilitates attachment and entry of the rhabdovirus, hepatitis B virus, gammaretrovirus (Bearzotti et al., 1999; Henderson et al., 2011; Jing Yang et al., 2006), human parvovirus B19 and Ebola virus (Schornberg et al., 2009; Weigel-Kelley et al., 2003) to the infected cells. IAV was found to promote bacterial attachment to the cell by upregulating the expression of fibronectin (Li et al., 2015). In another study, FN-1 has been demonstrated as an essential component for IAV binding to the 2,6-linkage sialic acid and entry in human lung cells (Leung et al., 2012). However, different strains of low and high pathogenic IAV were found to be significantly affecting FN-1 interacting proteins expression (Simon et al., 2015). A 96-well-based reverse-transfection siRNA screen revealed that several of these FN-1 interacting proteins (BST1, CLIC1, FUBP1, PSMA2, EIF4A3, and HSPA5) reduced and some increased (CLF1, CD81, MCM7) virus replication >3-fold (Figure 8). The results suggest that these proteins are important for IAV infection in humans. So far, no study has investigated the role of these proteins in the replication of influenza virus. In my thesis, I want to investigate the role of CLIC1, PSMA2, and HSPA5 (based on the reagents availability) in IAV replication steps and in the cellular signaling pathways that are required during IAV infection.

1.14 Significance of the study

IAV has a highly mutable genome that is made up of 8 segments of (-) sense single-stranded RNA (Sorrell et al., 2007). The genetic flexibility of the virus allows it to mutate quickly and develop resistance to antiviral treatments and vaccines. As a result, developing an effective vaccine has been challenging, and treatment has proven increasingly complicated (Shao et al., 2017). Because viruses are intracellular parasites, they utilize host cell proteins to complete their replication cycle and avoid detection by the immune system during infection. Therefore, virus reproduction inside the host cell could possibly be inhibited by disrupting the cell signaling

pathways critical for viral replication but not required by the cells. Comprehensive knowledge of host-virus interactions can demonstrate the precise mechanisms of IAV replication, which can be used to design strategies to prevent viral infections.

- To determine the function of fibronectin interacting proteins at different stages of IAV replication cycle.
- Understanding the role of these proteins in cellular signaling pathways and functions during viral infection will improve our knowledge of IAV disease mechanisms.
- The findings of this study may contribute to the generation of hypotheses for future research for the development of novel therapeutics or vaccines against IAV targeting host cellular proteins.

1.15 Hypothesis and Objectives

Hypothesis: IAV uses PSMA2, CLIC1 and HSPA5 proteins directly or indirectly through cellular signaling pathways to complete its replication cycle.

Specific Goals:

Aim-1: Optimization of KD of fibronectin interacting protein by siRNA.

Aim-2: Determine the effect of knocking down a target protein (PSMA2, CLIC1, or HSPA5) on the IAV replication cycle.

Aim-3: Bioinformatics analysis to understand the involvement of the PSMA2, CLIC1, or HSPA5 proteins in cellular function and signaling pathways required for viral replication.

CHAPTER 2. MATERIALS AND METHODS

2.1 Study Design.

To understand the role of the host proteins in the IAV replication cycle, siRNA was used to KD the expression of each of the target proteins (PSMA2, CLIC1, and HSPA5) in human lung epithelial cells and infected with the IAV (PR8 strain) virus. The impacts of PSMA2, CLIC1, or HSPA5 KD on IAV replication, viral protein synthesis, viral genome replication, intracellular localization of viral proteins, and host cellular protein expression were studied using plaque assays, Western blots, qRT-PCR, and SomaScan analysis, respectively (Figure 10). The proteomic data were analyzed using Ingenuity Pathway Analysis (IPA) software to determine cellular functions or signaling pathways that were impacted by the target protein KD during IAV replication. Finally, the role of PSMA2, CLIC1 and HSPA5 in the specific step/steps of IAV replication and its underlying mechanisms were predicted based on the overall results.

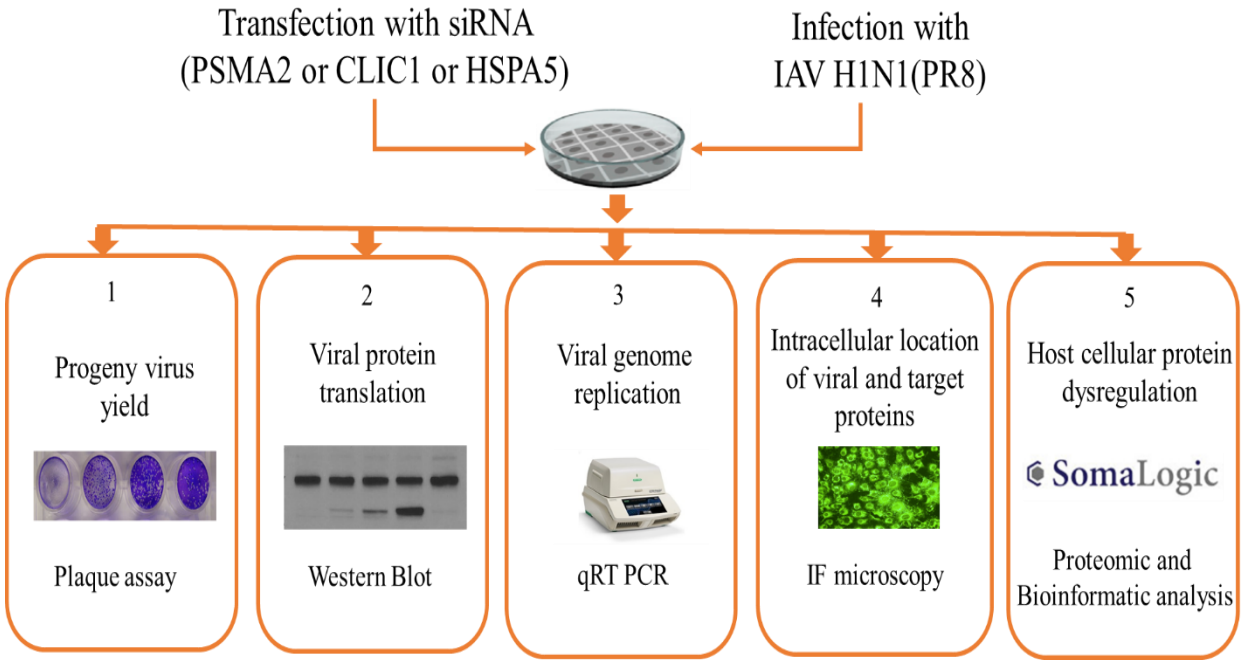


Figure 10: Flow diagram showing the experimental approaches used to determine the role of host proteins in the IAV replication cycle. The PSMA2, CLIC1 or HSPA5 KD cells were infected with IAV PR8 strain and determined the impact of KD on progeny virus yield, level of viral proteins, viral RNA, localization of viral proteins and host cellular protein dysregulation determined by plaque assay, Western blot, qRT-PCR, IF and proteomic analysis respectively.

2.2 Cells and Viruses

Human A549 lung epithelial cells were cultured in complete DMEM media (GIBCO, USA) containing Non-essential amino acids (1X), Sodium-pyruvate (2mM), L-glutamine (2mM), and supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Ontario, Canada) following the protocol as described in (Coombs et al., 2010). Similar to A549, MDCK cells were cultured in complete DMEM but supplemented with 5% FBS. Human fetal lung cells (MRC-5) were purchased from ATCC (Cat # CCL-171) and maintained in ATCC EMEM (Cat # 30-2003) supplemented with 10% FBS. The cells were passaged three times each week by trypsinization incubated at 37 °C with 5% CO2 in a humidified chamber. Human influenza virus strains A/Mexico/INDRE4487/2009 (H1N1;pdm-09), A/WSN/1933 (H1N1;WSN), A/NewCaledonia/20/1999 (N-cal) and mouse-adapted strain A/PR/8/34 (H1N1; PR8) were used in this study. MDCK cells were infected with an MOI of 0.01 (plaque-forming units (PFU)/cell), and viruses were harvested from the supernatant after 48 hours. The virus stocks were concentrated by centrifuging at 64,000 × *g* for two hours at 4°C, resuspended in phosphate-buffered saline (PBS) supplemented with 10% glycerol, and aliquots were frozen at -80 °C until used.

2.3 Infection and plaque assay

Human A549 cells were grown to 70-80% confluency, washed with PBS two times and infected with PR8, WSN, or pdm09 virus. To determine the impact of PSMA2, CLIC1 and HSPA5 KD, on viral replication, cells were infected at MOI 0.01. Supernatant from the virus-infected cells were collected at 0, 2, 4, 8,16, 24, 36 and 45 hours post-infection (hpi). Plaque assays were performed to determine the supernatant virus titers. Samples were serially 1:10 diluted in gel saline (containing 0.8% NaCl, 0.003% $CaCl_2$, 0.017% $MgCl_2.6H_2O$, 0.12% H_3BO_3, 0.005% $Na_2B_4O_7.10H_2O$ 0.03% Gelatin, type A) and 100 µL of each diluted sample was inoculated in duplicate into separate wells of 6-well plates containing monolayers of MDCK cells. The infected plates were rocked for one hour to allow virus attachment, then overlayed with FBS-free 1× DMEM media containing 0.8% Avicel, 2 mM sodium-pyruvate, 2 mM L-glutamine, and 1× MEM non-essential amino acids. The overlay media was also supplemented with antibiotics (gentamicin, 100 µg/ml and amphotericin B, 100 µg/ml) and 2.5 µg/ml trypsin. The infected cells were incubated for 72 hours (h) at 35°C. After that, the overlay media was removed, cells were washed with 1× PBS, and fixed with 2% formaldehyde for 30 minutes (min). The plaques were visualized

by staining with crystal violet for ≥ 1 h. The crystal violet stain was washed, plates were dried, and plaques were counted. The number of plaques was back-calculated to quantify PFU/ml.

2.4 Protein extraction and quantification

A549 cells in 6-well plates or 60mm dishes were transfected with siRNAs and infected with IAV at MOI of 3 PFU/cell (following the siRNA transfection and infection procedure described in other sections). Infections were harvested at different time points by scraping from the culture plates. To determine the impact of Opti-MEM on cellular protein expression, A549 cells were grown in either DMEM with 10% FBS or Opti-MEM. Cells were harvested every day for 4 days. A549 cells were transfected in DMEM with 10% FBS or Opti-MEM media and harvested every day for 4 days to compare transfection efficiency. The harvested cells were washed 3× in ice-cold PBS and were lysed by sonication in 60µl mammalian protein extraction reagent (M-PER, Cat. #78501, Thermo Fisher Scientific) detergent, supplemented with 1× HALT® Protease inhibitor (Cat. #78430, Thermo Fisher Scientific) solution. The cell lysates were centrifuged at 14,000×g for 10 minutes at 4°C to remove the insoluble cellular components. The protein concentrations in the clear supernatants were determined by BCATM Protein Assay (Pierce; Rockford, IL) and quantified using bovine serum albumin standards (Cat. # 23208, Thermo Fisher Scientific).

2.5 Immunoblotting

The expression of viral and host cellular proteins were detected by Western blot according to the procedure described elsewhere (Coombs et al., 2010). An equal amount (10-30ug) of protein samples were separated using 10 or 12% SDS-PAGE gels and transferred to 0.2µm nitrocellulose membranes. anti-PSMA1 (Invitrogen, Cat. PA1-963), anti-PSMA2 (Cell Signaling, Cat. 2455), Anti-PSMA6 (Invitrogen, Cat. PA576058), anti-Beta-Actin (Cell Signaling, Cat. 3700S), anti-STAT3 (Cell Signaling, Cat. 9139S), anti-CST3 (Cell Signaling, Cat. 4280), anti-CLIC1 (Cat. MABN46; EMD Millipore, Darmstadt, Germany), anti-HSPA5 (Cat. MABC675; Millipore), anti-GAPDH (Cat.2118L; Cell Signaling), and in-house prepared IAV mouse-anti-NS1 and mouse-anti-NP (Rahim et al., 2013) were used to detect specific proteins. Appropriate secondary horseradish peroxidase (HRP)-conjugated horse anti-mouse or anti-rabbit (Cell Signaling, cat.7076, cat.7074, respectively) were used to detect immune complexes. The working dilution for

the primary antibodies was 1:1000, while the working dilution for the secondary antibodies was 1:2500. Protein bands were developed with ECL reagents and captured by Amersham Imager 680 (GE Life Sciences, MA, USA). The difference in protein expression was determined by measuring band intensities with Image J 1.50i (NIH, USA). After normalization with the loading control (Beta-Actin or GAPDH), the data was analyzed and graphically presented by GraphPad Prism v 9.1.0 software.

2.6 Photomicrography

To visually observe the impact of siRNA transfection, or Opti-MEM media, A549 cells were photographed with a Canon-A700 digital camera at 200×magnification. Images were imported into Microsoft PowerPoint, and slight adjustments were made in brightness and contrast, which did not alter image context with respect to each other.

2.7 Immunofluorescent microscopy

A549 cells were grown on 6mm Multi-Spot Slides (Cat. 99-910-90: Fisher Scientific, USA) in complete DMEM media with 10% FBS at 37°C for 24 hours. Then the cells were treated with target siRNA (25nM PSMA2 or 50nM CLIC1 or 50nM HSPA5) or non-silencing/scrambled control (NSC) siRNAs for 48 hours and infected with IAV-PR8 at MOI 3. At 24 hours post-infection (hpi), each spot was washed 5× with PBS and fixed with 4% paraformaldehyde for 15min, then washed 5× with PBS and permeabilized with 0.1% Triton X-100 for 5min. The fixed cells were blocked overnight at 4 °C by 3% bovine serum albumin (BSA) in PBS. Cells were then incubated overnight with primary anti-PSMA2 (Cat. 2455; Cell Signaling), or anti-CLIC1 (Cat. MABN46; EMD Millipore, Damstadt, Germany), or anti-HSPA5 (Cat. MABC675; Millipore) or anti-NRF2 antibody (Cell Signaling, Cat. 12721S) in 3% BSA at 4 °C. After that, cells were washed 5× with PBS and 0.2% Tween 20 (PBT) and incubated with Alexa Fluor488-tagged anti-rabbit secondary antibody for 60 min. Finally, each spot on the slide was covered with DAPI mounting dye. The fluorescent images were visualized with a Zeiss Axio Observer Z1 inverted fluorescence microscope. ImageJ 1.53e (NIH, USA) was used for measuring the average fluorescence intensities of NRF2 in the nucleus. Data were analyzed, and the graphs were prepared by GraphPad Prism v 9.1.0.

2.8 siRNA transfection

KD of PSMA2, CLIC1 and HSPA5 protein expression was done by siRNA following the protocol described in (Rashid & Coombs, 2019). In summary, A549 cells were grown to 30 – 40% confluency in complete DMEM media with 10% FBS, washed twice before transfection with RNase-free PBS and overlayed with complete DMEM media with 10% FBS. ON-TARGET (OT) and SMARTpool (SP) siRNAs for PSMA2 (25nM), CLIC1 (50nM) and HSPA5 (50nM) NSC siRNA (50nM) (Dharmacon) as control, and Dharmafect (Cat. #T-2001; GE Healthcare Dharmacon, Lafayette, CO) were diluted in Opti-MEM media following the manufacturer's instructions. The diluted siRNA and Dharmafect were combined, incubated at room temperature for 20 min, and added directly into the A549 cell culture media. Culture dishes were incubated at 37 °C in 5% CO2. Cells were collected at 48 hours post- transfection (hpt) to investigate the impact of PSMA2, CLIC1 and HSPA5 KD on viral protein expression and RNA replication. Supernatants were collected at different time points up to 45 hpi to determine the impact on progeny infectious virus replication.

2.9 Cell viability

The impact of PSMA2, CLIC1 and HSPA5 KD on cell viability was determined by WST-1 (Roche) reagent according to the manufacturer's instructions. WST-1 assay measures the cleavage of tetrazolium salts by mitochondrial dehydrogenases enzyme present in live cells. Eight thousand A549 cells were seeded in each well of 96 well plates. After overnight incubation, cells were transfected with PSMA2 or CLIC1or HSPA5 or NSC siRNAs. At 48 and 72 hpt, 9µl of WST-1 reagent was added to each well and incubated at 37°C for 2 hours. Cell viability was calculated from the colorimetric changes in the media determined by a photo-densitometer. The percentage of cell viability was determined by comparing with time-matched non-silencing treated cells. Each experiment was done in 3 biological replicates with five technical replicates each time.

2.10 Proteasome 20S activity assay

Proteasome activity was assessed using a proteasome 20S assay kit according to the manufacturer's instructions (Sigma Aldrich, MAK172). In summary, 10000 A549 cells were seeded in each well of black clear bottom 96-well plates. After overnight incubation at 37 °C, cells were transfected with PSMA2 siRNA as described above. At 72 hpt, cells were washed 2× with

PBS, and 100 µl of "Proteasome Assay Loading Solution" was added to each well. The 96-well plates were incubated in the dark at 37^0C for 12 hours and fluorescence intensity was measured using a spectrophotometer at excitation = 490nm and emission = 525nm. The impact on 20s proteasome activity was determined by comparing the relative fluorescence units in PSMA2 KD cells to non-KD cells.

2.11 Reactive oxygen species (ROS) assay

The cellular ROS levels were determined by staining with 2′,7′-Dichlorofluorescin diacetate (DCF-DA, Cat. No. D6883, Sigma-Aldrich) following the manufacturer's instructions. A549 cells were cultured in 96 well plates and transfected with PSMA2 siRNA or NSC for 48h. Cells were washed 1× with PBS and incubated for 45min with 10 µM DCF-DA at 37 °C. After that, the cells were infected with IAV at MOI 3 and incubated at 37 °C in the dark. Fluorescence was measured (excitation 504, capture 529) at different time points post-infection. The experiment was performed in five replicates.

2.12. Impact of ROS scavenger and proteasome inhibitor on IAV replication

A ROS scavenger, N-Acetyl-L-cysteine (NAC) (Cat No. A7250) and a proteasome inhibitor, carbobenzoxy-Leu-Leu-leucinal (MG132, cat no. M8699) were purchased from Sigma-Aldrich. A549 and MRC5 cells were treated with different concentrations of MG132 and NAC, and the cytotoxicity was determined by WST-1 assay at 48 hours post-treatment. Treatment with 15mM NAC or 0.05 µM MG-132 showed >90% cell viability. To understand the effect of NAC and MG-132, cells were infected with IAV PR8 strain with MOI 0.01 and NAC/MG132 was added in the overlay media. Supernatants were collected at 45 hpi, and virus titers were determined by plaque assay.

2.13 Impact of CLIC1 and HSPA5 inhibitors on IAV replication

In A549 and MRC-5 cells, the effects of Epigallocatechin-3-gallate (EGCG), an HSPA5 inhibitor (Cat# E4143, Sigma-Aldrich), and 5-nitro-2-(3-phenylpropyl-amino) benzoic acid (NPPB), a CLIC1 inhibitor (Cat# sc-201542, Santa Cruz Biotechnology, USA), on IAV replication were evaluated. First, A549 and MRC5 cells were treated for 48 hours in serum-free medium with several concentrations of EGCG (0-1000µM) and NPPB (0-250µM) to find the highest

concentration of the drug with low (>80% cell viability) cytotoxicity. The WST-1 test was used to assess the drugs' cytotoxic effect. After 48 hours of treatment, 250 µM and 15.6 µM of EGCG showed cell viability of more than 80% in A549 and MRC5 cells, respectively. However, after 48 hours of treatment, 62.5 µM and 31.3 µM of NPPB showed cell viability of more than 80% in A549 and MRC-5 cells, respectively. To test the impact of the drugs on IAV replication, two or three different concentrations (below 20% toxicity) were tested. The A549 or MRC-5 cells were first pre-treated with the drugs for 2 hours before being infected with IAV PR8 or N-Cal strains. After one hour of virus absorption, the cells were overlayed with serum-free DMEM media, containing the same concentration of the drug and incubated at 37°C. The overlay media was also supplemented with antibiotics (gentamicin, 100 µg/ml and amphotericin B, 100 µg/ml) and 2.5 µg/mL trypsin. At 45 hpi, virus samples from the supernatant were collected and titrated by plaque assay.

2.13 SOMAscan® analyses

To determine the impact of PSMA2 KD on the cellular proteome during IAV infection, cell lysates were collected from NSC, NSC+PR8, PSMA2 KD, and PSMA2 KD+PR8 cells at 24 hpi and analysed by the SOMAscan® version 1.3K platform, which can simultaneously measure 1307 proteins in up to 92 samples. During the SOMAscan assay, each biologic sample was mixed with SomaLogic's proprietary SOMAmers®. Each of the SOMAmers can selectively recognize and bind to a specific human protein (Candia et al., 2017; Gold et al., 2010). After mixing and binding, each sample was added to an individual 96-well. This allows for the assessment of up to 92 samples and 4 controls at the same time. The SOMAmers are then washed, released, hybridized to DNA microarrays, and quantified (Brody et al., 2010; Gold et al., 2010). The expression values are generated as relative fluorescent units (RFU) which are directly proportional to the amounts of target proteins in the initial samples, as confirmed by a standard curve generated for each protein-SOMAmer pair. RFU were Log2 converted and analyzed as described (Rashid et al., 2020).

2.14 RNA extraction and real-time PCR

After KD of PSMA2 or CLIC1or HSPA5, A549 cells were infected with IAV-PR8 at MOI 3. At 24 hpi, cells were harvested, washed with cold PBS and total cellular mRNA was extracted with RNeasy Mini Kit (QIAGEN). cDNA was synthesized by Go Script ^{TM} Reverse Transcription

System kit (Promega) from 250 ng of purified mRNA. The qRT-PCR was performed using Platinum™ SYBR™ Green qPCR SuperMix-UDG kit (Thermo Fisher). The final volume of the master mix was 25ul, consisting of 12.5µl Platinum™ SYBR™ Green qPCR SuperMix (2X), 0.5 µl ROX Reference Dye, 0.5µl each of 10µM forward and reverse primers listed below, 6µl H2O, 5µl (10ng) template cDNA. The PCR was performed in three biological replicates and two technical replicates for each sample. All PCR reactions were done on QuantStudio™ 3 Real-Time PCR System (Applied Biosystems). The PCR cycle condition was 50°C for 2 min, 95°C for 2 min, and 40 cycles of (95°C for 15 sec. and 60°C for 30 sec. The Ct values were normalized to 18S rRNA control and compared to non-targeting siRNA control. The 5' to 3'sequences of the primers were PR8-NS1 (Fwd: CTTCGCCGAGATCAGAAATC; Rev: TGGACCATTCCCTTGACATT) PR8-NP (Fwd: AGAGGGTCGGTTGCTCACAA; Rev: TGGCTACGGCAGGTCCATA) and PR8-HA (Fwd: CATTCCGTCCATTCAATCC; Rev: AACCATACCATCCATCTATC)

2.15 Statistical and bioinformatics analyses

The SOMAScan-generated proteomic data are expressed in RFU. The RFU expression values were converted to Log2 values. The log2 expression value of each protein in the infected/knockdown condition was subtracted from the controlled (mock/ scrambled siRNA treated) condition, denoted as delta log2 value. The delta log2 value was converted to fold-changes for each of the proteins to determine the expression change caused by the viral infection or protein KD. Average expression change was calculated from three different biological replicates. To quantify the P-value from the fold-changes, Z-score analysis and Students' T-test (2 tails) were done (Coombs et al., 2010). The significantly dysregulated proteins (P-value <0.05) with fold change above 1.5 or below -1.5 (Table 2) were further analyzed by Ingenuity Pathway Analysis (IPA) and STRING: functional protein association networks. Z-score values $\geq 1.96\sigma$ or $\leq -1.96\sigma$ were considered significant. One-way ANOVA was performed to analyze the Western blot data for calculating the p-value. P <0.05 was considered significant. Heatmaps were plotted using MORPHEUS (Broad Institute, Cambridge, MA, USA), and Fig. 7 was designed with "BioRender (https://biorender.com/) free online software.

CHAPTER 3. RESULTS

3.1. Optimization of concentrations and conditions of siRNA transfection for PSMA2, CLIC1 and HSPA5 expression knockdown.

The results of this section are adapted from the following article:

Rashid, M.U. and Coombs, K.M., 2019. **Serum-reduced media impacts cell viability and protein expression in human lung epithelial cells.** Journal of Cellular Physiology, 234(6), pp.7718-7724.

Lay summary: During the optimization of siRNA KD of host proteins, I found that the transfection medium, Opti-MEM, had a negative impact on cell survival, morphology, and host protein expression. Then, as an alternative to the serum reduced medium (Opti-MEM), I optimized the siRNA transfection conditions by utilizing DMEM medium with serum.

3.1.1 Impact of Opti-MEM on A549 cells viability, morphology, and protein expression.

Suppression of protein expression by siRNA transfection often takes 24 to 96 hours, depending on the cell type and target protein (McNaughton et al., 2009). Serum-free or serum-reduced media are recommended for transfection to synchronize the cell cycle and reduce serum protein interference during the transport of genetic material into the cells (Khammanit et al., 2008). To understand the impact of Opti-MEM on cell viability and morphology, A549 cells were grown in Opti-MEM for 4 days, and cell morphology was monitored under the microscope. As a control of optimal growth conditions, A549 cells were also cultured in DMEM medium with 10% FBS. There was no visible change observed in the cells growing in two different media after 24 hours. However, after Day 2, more rounded and floating cells were visible in the Opti-MEM culture dishes than in the DMEM (Figure 11A). A549 are epithelial cells with a distinctive rectangular shape, and rounded morphology indicates that the cells are stressed and maybe dying. Therefore, I monitored the cell viability of A549 cells after growing in two different media. Cell viability of the cells growing in the Opti-MEM was significantly lower throughout the experiment from Day 1 to Day 4 than in DMEM (Figure 11B).

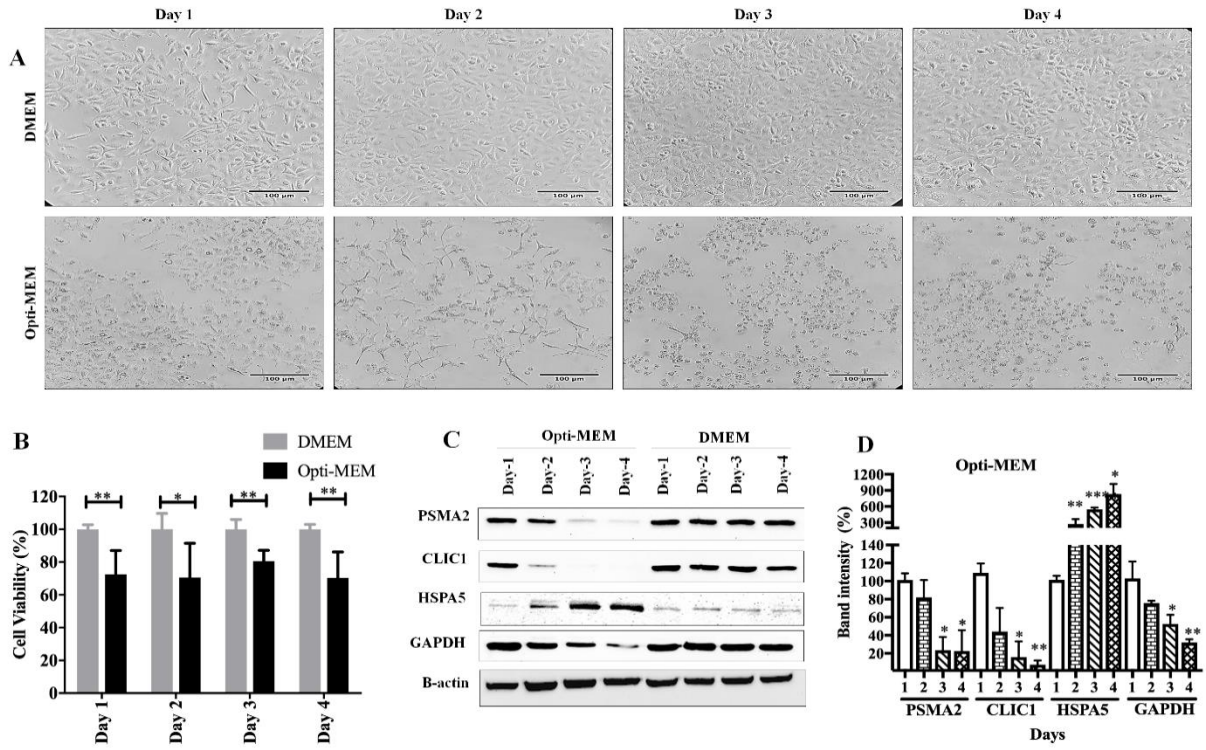


Figure 11. Impact of Opti-MEM on A549 cells viability, morphology, and protein expression. **A**. Observation of cells under the microscope in Opti-MEM and DMEM media for 4 days. The scale bar is 100 μm. **B.** A549 cell viability in Opti-MEM and DMEM media, determined by WST-1 assay. **C**. Expression of cellular proteins in Opti-MEM and DMEM media by Western blot. **D.** Quantification of PSMA2, CLIC1, HSPA5 and GAPDH expression in Opti-MEM compared to DMEM from Western blot. * = P< 0.05, ** = P< 0.01,***= P<0.001.

Next, I wanted to know if growing cells in Opti-MEM medium could impact the expression of CLIC1, PSMA2, and HSPA5 proteins. A549 cells were grown in Opti-MEM for 4 days, and expression of CLIC1, PSMA2, and HSPA5 proteins was determined by Western blot every day after harvesting the cell lysates. Cells were also grown in DMEM medium with 10% FBS supplement as a control to determine the usual expression pattern of the proteins in A549 cells. The expression of these three proteins in DMEM was stable throughout the experimental period. In A549 cells the expression of the PSMA2 and CLIC1 proteins had significantly decreased after three days of growth in Opti-MEM, but the expression of the HSPA5 protein had significantly increased after two days (Figure 11C,D). Furthermore, GAPDH, a widely used loading control, was shown to have a significant decrease in expression after Day 3 in Opti-MEM. (Figure 11C,D).

3.1.2 Efficacy of CLIC1 knockdown in DMEM medium compared with Opti-MEM.

Since A549 cell viability and cellular protein expression were affected in Opti-MEM media, I wanted to know if similar amounts of KD could be achieved by siRNA transfection in complete DMEM media (supplemented with all nutrients and 10% FBS). So, siRNA transfection was performed in A549 cells targeting CLIC1 and PSMA2 genes in complete DMEM medium. KD of the target proteins in Opti-MEM medium was used to evaluate the transfection efficacy by side-by-side comparison with DMEM. NSC was used as a control in both media. Protein expression or KD was monitored by Western blot every day up to 4 days after transfection. The cell morphology was adversely affected in Opti-MEM media but was unaffected in DMEM media after CLIC1 KD (Figure 12A). However, cell viability of A549 cells after CLIC1 KD was significantly greater in DMEM compared with Opti-MEM (Figure 12B). Compared to NSC, a significant amount of CLIC1 KD was achieved by the CLIC1 siRNA in A549 cells after 2 days of transfection in both types of media (Figure 12C,D). Statistically, there were significant differences in KD efficacy on day 3 and day 4 between Opti-MEM and DMEM (Figure 12D), because of the reduction of CLIC1 protein expression in the control cells. Overall, a transient KD of CLIC1 was achieved in DMEM media with less impact on cell viability and morphology than in Opti-MEM.

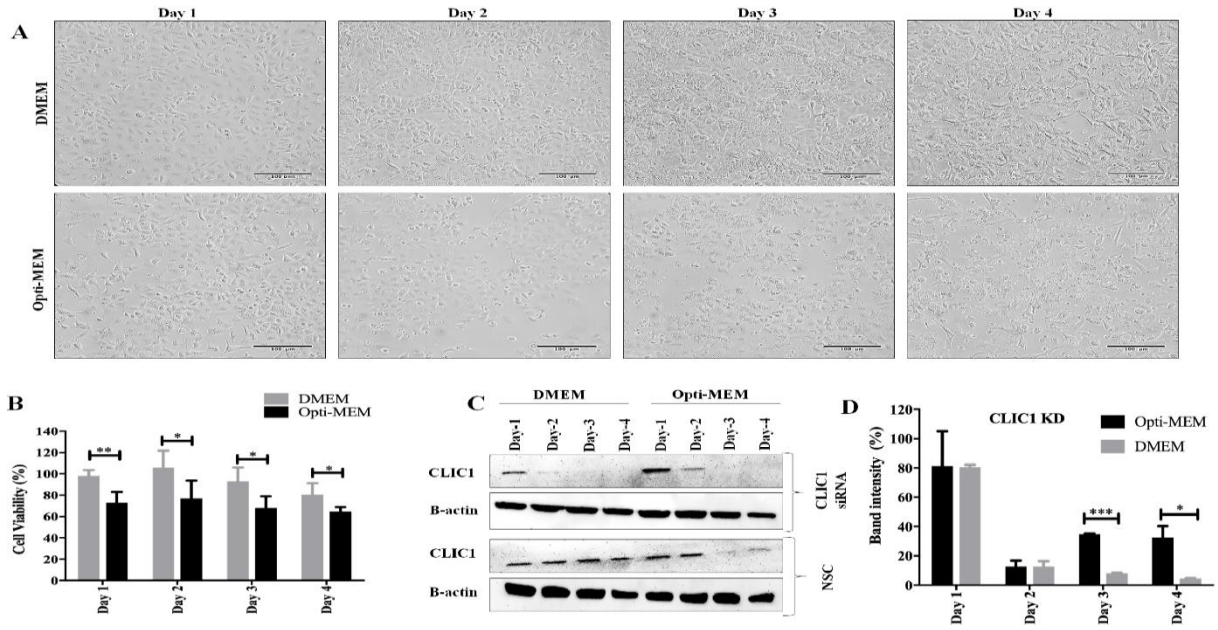


Figure 12. CLIC1 knockdown efficacy in DMEM medium compared to Opti-MEM. **A.** A549 cell morphology in DMEM medium and Opti-MEM medium after CLIC1 protein KD. Visualized under the microscope. The scale bar is 100μm. **B.** A549 cell viability after CLIC1 KD in DMEM and Opti-MEM medium, determined by WST-1 assay. **C.** Knockdown efficacy of CLIC1 siRNA

in DMEM and Opti-MEM medium determined by Western blot. **D.** Quantification of CLIC1 KD efficacy siRNA in Opti-MEM from Western blot images. KD= Knockdown. NSC= non-silencing Control (Scrambled siRNA). * = P< 0.05, ** = P < 0.01,***= P<0.001.

3.1.3 Efficacy of PSMA2 knockdown in DMEM medium compared to Opti-MEM.

After PSMA2 transfection, cell viability was significantly lower in A549 cells grown in Opti-MEM than in DMEM (Figure 13B). After two days of transfection in DMEM medium, a significant amount of PSMA2 knockdown was obtained in A549 cells, which was similar to the KD level after 4 days of transfection in Opti-MEM (Figure 13C,D). However, at 4 days post transfection (dpt), a significantly greater knockdown was achieved in DMEM media than Opti-MEM (Figure 13D).

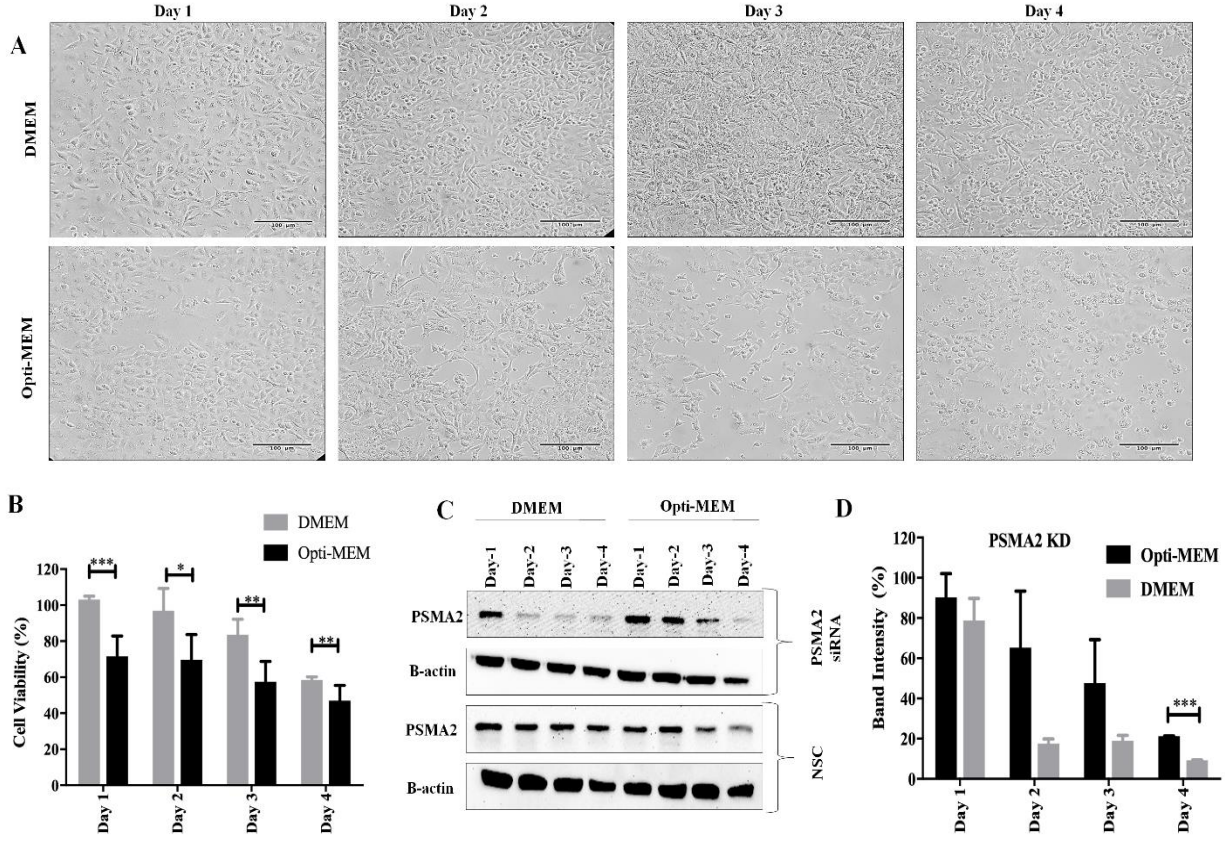


Figure 13. PSMA2 knockdown efficacy in DMEM medium compared to Opti-MEM. **A.** A549 cell morphology in DMEM medium and Opti-MEM medium after PSMA2 protein KD; visualized under the microscope. The scale bar is 100 μm. **B.** A549 cell viability after PSMA2 KD in DMEM and Opti-MEM media, determined by WST-1 assay. **C.** Knockdown efficacy of PSMA2 siRNA in DMEM and Opti-MEM media determined by Western blot. **D.** Quantification of PSMA2 KD efficacy siRNA in Opti-MEM from Western blot images. * = P < 0.05, ** = P< 0.01,***= P<0.001.

3.1.4 Optimization of PSMA2 knockdown by siRNA treatment

To KD the expression of PSMA2, cells were initially transfected with 25nM of SP siRNA targeted against PSMA2 for 48 hours based on our previous study (Rashid & Coombs, 2019). However, cell viability was significantly affected by siRNA treatment. Therefore, A549 cells were transfected with each of the four OT siRNAs that constitute the SP siRNA to assess the adverse effects of each OT siRNA. Cell morphology and cytopathic impact of siRNA treatment were determined by microscopic observation and cell viability assay. The OT3 siRNA treatment impacted A549 cell morphology (Figure 14A) and significantly reduced cell viability (Figure 14B), but OT1, OT2, and OT4 siRNA had no effect on cell viability or morphology. Each individual OT, and the SP, siRNAs significantly reduced PSMA2 expression (Figure 14C, D). Thus, I created a new SP (nSP), consisting of all OT except OT3, and used this nSP in all subsequent experiments. To determine KD kinetics, I transfected A549 cells with 25nM nSP and PSMA2 expression was monitored every day up to day 3 (Figure 1E). On days 1, 2, and 3, PSMA2 expressions were reduced to 80%, 20%, and 7.5%, respectively (Figure 14E, F). PSMA2 KD was further confirmed by IF microscopy (Figure 14G).

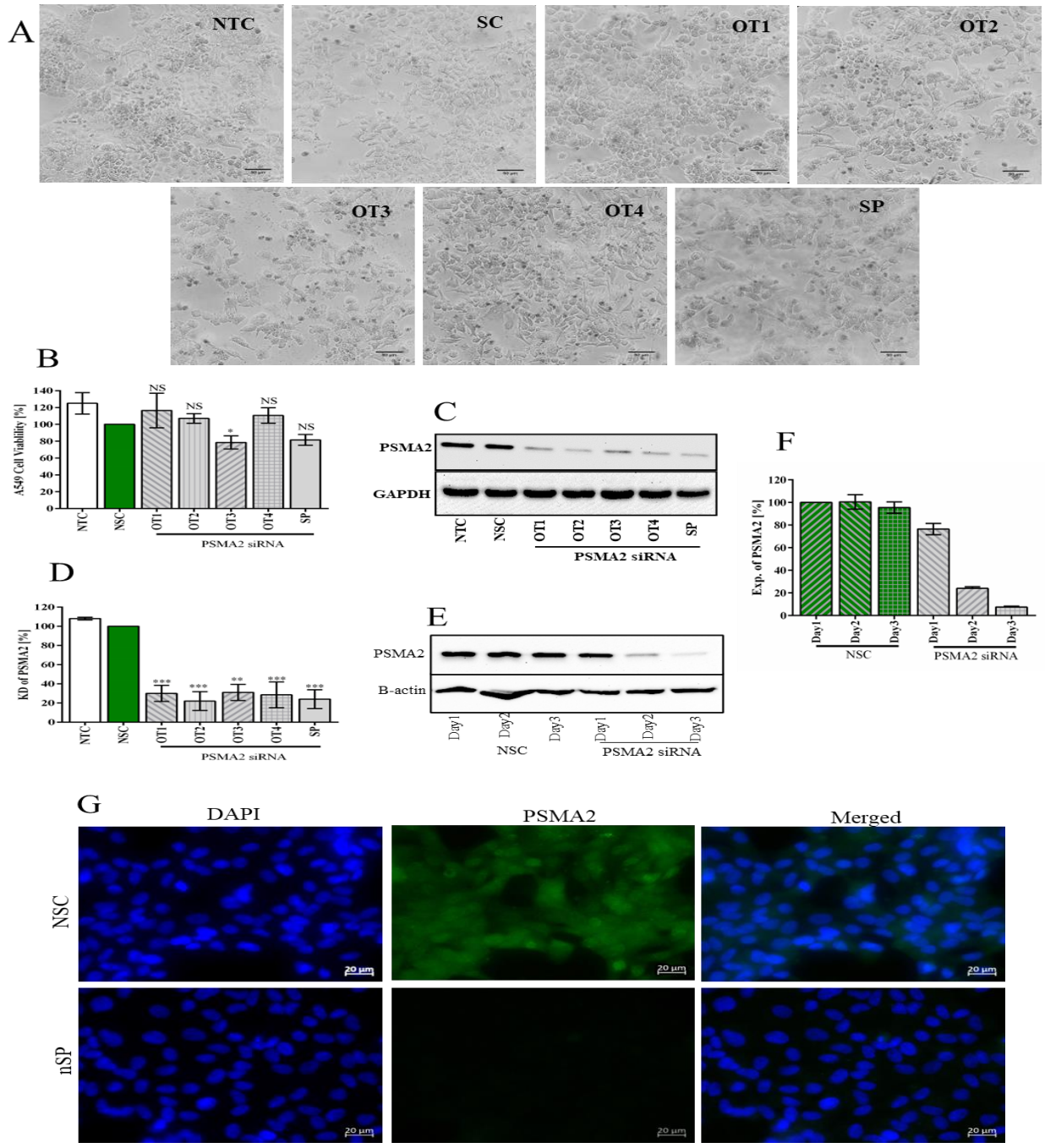


Figure 14. Optimization of PSMA2 Knockdown by siRNA treatment in A549 cells. A. Photomicrographs of cytopathic impact of siRNA KD, scale bars are 50 nm. **B**. Cell viability after 48h of siRNA treatment. **C**. Expression of PSMA2 determined by Western blot after 48h treatment with on-target (OT) siRNAs. **D**. Quantitative expression of PSMA2 KD from Western blots. **E.** Expression of PSMA2 over time after treatment with PSMA2 SMARTpool (nSP: contains only OT1, OT2, and OT4) siRNA. **F.** Quantitative expression of PSMA2 KD from Western blot images, from three replicates. **G.** Knock-down of PSMA2 was confirmed in PSMA2 siRNA-treated cells by IF microscopy at 72hpt (scale bar is 20 µm). Cell nuclei were visualized by DAPI. NTC= non-treated control, NS= not significant. *: P <0.05, **: P < 0.01, ***: P< 0.001.

3.1.5 Optimization of CLIC1 Knockdown by siRNA treatment.

To KD the expression of CLIC1, A549 cells were transfected with 50nM of four OT and SP siRNA targeted against CLIC1 for 48 hours. All of the OT and SP siRNA caused a significant reduction of CLIC1 proteins (Figure 15A,B) without any significant impact on cell viability (Figure 15C). The A549 cells were further treated with 50nM CLIC1 SP siRNA for four days to determine the KD stability over time. On days 1, 2, 3 and 4 CLIC1 expressions were reduced to 46%, 33%, 21% and 11%, respectively (Figure 15D,E). After 4 days of transfection, the morphology of A549 cells was not visually different between NSC and CLIC1 siRNA treatment (Figure15F). The CLIC1 protein knockdown was further confirmed by IF microscopy (Figure 15G).

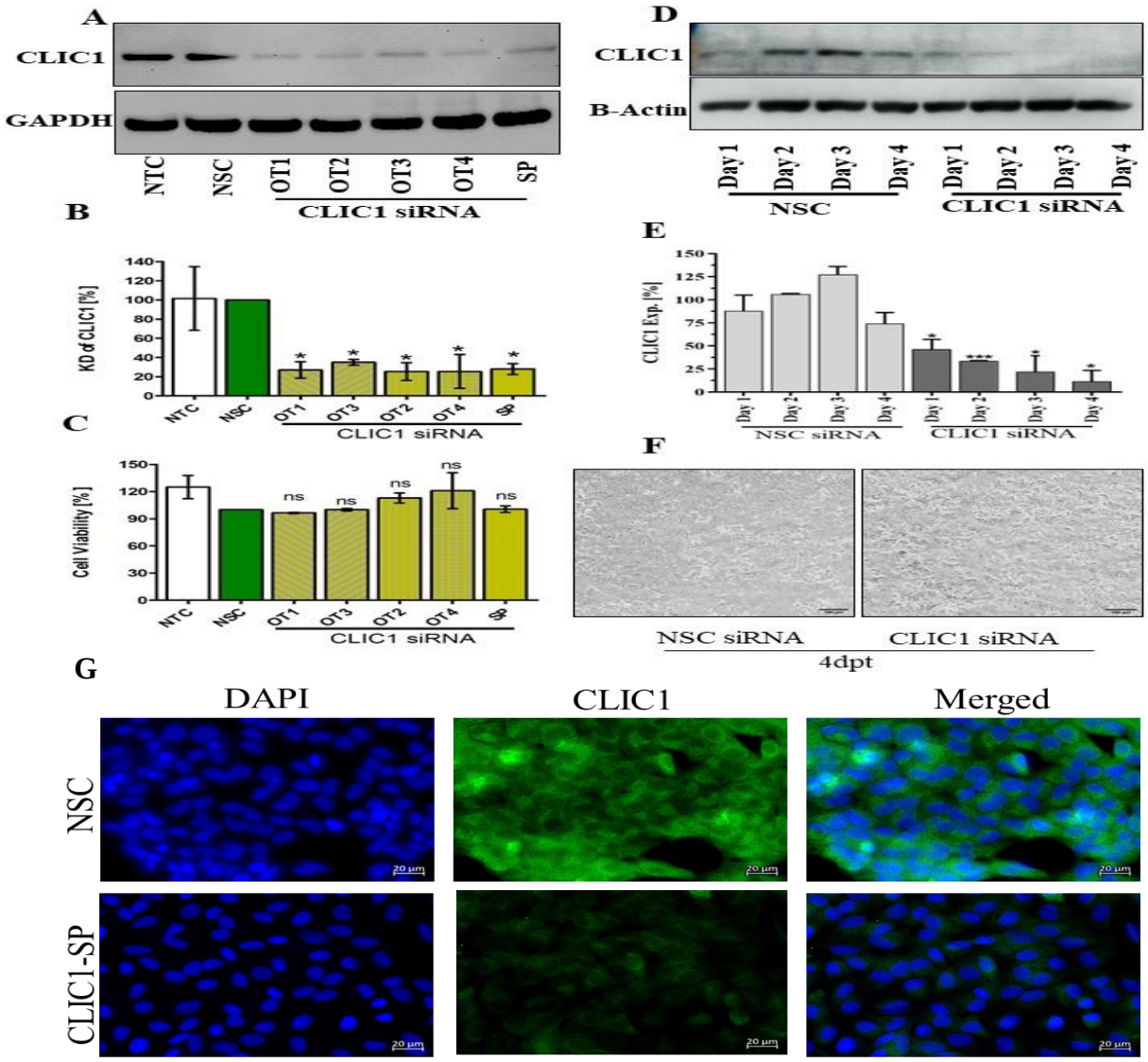


Figure 15. Optimization of CLIC1 knockdown by siRNA transfection in A549 cells. A. Expression of CLIC1 protein detected by western blot after 48hours of treatment with 50nM OT and SP CLIC1 siRNA. **B.** Quantitative expression of CLIC1 from Western blots after OT and SP CLIC1 siRNA treatment. **C.** Cell viability of A549 cells after 48h of siRNA transfection. **D**. Expression of CLIC1 detected by western blot up to 4days after 50uM SP CLIC1 siRNA treatment. **E**. Quantitative expression of CLIC1 from Western blot images after treatment with 50nM CLIC1 siRNA up to 4dpt. **F**. Photomicrographs of A549 cells showing the impact of siRNA treatment on A549 cell phenotypes after 4dpt with 50nM CLIC1 siRNA treatment, scale bars are 100µm. **G**. CLIC1 KD was confirmed in CLIC1 siRNA-treated cells by IF microscopy (scale bar is 20 µm). DAPI was used to visualize the cell nuclei. *: P <0.05, **: P< 0.01, ***: P < 0.001.

3.1.6 Optimization of HSPA5 Knockdown by siRNA treatment.

To KD the expression of HSPA5, A549 cells were transfected with 50nM of four OT and SP siRNA targeted against HSPA5 for 48 hours. All the HSPA5-OT and SP siRNAs caused a significant reduction of HSPA5 proteins (Figure 16A, B) without any significant impact on cell viability (Figure 16C). The A549 cells were further treated with HSPA5 SP siRNA (50nM) for four days to determine the KD stability over time. On days 1, 2,3 and 4 HSPA5 expressions were reduced to 28%, 20%, 18 and 13%, respectively (Figure 16D, E). After 4 days of transfection, the morphology of A549 cells was not visually different between SC and HSPA5 siRNA treatment (Figure 16F). The HSPA5 protein KD was further confirmed by IF microscopy (Figure 16G).

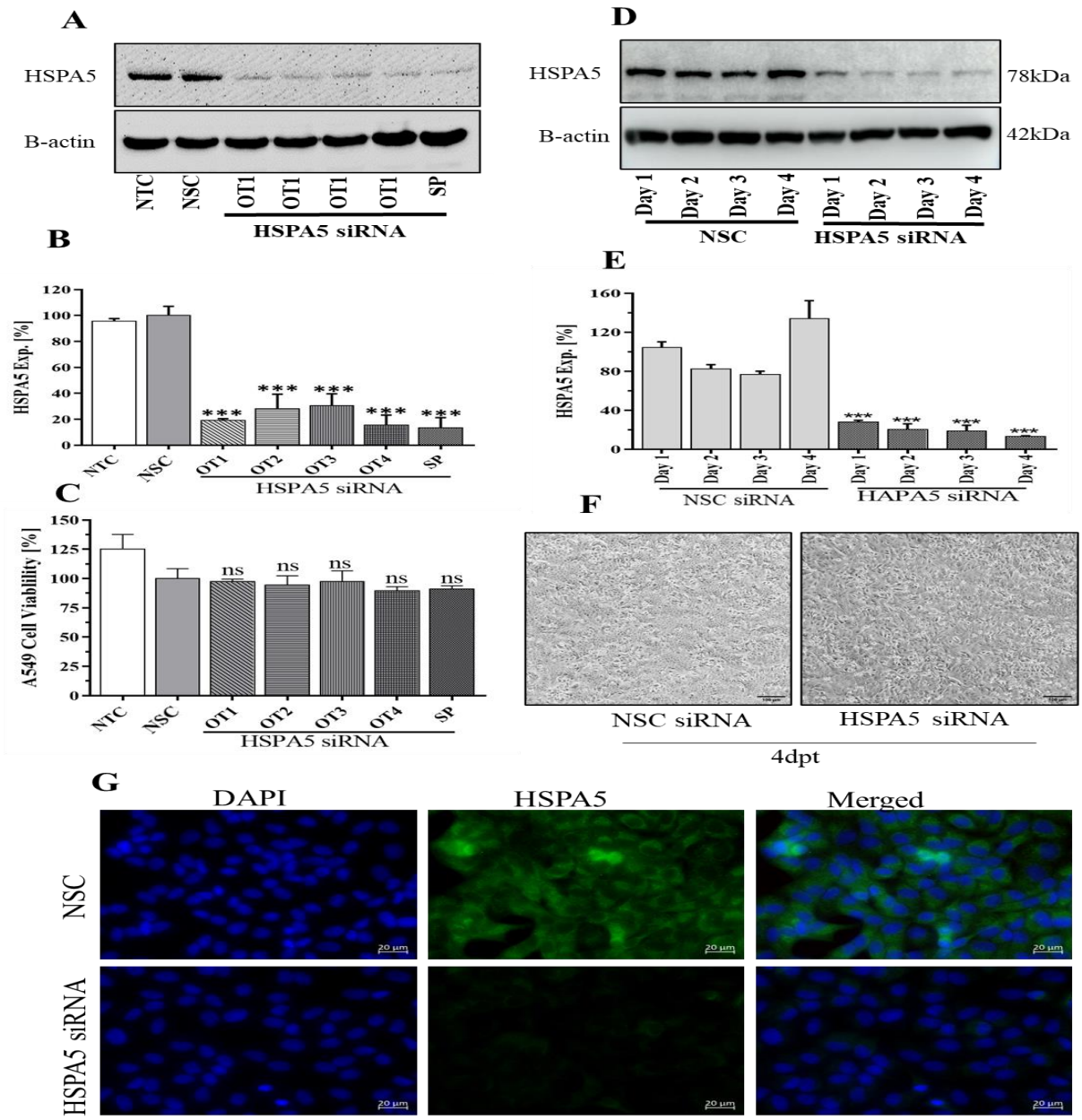


Figure 16. Optimization of HSPA5 knockdown by siRNA transfection in A549 cells. A. Expression of HSPA5 protein detected by Western blot after 48hours of treatment with 50nM on-target and SMARTpool HSPA5 siRNA. **B.** Quantitative expression of HSPA5 from Western blots after OT and SP HSPA5 siRNA treatment. **C**. Cell viability of A549 cells at 48hours post siRNA transfection. **D.** Expression of HSPA5 detected by Western blot up to 4days after 50nM SP HSAP5 siRNA treatment. **E.** Quantitative expression of HSPA5 from Western blot images after treatment with 50nM HSPA5 siRNA up to 4dpt. **F**. Photomicrographs of A549 cells showing the impact of siRNA treatment on A549 cell phenotypes after 4dpt with 50µM HSPA5 siRNA treatment, scale bars are 100 µm. **G.** HSPA5 KD was confirmed in HSPA5 siRNA-treated cells by IF microscopy (scale bar is 20 µm). Cell nuclei were visualized by DAPI. Exp= expression. *: P <0.05, **: P< 0.01, ***: P-value < 0.001.

3.2 Influenza A virus uses PSMA2 for downregulation of NRF2-mediated oxidative stress response

The results of this section were adapted from the following article:

Rashid, Mahamud-ur, Ang Gao, and Kevin M. Coombs. "***Influenza A virus*** **uses PSMA2 for downregulation of NRF2-mediated oxidative stress response**." *Journal of virology* (2022): jvi-01990.

Lay summary: PSMA2 is a fibronectin interacting protein that was highly expressed in IAV infected cells. However, the role of PSMA2 in IAV replication cycle is not clearly understood. Therefore, in this study I explored the association of this protein in IAV replication steps and the cellular signaling pathways during human lung cell infection.

3.2.1 PSMA2 is required for replication of IAV virus.

I focused on PSMA2 because of its key role in the proteasome and UPS, because little is known about its role in viral replication, and because of reagent availability. To understand the role of PSMA2 in IAV replication cycle initially, I determined the impact of PSMA2 KD on progeny virus replication. A549 cells were infected with PR8 after PSMA2 KD. The supernatant was collected at different time points up to 45 hpi. The progeny virus titer was determined by plaque assay. PSMA2 KD caused a significant reduction of progeny viruses in the supernatant at 45 hpi (Figure 17A). PSMA2 KD caused statistically non -significant decrease in cell viability (Figure 17B). The virus titer in the supernatant was normalized to cell viability; this indicated about 90% reduction of virus titer from PSMA2 KD cells (Figure 17C). The impact of PSMA2 KD was not restricted only to the PR8 strain but also caused a significant reduction in pdm-09 and WSN virus replication (Figure 17D). These data indicate that PSMA2 is required for the successful completion of IAV replication.

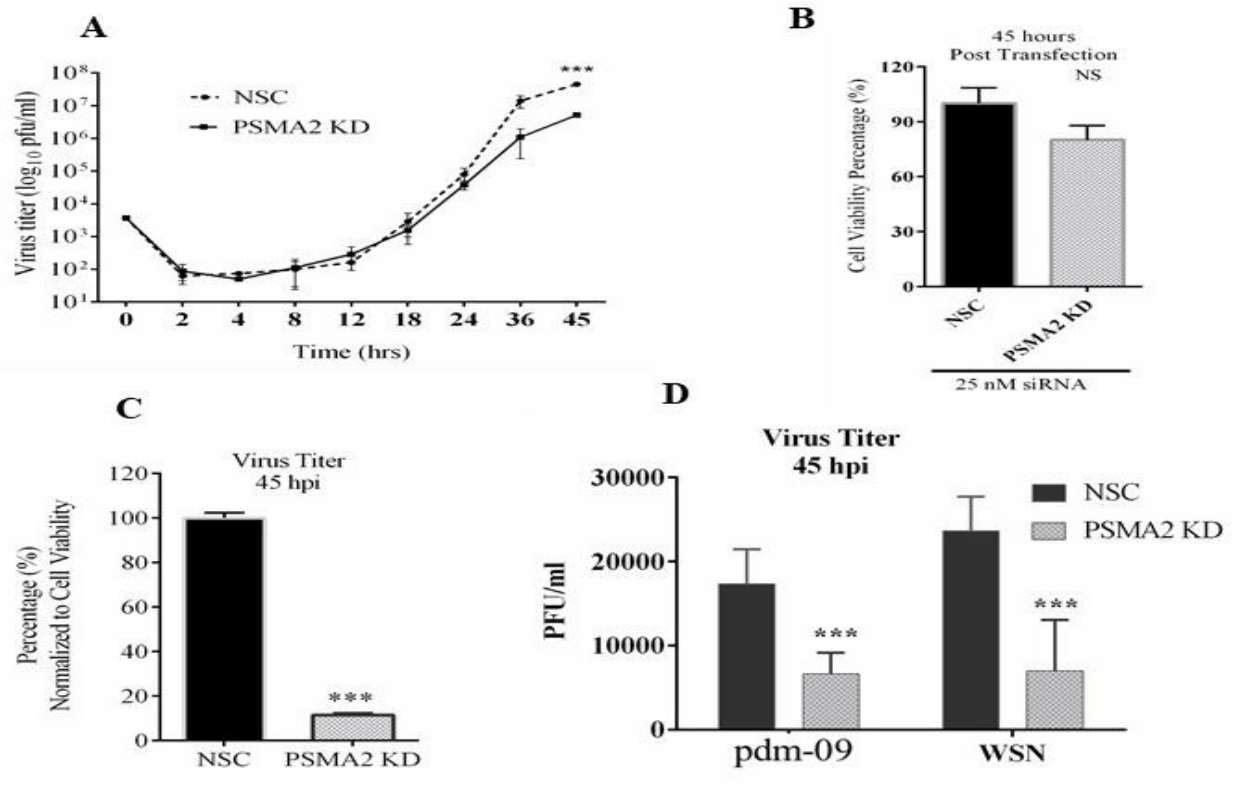


Figure 17. PSMA2 is required for replication of IAV. A549 cells were treated with either non-silencing siRNA (NSC) or PSMA2 siRNA for 48 hours and infected with IAV PR8 at an MOI of 0.01. Supernatants from the infected cells were collected at 0, 2, 4, 8, 12, 18, 24, 36, and 45 hpi. Similarly, NSC and PSMA2 KD cells were infected with IAV strains pdm-09 and WSN and supernatants were collected at 45 hpi. Virus titers were determined by plaque assay. **A.** IAV (PR8 strain) titer in the PSMA2 KD cell supernatant compared to the control (NSC) over time. **B**. Viability of cells measured by WST-1 assay at 45 hours post-siRNA transfection. **C.** Percentage of virus titer in PSMA2 KD cell supernatant at 45 hpi compared to the control and normalized with cell viability. **D.** Impact of PSMA2 KD on IAV pdm-09 and WSN strains. ***: P-value < 0.001.

3.2.2 PSMA2 KD does not impact translation of viral proteins and transcription of vRNAs but inhibits late stages of replication.

Since IAV progeny virus production was significantly reduced in PSMA2 KD cells, I investigated the specific step(s) in virus replication that were affected by PSMA2 KD. First, I assessed the impact on viral protein translation. PSMA2 KD and NSC A549 cells were infected with PR8 at MOI 3. The infected cells were harvested at 12, 24, and 48 hpi. IAV NS1 and NP proteins were detected by Western blot from cell lysates (Figure 18A). Although PSMA2 KD caused a significant impact on progeny virus yield, it did not impact viral protein synthesis (Figure

18C,D,E). As PR8 is a lab-adapted strain, I also tested PSMA2 KD effects on the translation of other human IAV strains. Like PR8, I also did not observe any significant differences in pdm09 or WSN viral protein translation (Figure 18B). A549 cell viability was not significantly affected by PSMA2 KD even after 72hpt (Figure 18F).

I next tested the impact of PSMA2 KD on the transcription of viral RNAs (vRNA) by qRT-PCR. PSMA2 KD and NSC cells were infected at MOI 3 and RNA was extracted from the cells at 24 hpi. cDNA was prepared by reverse transcription and qPCR was performed targeting NS1, NP and HA vRNAs. As with protein translation, PSMA2 KD did not have any significant impact on any of the targeted vRNA transcription processes (Figure 18G).

To further assess the impact of PSMA2 KD on the localization of viral proteins, PSMA2 KD and NSC cells were infected at MOI 3 and 24 hpi cells were fixed. Immunofluorescence (IF) microscopy was done targeting IAV NP protein. In PSMA2 KD cells, I observed that NP protein intensity was higher in many cells than in control NSC-treated infected cells (Figure 18H).

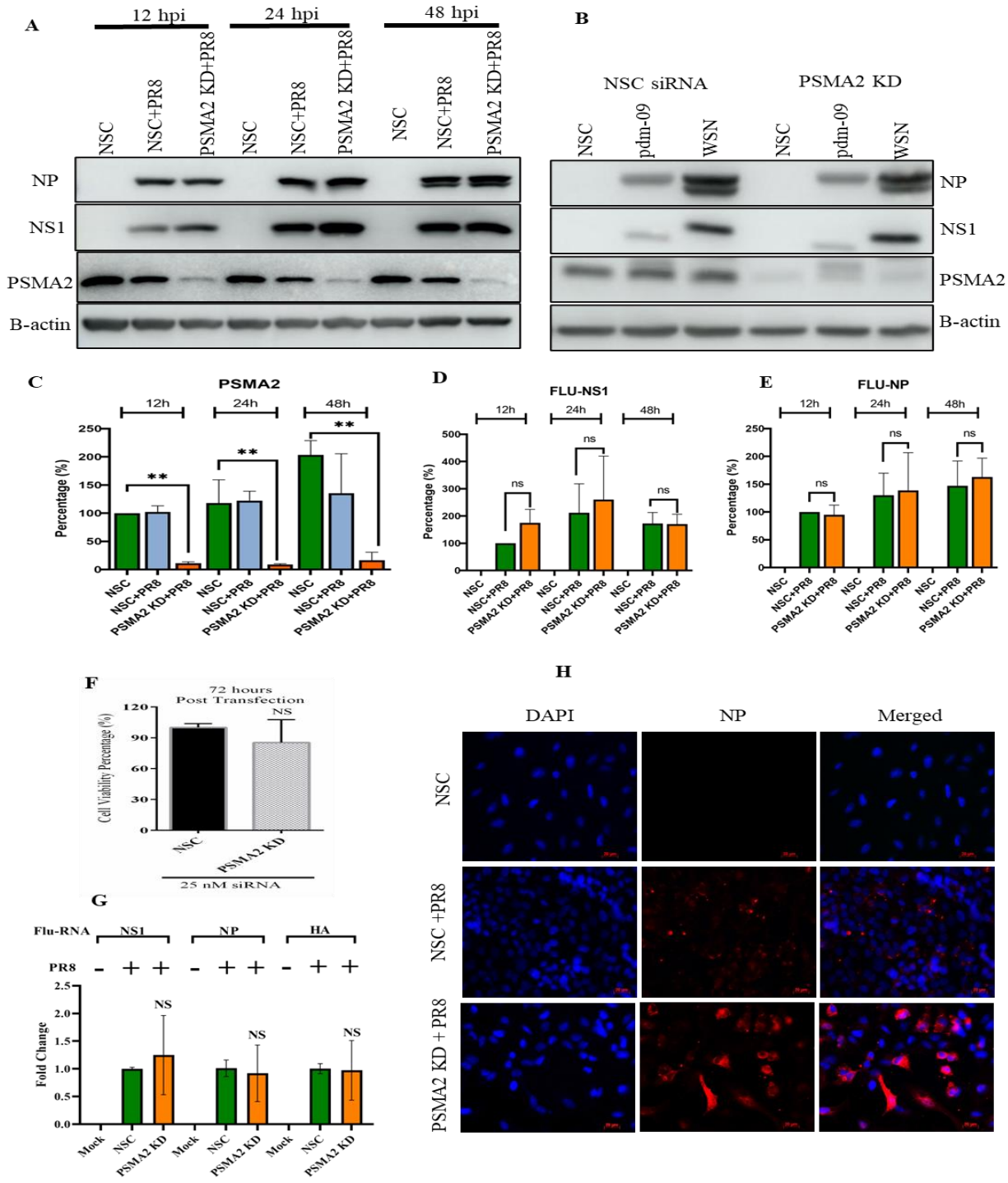


Figure 18. PSMA2 KD does not impact translation of viral proteins and transcription of vRNAs but impacts maturation. A549 cells were treated with either NSC siRNA or PSMA2 siRNA (PSMA2 KD) for 48 hours and infected with IAV PR8, pdm09, or WSN at an MOI of 3. Cell lysates were collected at 12, 24, and 48 hpi from PR8-infected cells and at 24hpi from pdm09-infected and WSN-infected cells for analyzing the expression of viral proteins by Western blotting. After 24 hpi, cells were fixed on slides to measure viral protein localization by IF microscopy. Viral RNAs were collected at 24 hpi, and the comparative vRNA transcripts were determined by

qRT-PCR. **A.** Expression of IAV PR8 NP and NS1 proteins in PSMA2 cells at 12, 24, and 48 hpi. **B.** Expression of viral proteins in PSMA2 KD at 24 hpi after infection with pdm09 and WSN strains. **C-E**. Quantitative densitometry analysis of Western blot images to determine knockdown of PSMA2 expression (**C**), IAV NS1 protein expression (**D**), and Flu-NP protein expression (**E**). F. Impact of PSMA2 KD on cell viability measured by WST-1 assay at 72 hours after transfection by PSMA2 siRNA. **G.** IAV NS1, NP, and HA vRNA transcripts in PSMA2 KD cells compared to mock-infected cells and NSC control. H. IF images showing the expression of IAV NP protein in infected PSMA2 KD cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

3.2.3 Proteomic analysis to understand the impact of PSMA2 KD on IAV replication.

To better understand the role of PSMA2 in the IAV replication cycle, I further evaluated the impact of PR8 in PSMA2 KD cells by measuring dysregulation of the cellular proteome. Cellular proteomic dysregulation was determined by the SOMAScan platform, which can perform quantitative measurements of 1307 proteins simultaneously from the same sample (Candia et al., 2017). The impact of PR8 infection alone, PSMA2 KD alone, and PSMA2 KD+PR8 together were determined by comparing the cellular proteomes in PR8 infected versus NSC, PSMA2 KD versus NSC, and PSMA2 KD+PR8 versus PSMA2 KD alone, respectively. PSMA2 KD, PR8 infection and PSMA2 KD+PR8 infection caused significant dysregulation of 272, 218, and 149 proteins (Table 1), respectively.

Table 1. Numbers of significantly dysregulated proteins by PSMA2 KD, PR8 infection and PR8 infection in PSMA2 KD cells

Range of Fold change	PSMA2 KD	Total Significant	PR8	Total Significant	PSMA2 KD +PR8	Total Significant
and F.C. > 1.00	140	272	76	218	50	149
and F.C. < 1.00	132		142		99	
and F.C. > 1.10	121	202	68	197	36	121
and F.C. < -1.10	81		129		85	
and F.C. > 1.20	88	120	43	144	19	86
and F.C. < -1.20	32		101		67	
and F.C. > 1.30	56	84	31	116	17	71
and F.C. < -1.30	28		85		54	
and F.C. > 1.50	32	52	21	71	15	46
and F.C. < -1.50	20		50		31	
and F.C. > 1.60	22	39	17	57	13	42
and F.C. < -1.60	17		40		29	
and F.C. > 2.00	3	10	11	33	7	42
and F.C. < -2.00	7		22		35	
and F.C. > 2.50	1	1	8	22	4	13
and F.C. < -2.50	0		14		9	

Significance was determined by T-test and Z-score as detailed in Materials and Methods from three biological replicates. The complete list of proteins dysregulated ≥ 1.5-fold in either direction is listed in Table 2.

However, by employing a cut off value ≥ +1.5 or ≤ -1.5 fold-change and P-value <0.05, a total of 52 (32 up-regulated and 20 downregulated) proteins from PSMA2 KD, 71 (up-regulated n=21, downregulated n=50) proteins from PR8 infection and 46 (up-regulated n=15, downregulated n=31) proteins from PSMA2 KD+PR8 infection were selected for further bioinformatics analysis (Table 2).

Table 2. Significantly dysregulated proteins by PSMA2 KD, PR8 infection and PR8 infection in PSMA2 KD cells

Type of Protein	Symbols	Entrez Gene Name	PSMA2 KD (FC)	P-value	PR8 (FC)	P-value	PSMA2 KD+PR8 (FC)	P-value	Location
Cytokines	CXCL8	C-X-C motif chemokine ligand 8	-1.1	8.6E-03	10.3	2.2E-05	3.2	2.7E-03	Extracellular Space
	IFNL1	interferon lambda 1	1.1	2.4E-01	2.9	3.5E-02	1.9	3.7E-02	Extracellular Space
	CCL13	C-C motif chemokine ligand 13	-1.0	8.6E-01	-1.4	9.4E-02	-1.6	3.1E-02	Extracellular Space
	CCL5	C-C motif chemokine ligand 5	1.0	8.7E-01	6.4	3.6E-03	7.5	3.0E-03	Extracellular Space
Enzymes	UBB	ubiquitin B	3.5	9.2E-03	1.1	5.4E-01	-1.4	1.2E-01	Cytoplasm
	TGM3	transglutaminase 3	2.0	2.9E-02	-1.1	8.4E-01	-1.4	1.4E-01	Cytoplasm
	UFC1	ubiquitin-fold modifier conjugating enzyme 1	1.6	6.6E-03	1.2	3.1E-01	1.0	4.0E-01	Cytoplasm
	EIF4A3	eukaryotic translation initiation factor 4A3	1.6	2.0E-03	1.1	1.6E-01	1.0	8.4E-01	Nucleus
	TYMS	thymidylate synthetase	1.6	1.7E-02	-1.1	1.2E-01	-1.8	2.8E-02	Nucleus
	AKR1A1	aldo-keto reductase family 1 member A1	1.2	4.3E-02	-1.7	3.8E-02	-1.1	5.6E-01	Cytoplasm
	RPS3	ribosomal protein S3	-1.9	2.9E-04	1.3	6.9E-01	1.4	1.6E-01	Cytoplasm
	PPIF	peptidylprolyl isomerase F	1.3	8.6E-02	1.7	2.9E-02	1.3	6.0E-02	Cytoplasm
	PPID	peptidylprolyl isomerase D	1.4	1.4E-01	-1.5	1.0E-02	-1.4	3.3E-01	Cytoplasm
	CNTN1	contactin 1	-1.1	3.5E-01	-2.9	1.6E-02	-2.9	1.3E-02	Plasma Membrane
	HAT1	histone acetyltransferase 1	2.4	3.7E-01	-2.4	2.1E-04	-3.0	1.0E-05	Nucleus
	TOP1	DNA topoisomerase I	-1.0	9.5E-01	1.5	1.8E-03	1.6	3.4E-01	Nucleus
Growth factors	DKK1	dickkopf WNT signaling pathway inhibitor 1	-2.0	2.9E-02	-3.6	5.5E-03	-3.5	1.3E-02	Extracellular Space
	BMP6	bone morphogenetic protein 6	1.2	6.2E-02	-1.6	3.2E-02	-1.4	9.0E-02	Extracellular Space
	FGF6	fibroblast growth factor 6	-1.2	2.2E-01	-1.6	5.6E-03	-1.4	9.8E-02	Extracellular Space
	NRG1	neuregulin 1	1.1	8.1E-01	-1.6	4.7E-02	-1.6	4.0E-01	Plasma Membrane
	GRN	granulin precursor	1.0	9.8E-01	-2.1	4.6E-03	-1.6	6.9E-02	Extracellular Space
Kinases	PDPK1	3-phosphoinositide dependent protein kinase 1	1.7	2.2E-02	-1.5	6.1E-02	-1.5	1.8E-01	Cytoplasm
	AK1	adenylate kinase 1	1.7	1.6E-02	-1.1	5.8E-01	-1.0	6.3E-01	Cytoplasm
	MAP2K3	mitogen-activated protein kinase kinase 3	1.7	8.1E-03	1.3	4.3E-02	1.1	5.0E-01	Cytoplasm
	SPHK1	sphingosine kinase 1	1.6	7.0E-03	1.3	1.1E-01	-1.1	5.5E-01	Cytoplasm
	EPHA2	EPH receptor A2	1.6	1.5E-02	3.0	4.7E-03	1.7	1.1E-01	Plasma Membrane
	CSNK2A2	casein kinase 2 alpha 2	1.6	6.3E-03	-1.4	8.6E-02	-1.7	2.9E-02	Cytoplasm
	CSNK2B	casein kinase 2 beta	1.6	6.3E-03	-1.4	8.6E-02	-1.7	2.9E-02	Cytoplasm

	MAPK8	mitogen-activated protein kinase 8	1.6	1.4E-02	-1.2	3.5E-01	-1.4	2.5E-01	Cytoplasm
	WNK3	WNK lysine deficient protein kinase 3	1.6	8.7E-03	-1.1	3.2E-01	-1.1	4.0E-01	Plasma Membrane
	PRKCI	protein kinase C iota	1.5	1.7E-02	1.4	9.7E-02	1.2	1.2E-01	Cytoplasm
	NAGK	N-acetylglucosamine kinase	1.3	4.0E-02	-1.5	3.5E-02	-1.2	2.8E-01	Cytoplasm
	PRKACA	protein kinase cAMP-activated catalytic subunit alpha	-1.5	1.7E-02	-1.2	3.8E-01	-1.1	3.9E-01	Cytoplasm
	CDK2	cyclin dependent kinase 2	-1.9	1.7E-02	-1.3	2.2E-01	1.4	4.8E-02	Nucleus
	EFNA2	ephrin A2	-1.4	5.5E-02	-1.7	4.8E-03	-1.4	9.9E-02	Plasma Membrane
	STC1	stanniocalcin 1	-1.3	5.7E-02	2.4	2.9E-03	1.6	3.6E-02	Extracellular Space
	MAPKAPK2	MAPK activated protein kinase 2	1.2	7.6E-02	-1.5	1.4E-02	-1.6	4.5E-02	Nucleus
	PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha	1.1	1.9E-01	-1.6	4.3E-02	-1.5	1.4E-01	Cytoplasm
	PIK3R1	phosphoinositide-3-kinase regulatory subunit 1	1.1	1.9E-01	-1.6	4.3E-02	-1.5	1.4E-01	Cytoplasm
	MET	MET proto-oncogene, receptor tyrosine kinase	-1.0	2.6E-01	-1.5	9.4E-02	-2.1	2.0E-02	Plasma Membrane
	CAMK2D	calcium/calmodulin dependent protein kinase II delta	1.3	3.2E-01	-1.5	4.2E-02	-1.4	1.8E-01	Cytoplasm
	CAMK2B	calcium/calmodulin dependent protein kinase II beta	1.2	4.4E-01	-1.6	6.0E-03	-1.5	2.3E-01	Cytoplasm
	EPHA3	EPH receptor A3	1.0	4.4E-01	1.6	2.0E-02	1.2	1.6E-01	Plasma Membrane
	FGFR1	fibroblast growth factor receptor 1	-1.0	4.5E-01	-2.1	3.3E-03	-2.8	4.3E-03	Plasma Membrane
	PRKCG	protein kinase C gamma	1.0	6.3E-01	-1.6	2.0E-02	-1.4	4.8E-02	Cytoplasm
	MAPKAPK3	MAPK activated protein kinase 3	-1.0	7.4E-01	-1.7	6.1E-02	-1.8	2.1E-03	Nucleus
Peptidases	PSMA1	proteasome 20S subunit alpha 1	1.6	2.3E-02	-1.2	6.6E-02	-1.9	6.4E-02	Cytoplasm
	IDE	insulin degrading enzyme	1.6	2.6E-02	1.1	4.8E-01	1.5	4.8E-01	Extracellular Space
	CTSA	cathepsin A	-1.4	4.4E-02	-2.1	4.8E-03	-1.7	2.4E-02	Cytoplasm
	CTSV	cathepsin V	-1.5	1.9E-02	-1.1	3.3E-01	1.0	9.3E-01	Cytoplasm
	PSMA2	proteasome 20S subunit alpha 2	-2.2	3.5E-03	-1.4	2.4E-02	-1.1	1.5E-01	Cytoplasm
	PCSK9	proprotein convertase subtilisin/kexin type 9	-2.2	9.3E-03	-5.2	7.2E-03	-4.3	2.4E-03	Extracellular Space
	C1R	complement C1r	1.0	6.4E-01	1.5	2.0E-01	1.9	8.7E-03	Extracellular Space
Phosphatase	PPP3CA	protein phosphatase 3 catalytic subunit alpha	1.5	2.5E-02	-1.3	1.7E-01	-1.2	3.0E-01	Cytoplasm

	PPP3R1	protein phosphatase 3 regulatory subunit B, alpha	1.5	2.5E-02	-1.3	1.7E-01	-1.2	3.0E-01	Cytoplasm
	PTPN6	protein tyrosine phosphatase non-receptor type 6	-1.0	4.0E-01	-1.5	1.2E-03	-1.5	5.2E-03	Cytoplasm
Transcription Regulators	STAT3	signal transducer and activator of transcription 3	1.8	1.3E-02	-2.1	7.9E-03	-2.5	2.3E-02	Nucleus
	ARID3A	AT-rich interaction domain 3A	1.8	1.2E-02	-1.1	4.6E-01	-1.3	3.2E-02	Nucleus
	TBP	TATA-box binding protein	1.7	5.4E-03	-1.5	5.8E-02	-1.6	7.3E-03	Nucleus
	STAT6	signal transducer and activator of transcription 6	1.2	4.2E-02	-1.7	2.1E-02	-1.4	1.5E-02	Nucleus
	NACA	nascent polypeptide associated complex subunit alpha	-1.4	5.8E-04	2.1	1.8E-02	2.2	1.6E-03	Cytoplasm
	EEF1B2	eukaryotic translation elongation factor 1 beta 2	-1.7	3.4E-02	1.1	4.9E-01	1.4	5.4E-02	Cytoplasm
	SMAD2	SMAD family member 2	1.0	2.0E-01	-1.6	3.5E-02	-1.6	7.8E-03	Nucleus
	AIP	aryl hydrocarbon receptor interacting protein	1.1	7.8E-01	-1.6	1.1E-02	-1.2	5.4E-01	Nucleus
	HMGB1	high mobility group box 1	1.0	8.9E-01	-1.5	1.4E-02	-1.2	1.6E-01	Nucleus
Transmembrane Receptors	FAS	Fas cell surface death receptor	2.3	4.8E-04	1.3	2.3E-02	-1.0	9.6E-01	Plasma Membrane
	ITGB1	integrin subunit beta 1	1.6	3.1E-02	1.1	6.2E-01	1.1	2.8E-01	Plasma Membrane
	TNFRSF21	TNF receptor superfamily member 21	-1.4	2.7E-02	-2.5	8.6E-04	-2.3	9.9E-03	Plasma Membrane
	GFRA1	GDNF family receptor alpha 1	-1.6	4.5E-02	-1.9	1.5E-02	-1.6	5.5E-02	Plasma Membrane
	TNFRSF1A	TNF receptor superfamily member 1A	-1.7	1.5E-02	-3.9	5.4E-03	-2.5	8.6E-03	Plasma Membrane
	SFRP1	secreted frizzled related protein 1	-2.1	1.2E-02	-1.2	6.6E-02	-1.3	2.2E-01	Plasma Membrane
	B2M	beta-2-microglobulin	1.1	6.3E-02	2.5	1.3E-04	2.3	3.1E-02	Plasma Membrane
	PLAUR	plasminogen activator, urokinase receptor	-1.0	1.0E-01	1.9	8.8E-03	1.5	8.2E-03	Plasma Membrane
	RTN4R	reticulon 4 receptor	-1.8	1.8E-01	-3.0	1.4E-02	-1.6	2.3E-01	Plasma Membrane
	TNFRSF10D	TNF receptor superfamily member 10d	-1.1	1.8E-01	3.6	1.0E-02	2.5	3.8E-03	Plasma Membrane
	KIR2DL4	killer cell immunoglobulin like receptor, two Ig domains and long cytoplasmic tail 4	-1.1	2.2E-01	-1.7	1.0E-02	-1.5	3.5E-02	Plasma Membrane
	MICB	MHC class I polypeptide-related sequence B	-1.2	2.5E-01	-1.7	3.0E-02	-1.9	2.7E-02	Plasma Membrane

	PLXNB2	plexin B2	1.1	3.8E-01	-1.3	3.8E-02	-2.0	1.2E-02	Plasma Membrane
	NRP1	neuropilin 1	1.0	9.6E-01	-2.2	1.8E-02	-2.4	1.9E-02	Plasma Membrane
Transporters	BPI	bactericidal permeability increasing protein	-1.2	1.1E-01	-1.6	4.5E-03	-1.4	2.4E-02	Plasma Membrane
	SNX4	sorting nexin 4	-1.1	3.5E-01	-1.6	2.0E-02	-1.4	1.8E-01	Cytoplasm
	ATP5PO	ATP synthase peripheral stalk subunit OSCP	-1.1	4.5E-01	1.6	2.7E-02	1.7	1.8E-02	Cytoplasm
	LCN2	lipocalin 2	-1.0	6.8E-01	-1.2	1.2E-01	1.9	6.3E-03	Extracellular Space
Others	GRB2	growth factor receptor bound protein 2	1.9	4.8E-03	1.0	9.3E-01	-1.6	1.1E-02	Cytoplasm
	CD55	CD55 molecule (Cromer blood group)	1.7	3.3E-03	1.6	5.6E-04	1.2	2.3E-01	Plasma Membrane
	L1CAM	L1 cell adhesion molecule	1.7	4.7E-02	1.3	5.0E-01	1.1	3.1E-01	Plasma Membrane
	NSFL1C	NSFL1 cofactor	1.7	2.2E-02	-1.1	3.9E-01	-1.3	7.7E-02	Cytoplasm
	SBDS	SBDS ribosome maturation factor	1.6	5.8E-03	1.1	7.0E-01	-1.3	2.8E-01	Nucleus
	ISG15	ISG15 ubiquitin like modifier	1.6	4.9E-03	7.2	4.5E-03	7.7	1.5E-02	Extracellular Space
	ITGA1	integrin subunit alpha 1	1.6	3.1E-02	1.1	6.2E-01	1.1	2.8E-01	Plasma Membrane
	SHC1	SHC adaptor protein 1	1.5	2.9E-02	-1.2	8.5E-02	-1.2	4.2E-01	Cytoplasm
	H2AZ1	H2A.Z variant histone 1	1.4	6.2E-03	-2.1	1.2E-02	-1.9	1.1E-02	Nucleus
	CST3	cystatin C	-1.4	3.1E-03	2.0	3.3E-03	1.7	1.9E-02	Extracellular Space
	FSTL1	follistatin like 1	-1.6	4.8E-03	1.1	2.1E-01	1.1	2.6E-01	Extracellular Space
	APP	amyloid beta precursor protein	-1.7	2.6E-02	-1.9	1.8E-02	-2.2	1.6E-02	Plasma Membrane
	MAPT	microtubule associated protein tau	-1.7	2.6E-02	-1.4	1.5E-01	1.1	7.2E-01	Plasma Membrane
	AMIGO2	adhesion molecule with Ig like domain 2	-1.8	3.0E-02	-2.6	1.9E-02	-1.9	1.2E-02	Plasma Membrane
	SERPINE2	serpin family E member 2	-1.9	2.4E-03	-2.6	1.7E-04	-1.8	7.4E-03	Extracellular Space
	CCNA2	cyclin A2	-1.9	1.7E-02	-1.3	2.2E-01	1.4	4.8E-02	Nucleus
	DKK4	dickkopf WNT signaling pathway inhibitor 4	-2.1	3.4E-02	-3.6	8.2E-03	-3.4	1.7E-02	Extracellular Space
	KIF23	kinesin family member 23	-2.3	2.0E-05	-2.6	8.7E-03	-1.3	4.7E-01	Cytoplasm
	UNC5D	unc-5 netrin receptor D	-2.3	2.2E-03	-4.3	9.5E-04	-2.2	3.2E-02	Plasma Membrane
	TGFBI	transforming growth factor beta induced	-1.3	9.4E-02	1.5	1.4E-02	-1.2	1.9E-01	Extracellular Space
	IGFBP2	insulin like growth factor binding protein 2	-1.1	1.5E-01	1.8	2.1E-02	1.2	1.0E-01	Extracellular Space
	MICA	MHC class I polypeptide-related sequence A	-1.5	2.0E-01	-1.8	4.9E-02	-1.7	1.5E-01	Plasma Membrane
	RSPO2	R-spondin 2	-1.2	2.1E-01	-1.7	1.8E-02	-1.5	5.1E-02	Extracellular Space

	H1-2	H1.2 linker histone, cluster member	1.4	2.1E-01	2.8	8.0E-03	3.2	4.5E-02	Nucleus
	GREM1	gremlin 1, DAN family BMP antagonist	-1.2	2.2E-01	-1.5	1.1E-02	-1.4	6.4E-02	Extracellular Space
	CFH	complement factor H	-1.2	2.4E-01	-1.8	5.7E-03	-1.4	1.3E-01	Extracellular Space
	LAMA1	laminin subunit alpha 1	1.2	3.6E-01	-4.4	1.2E-03	-5.0	1.0E-02	Extracellular Space
	LAMB1	laminin subunit beta 1	1.2	3.6E-01	-4.4	1.2E-03	-5.0	1.0E-02	Extracellular Space
	LAMC1	laminin subunit gamma 1	1.2	3.6E-01	-4.4	1.2E-03	-5.0	1.0E-02	Extracellular Space
	SERPINE1	serpin family E member 1	1.1	3.8E-01	6.0	3.8E-03	1.6	4.1E-02	Extracellular Space
	IGFBP6	insulin like growth factor binding protein 6	1.1	5.0E-01	2.0	5.3E-04	1.1	5.7E-01	Extracellular Space
	SLITRK5	SLIT and NTRK like family member 5	1.0	5.9E-01	-1.6	1.4E-02	-1.5	5.7E-02	Plasma Membrane
	MFGE8	milk fat globule EGF and factor V/VIII domain containing	-1.0	8.9E-01	-2.0	4.2E-02	-1.6	8.8E-02	Extracellular Space

***List of proteins with Fold change Up-regulated ≥ 1.5 (Red) or Down-regulated ≤ -1.5 (Blue) FC= Fold change. Green: P-value <0.05.**

Volcano plot analysis of dysregulated proteins indicated that many proteins were differentially dysregulated between PR8 infected and PSMA2 KD+PR8 infected cells (Figure 19A1, A2, A3). TGFBI, FAS, PLAU and CTSB proteins were significantly dysregulated by PR8 infection but had an opposite trend of expression in PSMA2 KD+PR8 (Figure 19B1). CDK2, CCNA2, CDKN1B, LCN2, TFPI, GRB2 proteins were significantly dysregulated by PSMA2 KD+PR8 infection but had an opposite trend of expression in PR8-infected non-KD cells (Figure 19B2). However, 68 proteins were significantly dysregulated by PR8 infection but were not affected in the PSMA2 KD +PR8 condition (Appendix Figure 1A). Alternatively, 21 proteins were significantly dysregulated in PSMA2 KD+PR8 cells but were not significantly impacted in PR8 infection (Appendix Figure 1B). Western blot was performed targeting STAT3, CST3 and PSMA2 proteins to validate the SOMAScan data (Figure 19C). Quantitative densitometry of Western blot images showed that all three proteins follow the same expression trend as determined by SOMAScan (Figure 19D).

However, IPA also predicted PR8 infection can cause dysregulation of 121 canonical pathways (upregulated 2, downregulated 119). In comparison, PSMA2 KD caused upregulation of 35 pathways and downregulation of only one. Interestingly, IPA could predict 61 (Upregulated 1 and downregulated 60) pathways were significantly reregulated by PSMA2 KD+ PR8 infection (Appendix Table 2). However, I found 46 canonical pathways were significantly downregulated by PR8 infection but were not significantly affected by PSMA2 KD+PR8 (Figure 19F). Among

them, 10 pathways were activated significantly in PSMA2 KD cells; there was no impact in PSMA2 KD+PR8 infection by IPA prediction. Three of these pathways may not be relevant for lung epithelial cells, as those are immune cell specific. The remaining seven pathways are Phospholipase C Signaling, NGF Signaling, ErbB4 Signaling, PAK Signaling, regulation of eIF4 and P7S6K signaling, Cholecystokinin/Gastrin-mediated Signaling, and NRF2-Mediated Oxidative Stress Response (Figure 19F). Based on relevance and the most number of significantly dysregulated proteins in the pathway, I selected NRF2-Mediated Oxidative Stress Response signaling for further investigation (Figure 20A).

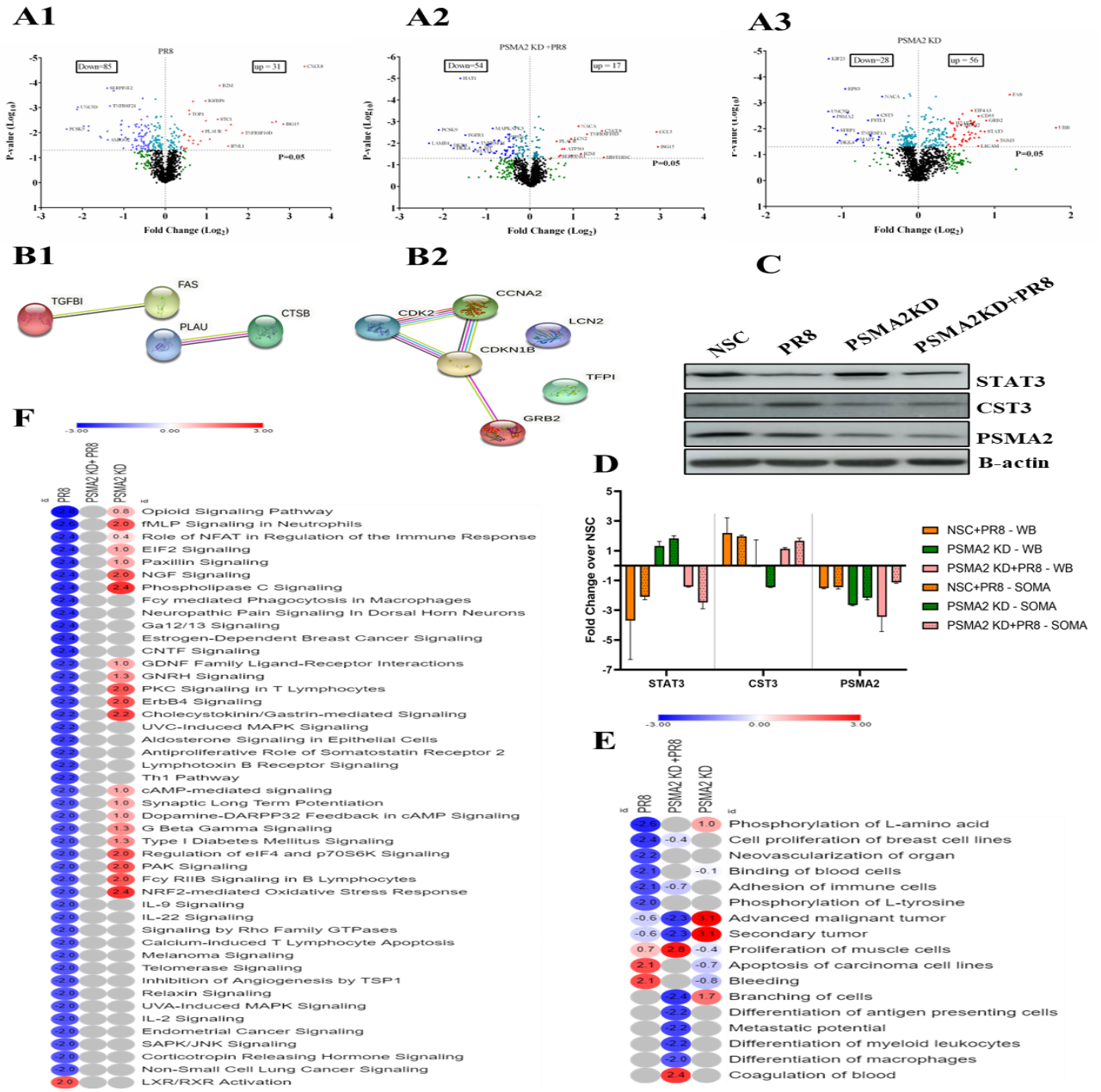


Figure 19. Proteomic analysis to delineate the impact of PSMA2 KD on IAV replication. NSC and PSMA2 KD cells were infected with IAV PR8 at an MOI of 3. Cell lysates were collected from uninfected NSC and PSMA2 KD cells and after infection with PR8 at 24 hpi. Uninfected NSC and PSMA2 KD cells were used as controls. Cell lysates were analyzed by the SomaScan platform, which can detect >1,300 predefined proteins simultaneously from each sample. The protein expression values were compared between the groups to determine whether the protein dysregulation was an experimental condition. PSMA2 KD versus NSC, NSC infected with PR8 versus NSC, and PSMA2 KD infected with PR8 versus PSMA2 KD comparisons were made to determine the impact of PSMA2 knockdown (PSMA2 KD), PR8 infection (PR8) and impact of PR8 infection in PSMA2 KD cells (PSMA2 KD+PR8), respectively. **A.** Volcano plot of proteins dysregulated in IAV PR8-infected (**A1**), PSMA2 KD (**A2**), and IAV PR8-infected PSMA2 KD (**A3**) cells. **B1.** Proteins significantly dysregulated by PR8 infection but with an opposite trend of expression in PSMA2 KD+PR8 cells. **B2**. Proteins significantly dysregulated by PSMA2 KD+PR8 infection but with an opposite trend of expression in PR8-infected cells. **C.** Validation SomaScan data by Western blot detection of STAT3, CST3, and PSMA2 proteins. **D.** Quantitative densitometry of Western blot images and comparison with SomaScan data for data validation. **E.** Heat map of the disease and functions significantly dysregulated by either PR8 or PSMA2 KD+PR8 but not by the others. **F.** Heat map of significantly dysregulated canonical pathways in PR8-infected cells; significance could not be predicted by IPA in PSMA2 KD+PR8 cells.

3.2.4. Influenza A virus utilizes PSMA2 for downregulation of NRF2-mediated oxidative stress response.

Based on the dysregulated proteins, IPA predicted that PR8 infection could cause significant downregulation of NRF2 mediated signaling pathways (Z-score= -2, Figure 20B1) but a significant upregulation by PSMA2 KD (Z-score= 2.44, Figure 20B2). However, during IAV infection in PSMA2 KD cells, IPA could not predict any significant impact on this pathway (Z-score= NS, Figure 20B3).

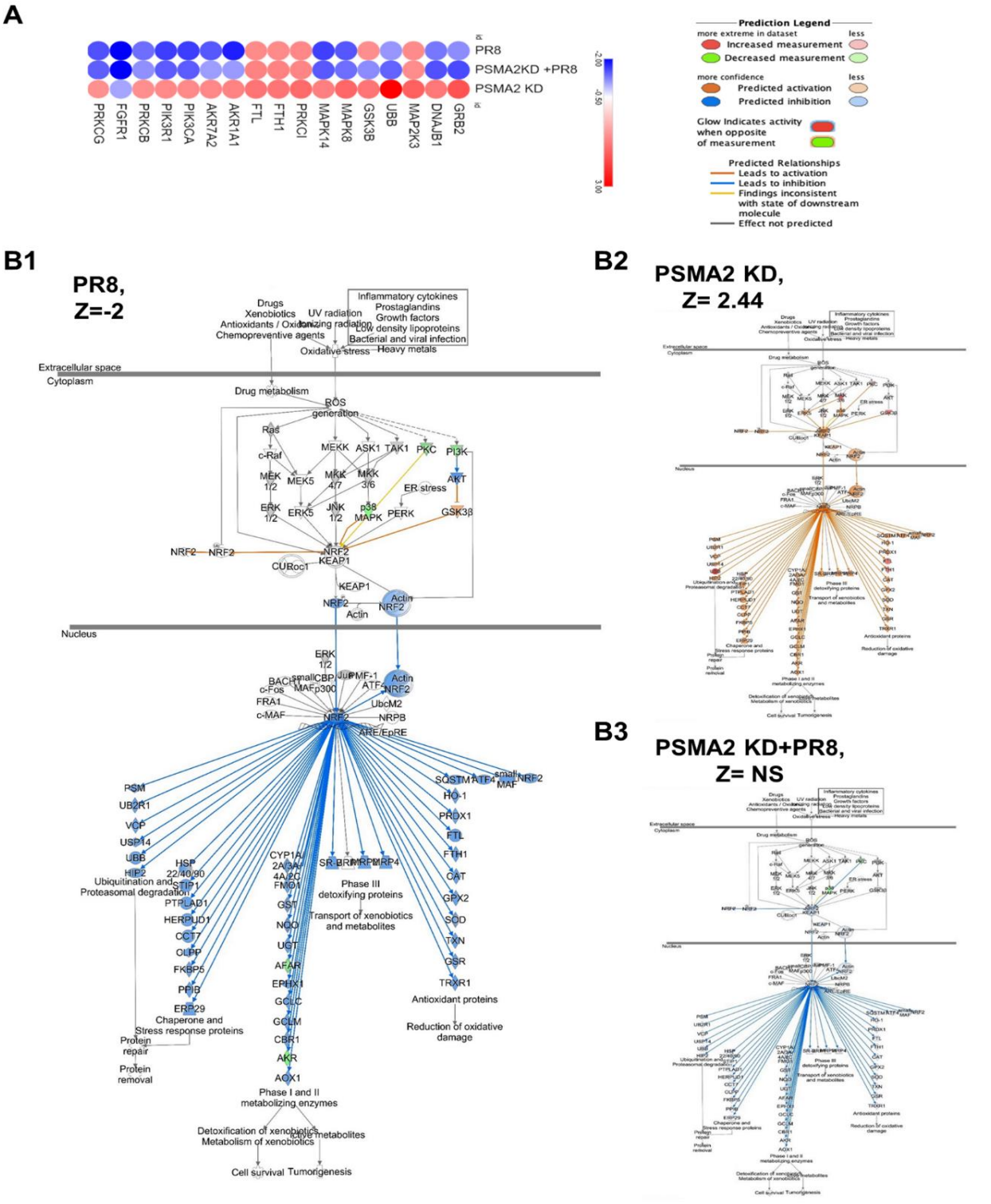


Figure 20. Influenza A virus utilizes PSMA2 for downregulation of NRF2-mediated oxidative stress response. A. Proteins associated with NRF2-mediated oxidative stress response pathway dysregulated by PR8 infection, PSMA2 KD, and PSMA2 KD+PR8. Red, upregulated; blue, downregulated. **B.** NRF2-mediated oxidative stress response signaling pathway activation

by PR8 infection (**B1**), PSMA2 KD (**B2**), and PR8 infection with PSMA2 KD (**B3**). Orange and blue indicate IPA-predicted activation and inactivation, respectively. mpi: minutes post-infection.

3.2.5. PSMA2 KD reduces proteasome activity but does not affect IAV replication in the presence of NAC.

Next, I examined the impact of PSMA2 KD on reactive oxygen species (ROS) concentration in IAV infected PSMA2 KD and control cells. IAV infection caused a significant decrease in ROS levels, whereas PSMA2 KD caused significant up-regulation of ROS levels. Interestingly, the ROS levels increases were even higher in PSMA2 KD cells during IAV infection (Figure 21A,B). To investigate the importance of PSMA2 in the proteosome, I determined the impact of PSMA2 KD on PSMA1 and PSMA6 expression and 20S proteasome activity. PSMA2 KD negatively impacted the expression of PSMA1 and PSMA6 (Figure 21C) and significantly reduced 20S proteasome activity (Figure 21D).

To further understand the role of PSMA2 in NRF2 mediated oxidative response pathway, I investigated the role of a proteasome inhibitor (MG132) and ROS scavengers (NAC) during IAV replication. MG132 caused significant inhibition of IAV in both A549 and MRC5 cells. In contrast, NAC caused significant enhancement of IAV replication in wild type and PSMA2 KD A549 cells (Figure 21E,F). Interestingly I did not observe any increase in IAV replication in wild-type MRC-5 cells after NAC treatment but was enhanced in PSMA2 KD cells (Figure 21F).

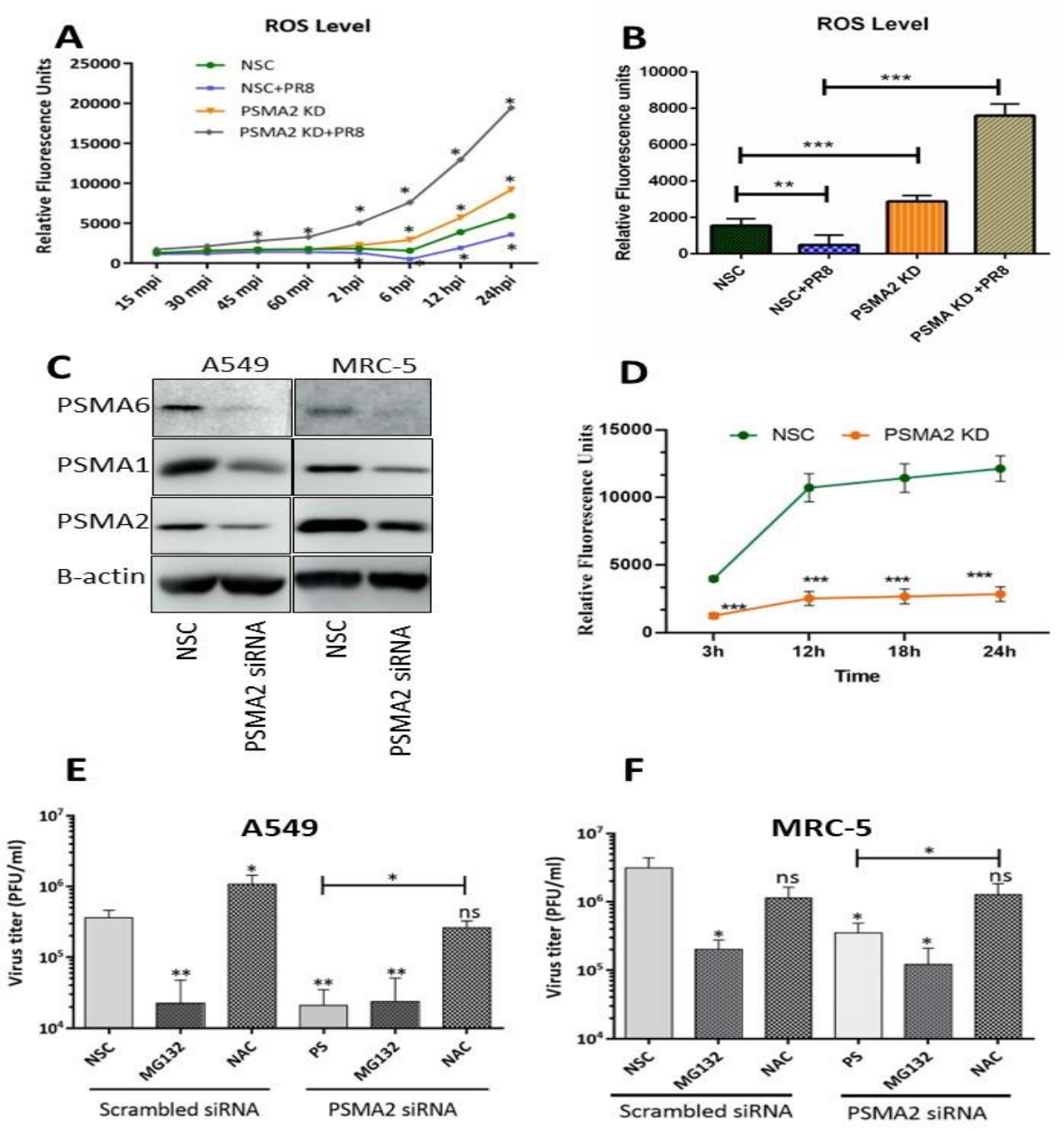


Figure 21. PSMA2 KD reduces the 20s proteasome activity and increases ROS levels in human lung cells inhibiting IAV replication. A and **B**. Change in reactive oxygen species (ROS) concentration over time (**A**) and at 6 hpi (**B**) by PR8 infection, PSMA2 KD, and PR8 infection in A549 cells. **C.** Expression of PSMA1 and PSMA6 in A549 and MRC-5 cells after PSMA2 KD. **D**. 20S proteasome activity in PSMA2 KD cells. **E.** Impact of MG132 and NAC on IAV replication in wild-type cells and after PSMA2 KD in A549 cells. **F.** Impact of MG132 and NAC on IAV replication in wild-type MRC-5 cells and after PSMA2 KD. All significance levels were calculated in comparison with NSC, without the bars compared with the horizontal lines. PS: PSMA2 KD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

3.2.6. PSMA2 is required for nuclear translocation of NRF2

NRF2 nuclear translocation is a critical step in the NRF2 mediated oxidative response pathway. Immune fluorescent microscopy showed that IAV infection caused substantial translocation of the NRF2 proteins into the nucleus. However, during IAV infection in PSMA2 KD cells, NRF2 nuclear translocation was not significant (Figure 22A, B).

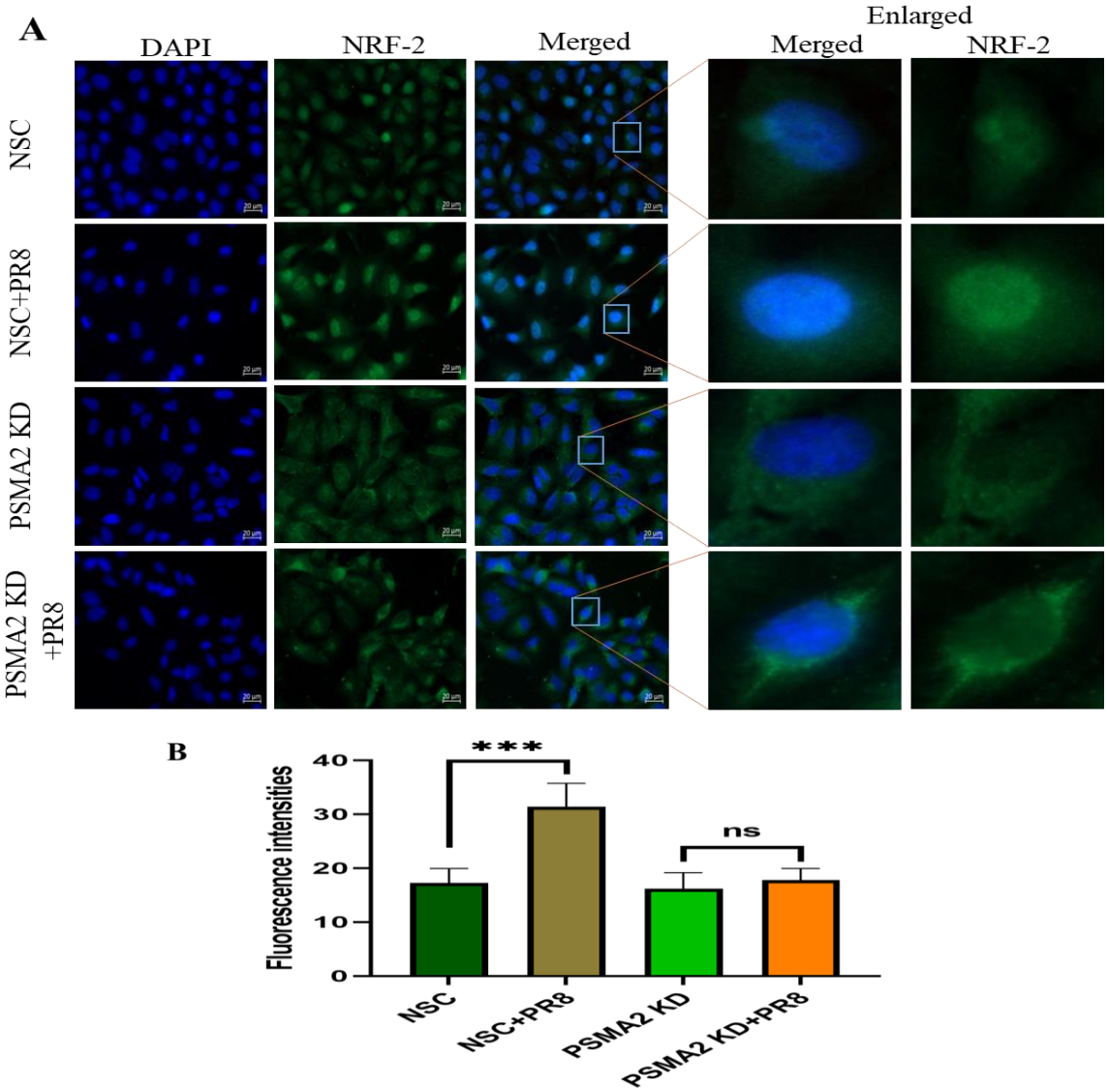


Figure 22. PSMA2 is required for nuclear translocation of NRF2. A. IF images showing the impact of PSMA2 KD on NRF2 nuclear translocation in IAV-infected A549 cells. **B.** Quantitative fluorescence intensity of NRF2 in the nucleus determined by ImageJ. The NSC shows the distribution of NRF2 in non-infected cells treated with scrambled siRNAs. NSC+PR8 indicates the nuclear translocation of NRF2 in IAV-infected cells. PSMA2 KD shows the distribution of NRF2 in non-infected, PSMA2-depleted cells. PSMA2 KD+PR8 shows the impact of PSMA2 KD on translocation of NRF2 in IAV-infected cells.

3.3 CLIC1 is a critical host protein for IAV replication.

3.3.1 Impact of CLIC1 KD on IAV replication

Initially, I determined the impact of CLIC1 KD on progeny virus replication. A549 cells were infected with PR8 after CLIC1 KD. The supernatants were collected at different time points up to 45hpi. The progeny virus titer was determined by plaque assay. CLIC1 KD caused a significant reduction of progeny viruses in the supernatant at 45hpi (Figure 23A). However, CLIC1 KD did not cause any significant impact on cell viability (Figure 23B). The virus titer in the supernatant was normalized to cell viability; this indicated about 60% reduction of virus titer from CLIC1 KD cells (Figure 23C). The impact of CLIC1 KD was not restricted only to the PR8 strain but also caused a significant reduction in pdm-09 and WSN virus replication (Figure 23D).

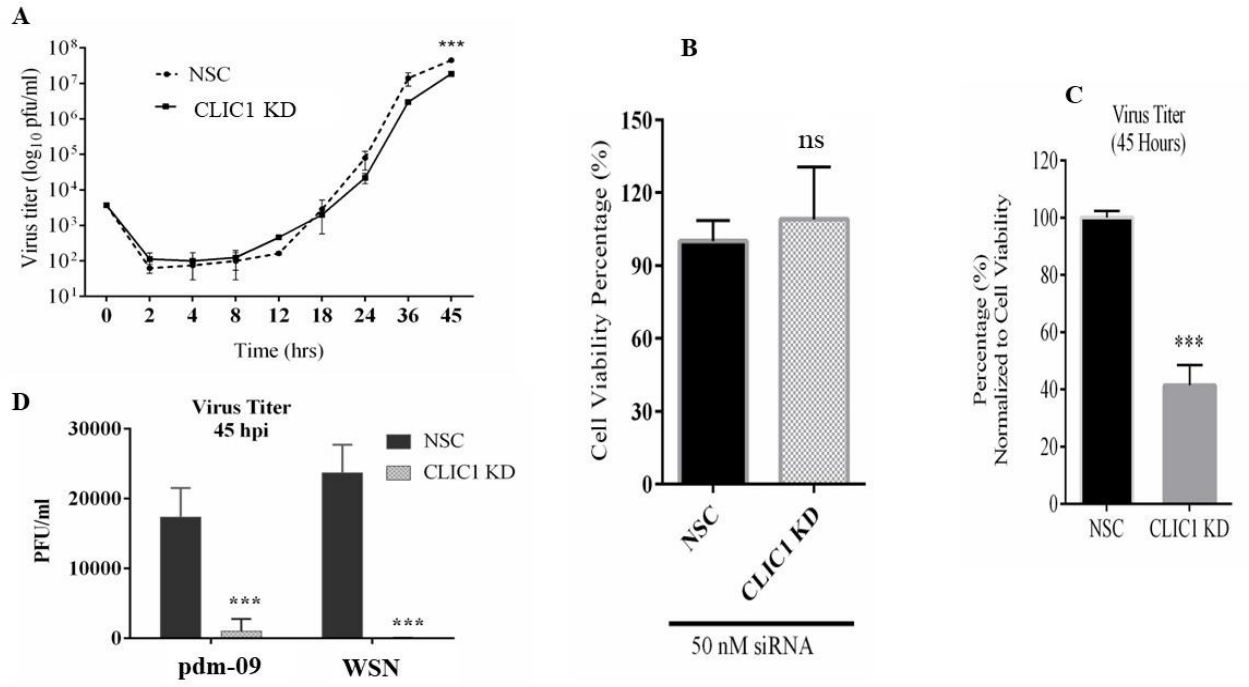


Figure 23. CLIC1 is required for replication of IAV virus. A549 cells were treated with either NSC or CLIC1 siRNA (CLIC1 KD) for 48 hours and infected with IAV-PR8 MOI 0.01. Supernatant from the infected cells was collected at 0,2,4,8,12,18,24,36 and 45hpi. Similarly, NSC and CLIC1 KD cells were infected with pdm-09 and WSN strains and supernatant was collected at 45hpi. The virus titer was determined by plaque assay. **A.** IAV (PR8 strain) titer in the supernatant of CLIC1 KD cells compared to NSC over time. **B.** Viability of cells was measured by WST-1 assay at 96 hours post siRNA transfection. **C.** Percentage of virus titer in CLIC1 KD cell supernatant at 45hpi compared to control and normalized with cell viability. **D.** Impact of CLIC1 KD on pdm-09 and WSN strain of IAV. hrs= hours. *: P <0.05, **: P < 0.01, ***: P < 0.001.

3.3.2 Chloride channel inhibitor (NPPB) suppresses the replication of IAV virus.

Knockdown of CLIC1 expression caused a significant impact on IAV replication, indicating that CLIC1 could be a important host factor for IAV replication. So, I wanted to examine if a chloride channel inhibitor 5-nitro-2-(3-phenylpropyl-amino) benzoic acid (NPPB) can also impact the replication of IAV**.** The NPPB inhibitor blocks the chloride ion channel. The toxicity of the different concentrations of the drug was tested in A549 (Figure 24A) and MRC5 (Figure 24B) cells. Based on the drug's cytotoxicity, 63.5, 90, and 125μM concentrations of NPPB were used in A549 cells and 15.6 and 31.3 μM in MRC5 cells to determine the impact of the drug on IAV replication. The chloride channel inhibitor significantly reduced the replication of PR8 (Figure 24C) and N-cal (Figure 24 D) strains of IAV in A549 cells. However, the lower concentration of NPPB did not affect the replication of PR8 in MRC5 cells (Figure 24E).

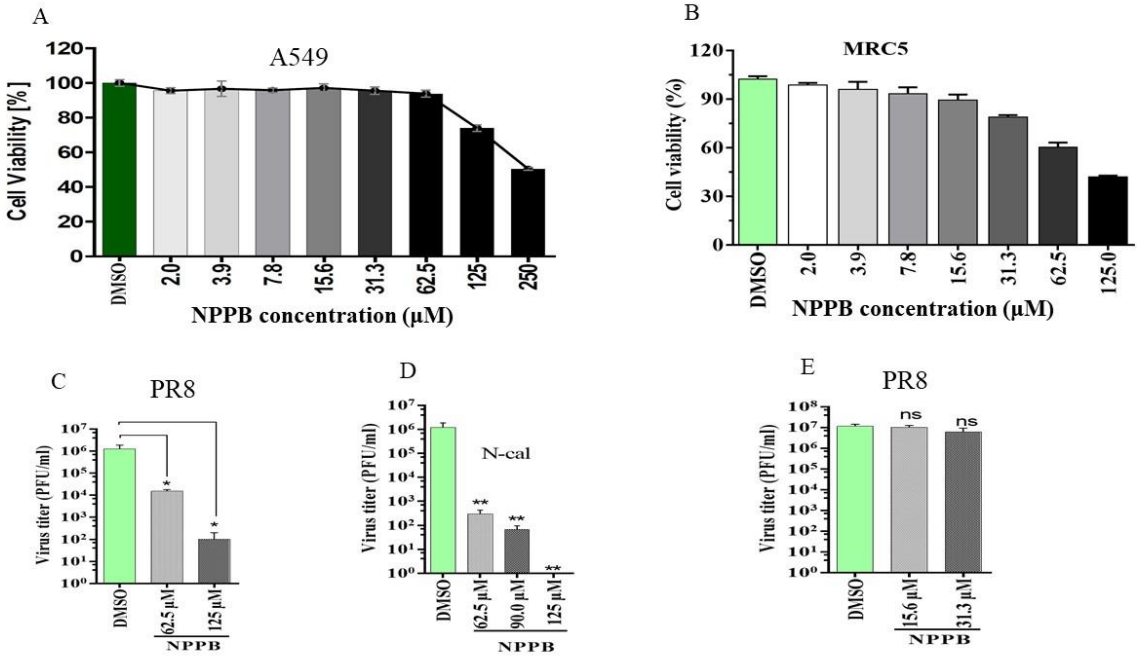


Figure 24: NPPB suppresses the replication of IAV virus. The cytotoxicity of NPPB was determined by treating **A.** A549 and **B.** MRC5 cells with different concentrations of the drug and cell viability was measured by WST-1 assay. To determine the impact of the NPPB drug on the virus replication, A549 cells were pre-treated with the drug and infected with MOI 0.01 IAV PR8 and N-Cal strains. The drug was also added to the overlay media after infection. PR8 and N-Cal was collected at 45 hours post-infection. Impact of NPPB on (**C**) PR8 and (**D**) N-cal replication in A549 cell. **E.** Impact of NPPB on PR8 replication in MRC5 cells. The light or dark green color bar represents the DMSO control, and the lower to higher darker grayscale gradient bars represent the low to a high concentration of NPPB drugs. *: P <0.05, **: P < 0.01, ***: P < 0.001.

3.3.3 Impact of CLIC1 KD on viral protein and vRNA expression.

Since IAV progeny virus production was significantly reduced in CLIC1 KD cells, I investigated the specific step(s) in virus replication affected by CLIC1 KD. First, I assessed the impact on viral protein translation. CLIC1 KD and NSC A549 cells were infected with PR8 at MOI 3. The infected cells were harvested at 12, 24, 36 and 48hpi. IAV NS1 and NP proteins were detected by Western blot from cell lysates (Figure 25A). A significant reduction of CLIC1 expression was confirmed by Western blot, which did not impact viral protein synthesis (Figure 25C,D). I next tested the impact of CLIC1 KD on the transcription of viral RNAs (vRNA) by qRT-PCR. CLIC1 KD and NSC cells were infected at MOI 3 and RNA was extracted from the cells at 24hpi. cDNA was prepared by reverse transcription and qPCR was performed targeting NS1, NP and HA vRNAs. CLIC1 KD caused a significantly higher level of all three targeted vRNA transcripts (Figure 25E). To further assess the impact of CLIC1 KD on the localization of viral proteins, CLIC1 KD and NSC cells were infected with MOI 3, and at 24hpi, cells were fixed. Immune fluorescent microscopy was done targeting IAV NS1 protein in CLIC1 KD cells. Interestingly the NS1 protein intensity was significantly low after IAV infection in CLIC1 KD cells compared to the non-silencing control cells (Figure 25F).

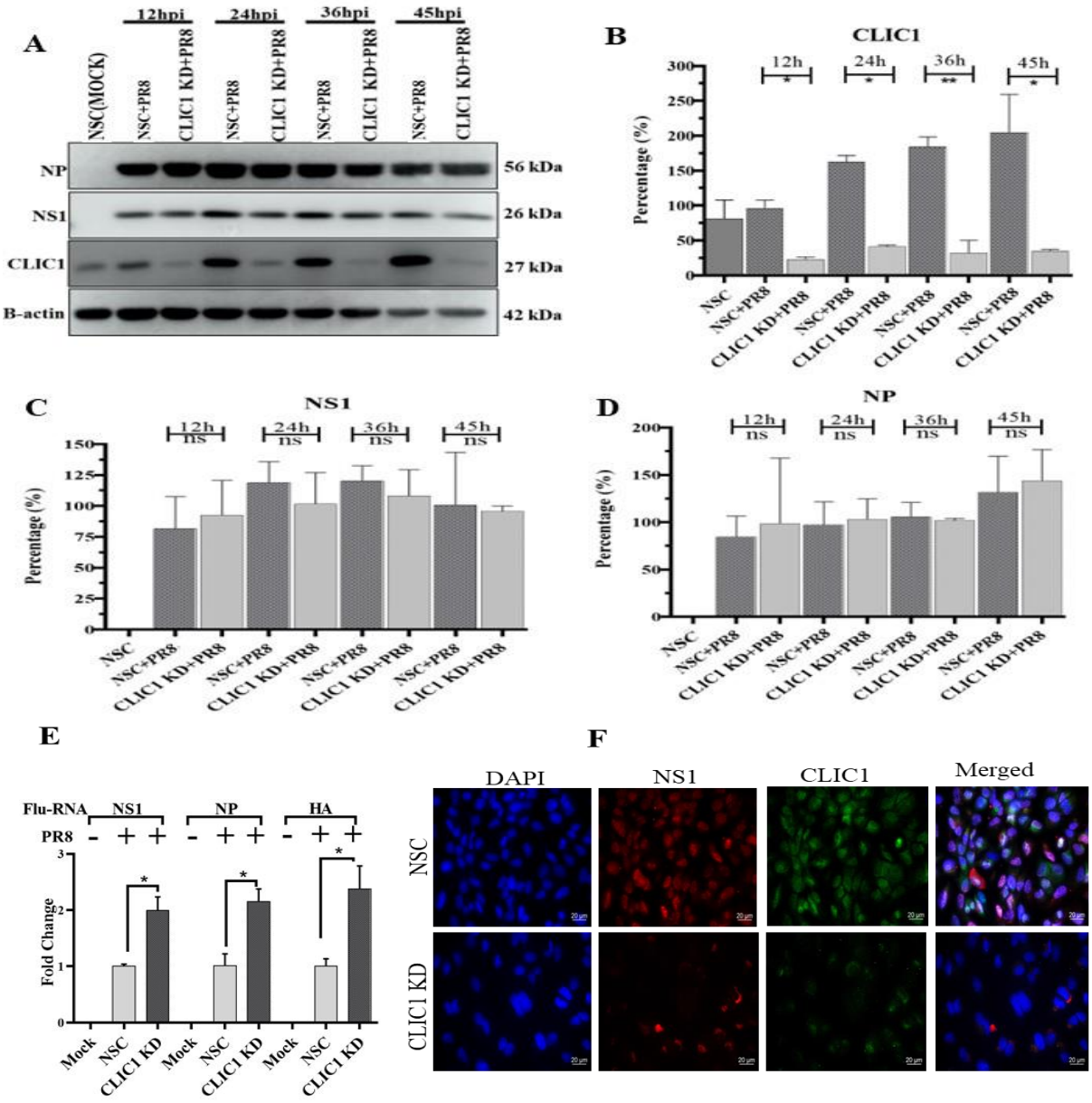


Figure 25. Impact of CLIC1 KD on the levels of IAV viral proteins and RNAs. A549 cells were treated with either NSC or CLIC1 siRNA (CLIC1 KD) for 48 hours and infected with IAV-PR8, at MOI 3. Cell lysates were collected at 12, 24,36 and 48hpi from PR8 infected cells for analyzing the expression of viral proteins by western blot. After 24hpi cells were fixed on slides to measure viral protein localization by IF microscopy. Viral RNAs were collected at 24hpi and the comparative vRNA transcripts were determined by qRT-PCR. **A.** Expression of IAV-PR8 NP and NS1 proteins in CLIC1 cells after 12,24,36 and 48 hpi. **B.** Quantitative densitometry analysis of Western blot images to determine B. CLIC1 expression. **C.** IAV-NS1 protein expression. **D.** Flu-NP protein expression. **E.** IAV-NS1, NP and HA vRNA transcripts in CLIC1 KD cells compared to mock-infected and NSC control. **F.** IF images showing the expression of NS1 protein in IAV infected CLIC1 KD cells. h= hours. *: P<0.05, **:P<0.1, ***:P<0.001.

3.4 HSPA5 is required for IAV replication.

3.4.1 Impact of HSPA5 KD on IAV replication

I first investigated the effect of HSPA5 KD on progeny virus replication. A549 cells were transfected with either NSC siRNA or HSPA5 siRNA for 48hours and infected with IAV PR8 strain. The supernatant was collected at different time points up to 45hpi. The progeny virus titer was determined by plaque assay. HSPA5 KD caused a significant reduction of progeny viruses in the supernatant at 45hpi (Figure 26A). HSPA5 KD did not cause any significant impact on cell viability (Figure 26B). The virus titer in the supernatant was normalized to cell viability; this indicated about 95% reduction of virus titer from HSPA5 KD cells (Figure 26C). The impact of HSPA5 KD was not restricted only to the PR8 strain but also caused a significant reduction in pdm-09 and WSN virus replication (Figure 26D).

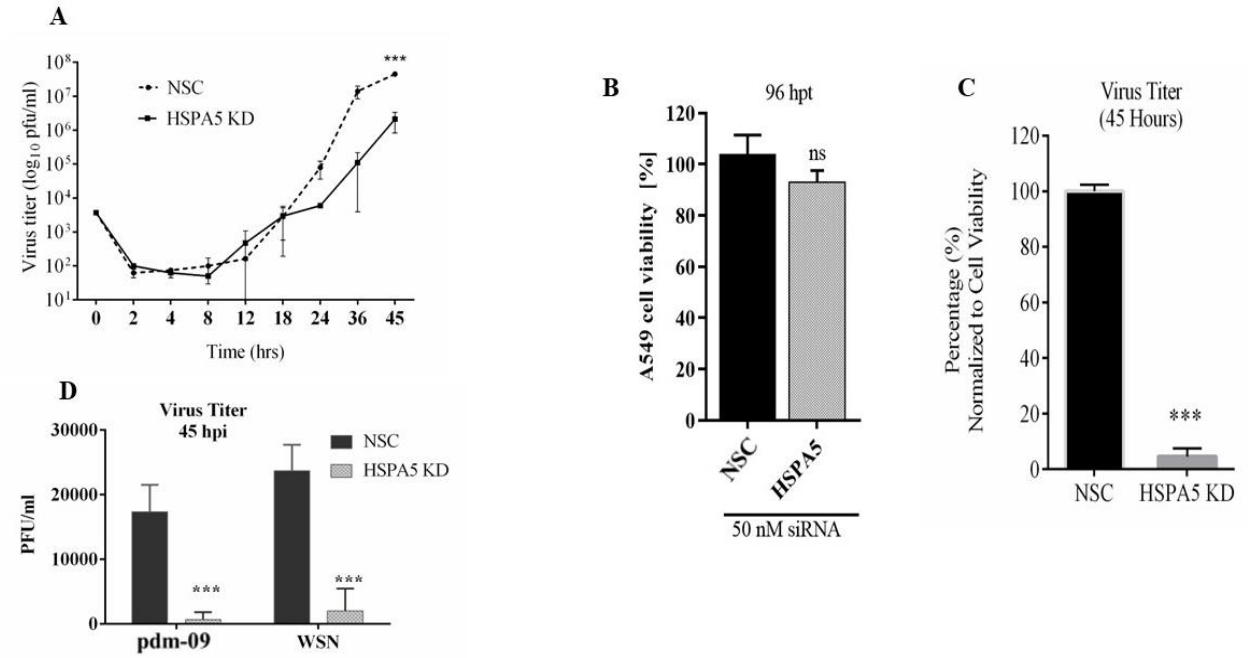
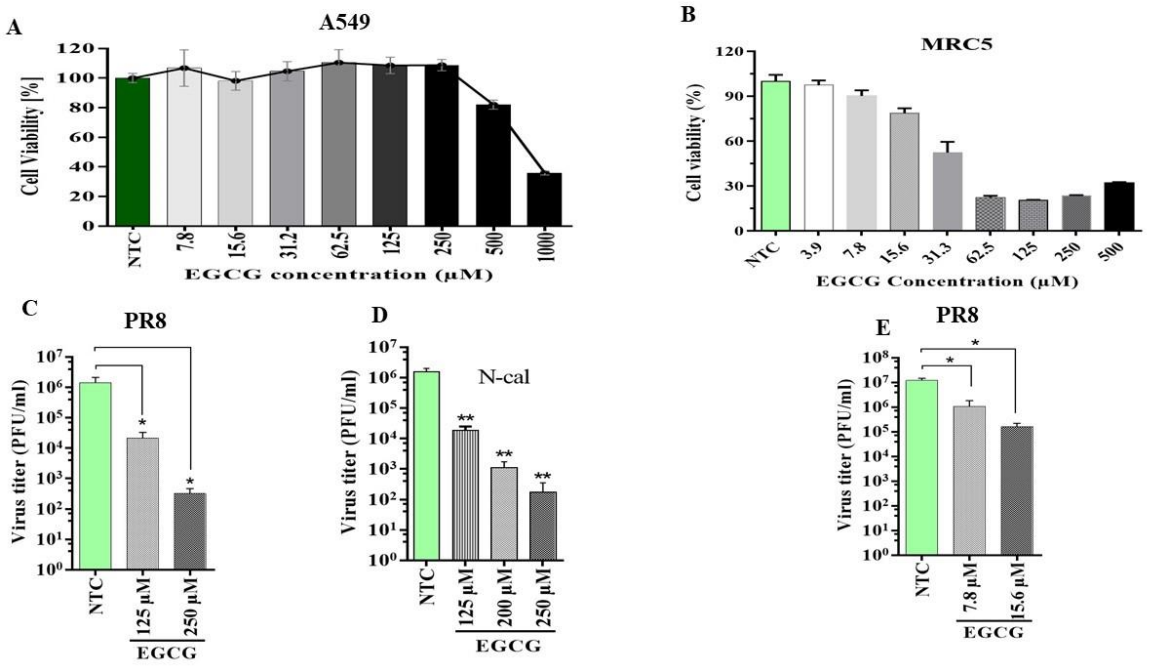


Figure 26. HSPA5 is required for replication of IAV virus. A549 cells were treated with either NSC or HSPA5 siRNA (HSPA5 KD) for 48 hours and infected with IAV-PR8 MOI 0.01. Supernatant from the infected cells was collected at 0,2,4,8,12,18,24,36 and 45hpi. Similarly, NSC and HSPA5 KD cells were infected with pdm-09 and WSN strains and supernatant was collected at 45hpi. Virus titer was determined by plaque assay. **A.** IAV (PR8 strain) titer in the Supernatant of HSPA5 KD cells compared to NSC over time. **B.** Viability of cells measured my WST-1 assay after 96 hours post siRNA transfection. **C.** Percentage of virus titre in HSPA5 KD cell supernatant at 45hpi compared to control and normalized with cell viability. D. Impact of HSPA5 KD on pdm-09 and WSN strains of IAV. *: P<0.05, **: P < 0.01, ***: P< 0.001.

3.4.2 Epigallocatechin-3-gallate (EGCG), a HSPA5 inhibitor, suppresses the replication of IAV virus.

HSPA5 knockdown had a significant impact on IAV replication, indicating that HSPA5 may be a critical host factor for IAV replication. As a result, I wanted to see if an HSPA5 inhibitor (EGCG) could affect IAV replication. The toxicity of various drug concentrations was tested in A549 (Figure 27A) and MRC5 (Figure 27B) cells. To determine the impact of the drug on IAV replication, 125, 200, and 250 μM were used in A549 cells and 7.8 and 15.6 μM in MRC5 cells, respectively, based on the drug's cytotoxicity. In A549 cells, EGCG significantly reduced the replication of the PR8 (Figure 27C) and N-Cal (Figure 27D) strains of IAV. However, the lower EGCG concentrations had a significant effect on PR8 replication in MRC5 cells (Figure 27E).

Figure 27: EGCG a HSPA5 inhibitor suppresses the replication of IAV virus.



A. and **B.** The cytotoxicity of EGCG was determined by treating (**A)** A549 and (**B)** MRC5 cells with different concentrations of the drug and cell viability was measured by WST-1 assay. To determine the impact of the EGCG drug on the virus replication, A549 cells were pre-treated with the drug and infected with MOI 0.01 PR8 and N-Cal strains. The drug was also added to the overlay media after infection. PR8 and N-cal was collected at 45 hpi. Impact of EGCG on **C.** PR8 and **D**. N-cal replication in A549 cell. **E.** Impact of EGCG on PR8 replication in MRC5 cells. The light or dark green color bar represents the NTC control, and the lower to higher darker grayscale gradient bars represent the low to a high concentration of EGCG drugs. *: P<0.05, **: P < 0.01, ***: P < 0.001.

3.4.3 Impact of HSPA5 KD on IAV viral protein translation and vRNAs transcription.

To assess the impact of HSPA5 KD on viral protein translation, HSPA5 KD and NSC A549 cells were infected with PR8 at MOI 3. The infected cells were harvested at 12, 24, 36 and 48 hpi. IAV NS1 and NP proteins were detected by Western blot from cell lysates (Figure 28A). Western blot confirmed a significant reduction of HSPA5 expression (Figure 28B). The NS1 expression was significantly reduced at 12hpi, whereas at 36hpi, the NP expression was significantly higher in HSPA5 KD cells compared to the control (Figure 28C,D). Next, the impact of HSPA5 KD on the transcription of vRNA was tested by qRT-PCR. HSPA5 KD and NSC cells were infected at MOI 3 and cells were harvested at 24hpi for RNA extraction. cDNA was prepared by reverse transcription and qPCR was performed targeting NS1, NP and HA vRNAs. HSPA5KD did not significantly impact the transcription of any of the three vRNA transcripts tested in this study (Figure 28E). To further assess the impact of HSPA5 KD on the localization of viral proteins, HSPA5KD and NSC cells were infected at MOI 3 and fixed after 24 hpi. Immune fluorescent microscopy was done targeting IAV NP protein in HSPA5 KD cells. NP protein intensity was significantly higher in HSPA5 KD and compared to the control after IAV infection (Figure 28F).

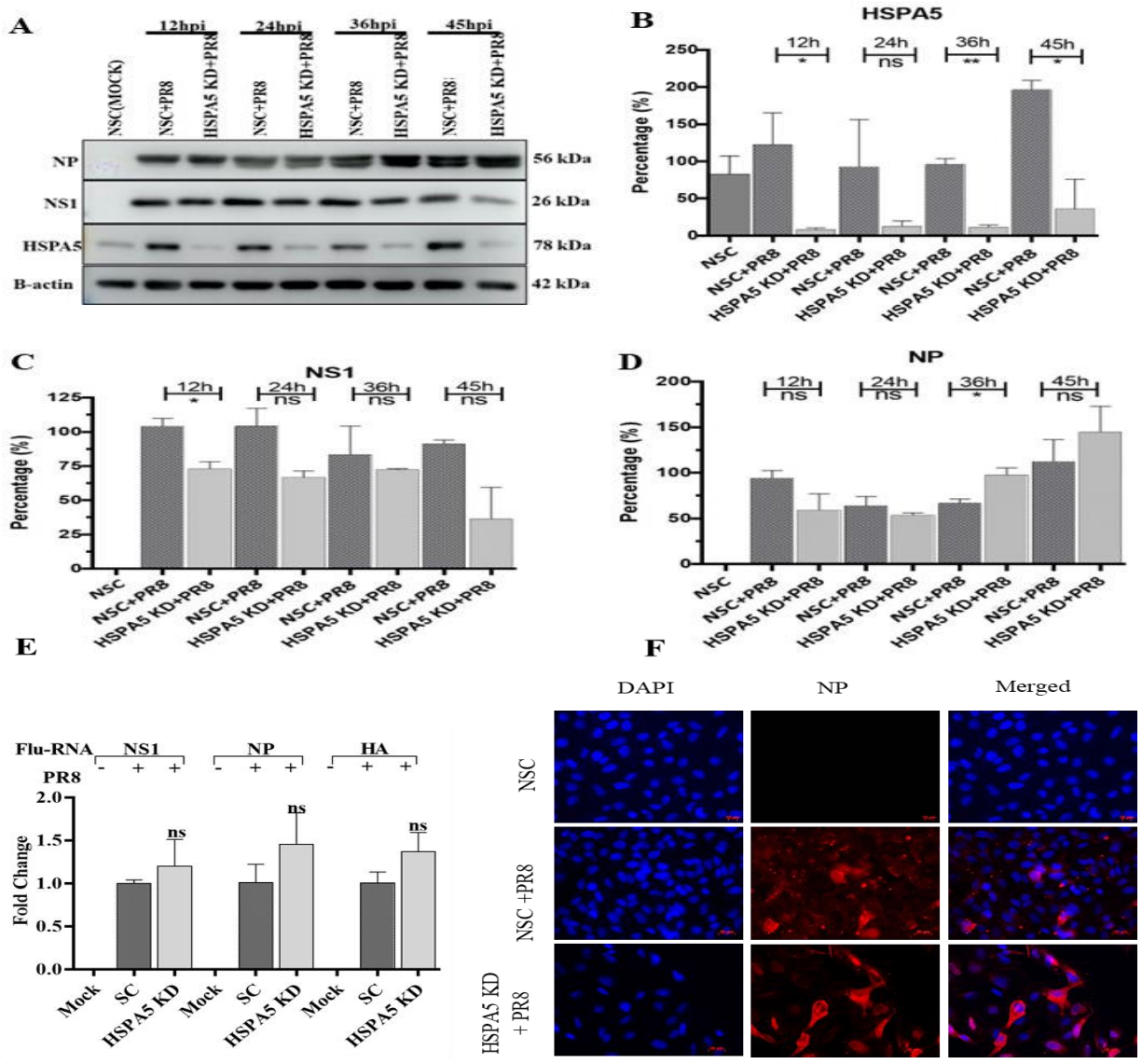


Figure 28. Impact of HSPA5 KD on IAV viral protein translation and vRNAs transcription. A549 cells were treated with either scrambled siRNA (NSC) or HSPA5 siRNA (HSPA5 KD) for 48 hours and infected with IAV-PR8, at MOI 3. Cell lysates were collected at 12, 24,36, and 48 hpi from PR8 infected cells for analyzing the expression of viral proteins by western blot. After 24hpi cells were fixed on slides to measure viral protein localization by IF microscopy. Viral RNAs were collected at 24hpi and the comparative vRNA transcripts were determined by qRT-PCR. **A.** Expression of IAV-PR8 NP and NS1 proteins in HSPA5 cells after 12, 24, 36 and 48hpi. **B.** Quantitative densitometry analysis of Western blot images to determine B. HSPA5 expression. **C.** IAV-NS1 protein expression. **D.** Flu-NP protein expression. **E.** IAV-NS1, NP and HA vRNA transcripts in HSPA5 KD cells compared to mock infected and NSC control. **F.** IF images showing the expression of NS1 protein in IAV infected HSPA5 KD cells. *: P<0.05, **:P<0.1, ***:P<0.001.

CHAPTER 4. DISCUSSION

IAV utilizes FN-1 for virus attachment via 2,6-sialic acid-binding and entry into human lung epithelial cells (Leung et al., 2012). However, different pathogenic strains of IAV were found to affect FN-1 interacting proteins expression significantly (Simon et al., 2015). PSMA2, CLIC1, and HSPA5 are three FN-1 interacting proteins whose expressions were upregulated by high pathogenic IAV (Simon et al., 2015). A preliminary siRNA screening experiment has shown that knockdown of these proteins significantly reduces the replication of IAV (Figure 8), indicating they might be critical for the viral replication cycle. Initially, the expression of these proteins were knocked down by siRNA treatment to understand the role of these proteins in the IAV replication cycle. Thus, I had to optimize the conditions and concentrations for siRNA treatment. During this process, I observed that Opti-MEM, a common serum-reduced media used for genetic material transfection, can impact the cell morphology, cell viability and expression of PSMA2, CLIC1 and HSPA5.

4.1 Serum-reduced media impacts cell viability and protein expression in human lung epithelial cells.

Fetal bovine serum (FBS) is one of the most commonly used supplements in eukaryotic cell culture media, but as a complex natural product, its composition is poorly defined and may vary between lots from the same manufacturer (Zheng et al., 2006). Maintaining consistent cell growth conditions is often very difficult in FBS-containing media and can lead to inconsistent or opposing results during bioassays (Krämer et al., 2005; Mannello & Tonti, 2007). Thus, serum is often eliminated from the media to remove the unknown factors to reduce analytical interference and provides more reproducible experimental conditions (Colzani et al., 2009; Lambert & Pirt, 1979; Mbeunkui et al., 2006).

However, serum starvation has been used as a tool for molecular mechanism studies, like autophagy, apoptosis (Bhutia et al., 2010; Terra et al., 2011; Zhao et al., 2010), cellular stress response (Arrington & Schnellmann, 2008; Levin et al., 2010) etc. Although serum starvation has been performed in hundreds of research studies, the impact of the condition is not well understood (Pirkmajer & Chibalin, 2011).

In this study, I have discovered that serum-reduced media (Opti-MEM) can adversely impact A549 cell viability and expression of PSMA2, CLIC1 and HSPA5 proteins (Figure 11).

Transfection of A549 cells with PSMA2 and CLIC1 siRNAs was more efficient in DMEM (with 10% FBS) than in Opti-MEM media. Successful knockdown of CLIC1 and PSMA2 were achieved in DMEM media with better cell viability and less impact on cell morphology than in Opti-MEM media (Figure 12, Figure 13).

However, understanding the cellular responses impacted by serum starvation is critical for data interpretation and reproducibility. Many cellular signaling pathways, including EGFR-MAPK-Stat, PTEN-PI-3 ERK1/2, phospho-ACC, and Akt signaling, have been demonstrated to be dysregulated by FBS starvation in glioma and adenocarcinoma cell lines (Levin et al., 2010; Pirkmajer & Chibalin, 2011). Similar to what I observed in the A549 cell in this study, the expression of GAPDH was also found unstable after serum starvation in primary human myotubes and HEK293 cells (Pirkmajer & Chibalin, 2011). GAPDH was also found as a non-reliable reference marker in a colitis mouse model (Eissa et al., 2017). Therefore, careful consideration of the impact of the media or the experimental condition is crucial for choosing the appropriate loading control. Serum starvation dysregulates different cellular signaling pathways, which might affect the expression of different cellular proteins. A proteomic study of serum-starved cells by mass spectrometry might help us to get a deeper understanding of the impact on cell signaling pathways and cellular functions.

An appropriate experimental condition is critical for data reproducibility and the undesirable impact of the media may lead to misinterpretation of the data. A549 cells were stressed, and cellular protein expression was destabilized after growing in the Opti-MEM. Consideration of these facts is necessary while using Opti-MEM as a culture or transfection medium. While Opti-MEM medium may still be used for dilution of siRNA and transfection reagents, DMEM medium with all nutrients and FBS is a preferable choice for transfection experiments in A549 cells. However, since this study evaluated only three proteins and one cell type, it is not possible to comment on the effects on other cellular proteins or cell types.

4.2 Optimization of PSMA2, CLIC1 and HSPA5 knockdown by siRNA treatment

The SP siRNAs contain a mixture of 4 siRNAs targeting a single gene and are recommended by the suppliers because of their higher potency over OT siRNA**.** However, using too much siRNA poses the risk of inducing off-target effects (Neumeier & Meister, 2021). Thus, I tested the efficacy and cytotoxicity of the SP and OT siRNAs for knocking down each of the

three target proteins (PSMA2, CLIC1 and HSPA5). The OT3 siRNA targeting PSMA2 reduced the cell viability and impacted cell morphology (Figure 14). The cytotoxic effect of the PSMA2 OT3 siRNA could have off-target effects on A549 cells. Thus, I removed the OT3 siRNA from the PSMA2 SP-mixture and used it in subsequent experiments. PSMA2 is a critical structural subunit of the cellular 20S proteasome, which is involved in a wide range of cellular processes (Tanaka, 2009). Knocking down PSMA2 expression may adversely impact normal cellular functions. Thus, the impact of PSMA2 KD needs to be carefully monitored, and minimal siRNA concentration and shorter treatment time should be considered for knockdown PSMA2. Interestingly, all of the OT and SP siRNAs targeting CLIC1 (Figure 15) and HSPA5 (Figure 16) successfully KD the proteins without impacting cell viability or cell morphology. However, in this study, I only evaluated the impact of siRNA knockdown of PSMA2, CLIC1 and HSPA5 based on cell viability and morphology. Proteomic analysis of the cell lysates after each target protein's knockdown may help us understand the impact more clearly.

4.3 Influenza A virus utilizes PSMA2 for downregulation of NRF2-mediated oxidative stress response.

4.3.1 PSMA2 knockdown alters IAV-mediated host proteomic responses

By proteomic analysis, I found that transforming growth factor beta-induced (TGFBI), Fas cell surface death receptor (FAS), plasminogen activator urokinase (PLAU) and cathepsin B (CTSB) proteins were significantly upregulated by PR8 infection, but were regulated somewhat in the opposite direction in PSMA2 KD cells during IAV infection. TGFBI is involved in cell movement and transformation, but its role in viral replication is not well understood (Ween et al., 2012). In contrast, FAS (Fujimoto et al., 1998; Takizawa et al., 1995), PLAU(Ali et al., 2021) and CTSB (Coleman et al., 2018) were previously identified as critical factors for IAV replication. The interaction of PSMA2 with FAS, PLAU and CTSB proteins in IAV needs to be investigated. However, lipocalin 2 (LCN2), cyclin-dependent kinase 2 (CDK2) and cyclin A2 (CCNA2) were significantly upregulated, and tissue factor pathway inhibitor (TFP1), growth factor receptor-bound protein 2 (GRB2) and cyclin-dependent kinase inhibitor 1B (CDKN1B) were significantly downregulated in PSMA2 KD+PR8 infection, but oppositely regulated and not significant in PR8 infection. LCN2 is a key regulator of inflammation during mycobacterial infection (Guglani et al.,

2012) and deactivates macrophages (Warszawska et al., 2013) during viral infection. CDK2 and CCNA2 play vital roles in regulating the eukaryotic cell division cycle (Bártová et al., 2004; Pagano et al., 1992) but IAV tries to arrest the cell cycle during infection (Jiang et al., 2013). GRB2 and CDKN1B are also involved in regulating cell proliferation and cell cycle control (Frelin et al., 2017; Kiyokawa et al., 1996). Thus, further study is necessary to understand the role of PSMA2 in inflammation and cell cycle regulation during infection.

IPA analysis also showed that phosphorylation of L-tyrosine was significantly downregulated by PR8 infection but not reflected during PSMA2 KD+PR8 infection (Figure 16E). Regulation of viral protein phosphorylation is critical for viral replication (Cui et al., 2019; Dawson et al., 2020; Kamata & Watanabe, 1977; Patil et al., 2021) and activation of cellular signaling pathways (Ardito et al., 2017). In addition, canonical pathway analysis showed that FMLP signaling in neutrophils, PKC signaling in T lymphocytes and FCy RIIB signaling in B lymphocytes were downregulated by PR8 infection but upregulated by PSMA2 KD, resulting in no impact during PSMA2 KD+PR8 infection (Figure 16C). One limitation of this study is that it was performed in an in-vitro setting using a transformed lung epithelial cell line. Further investigation is necessary to understand the role of PSMA2 in the regulation of immune cell differentiation and activation of signaling pathways in immune cells during IAV infection using an in-vivo experimental model.

4.3.2 PSMA2 promotes IAV maturation

PSMA2 is one of the critical alpha subunits of the 20S proteasome that builds the substrate entrance gate. The 20S proteasome is an essential component of the 26S proteasome. Both of these proteasomes are pivotal components in the UPS and are mainly involved in cellular proteolytic modification and recycling of defective proteins (Kleiger & Mayor, 2014). During a viral infection, the UPS works as a double-edged sword. Mouse minute virus (Ros et al., 2002; Ros & Kempf, 2004) and murine coronavirus (Yu & Lai, 2005) take the help of UPS to enter into the host cells, where as HIV needs to release from cytoplasm (Patnaik et al., 2000; Schubert et al., 2000; Strack et al., 2000). However, host cells may use it to eliminate the virus (Luo, 2016). The proteasome activity was found important for replication of different viruses including entry of herpes simplex virus (Delboy et al., 2008), budding of rhabdoviruses (Harty et al., 2001), DNA replication of vaccinia virus (Satheshkumar et al., 2009), genome replication of West Nile virus (Gilfoy et al., 2009), and RNA replication and protein synthesis of coxsackievirus (Si et al., 2008). A previous

study showed that protease inhibitors can affect the entry of the IAV WSN strain (Khor et al., 2003). Although PSMA2 knockdown caused a significant reduction in proteasomal activity, it did not affect the viral entry of IAV PR8 virus in our study.

PSMA2 KD caused a significant reduction of infectious IAV viruses in the supernatant compared to non-KD control (Figure 17A). The translation of viral protein and transcription of vRNAs did not appear to be affected by PSMA2 KD (Figure 18A,D,E). This indicates that earlier steps in the IAV replication cycle, e.g., attachment, endocytosis, fusion, nuclear transport and mRNA synthesis, were also unaffected. Although viral protein expression was not affected by PSMA2 KD, I observed higher NP protein abundance inside the PSMA2 KD cells (Figure 18H). In summary, these data suggest that PSMA2 is involved in the maturation steps of IAV replication (Figure 29I). As a component of the proteasome, PSMA2 is involved in the processing and modification of cellular proteins; thus, it might be used by IAV for processing viral proteins and is required for virus particle assembly or release.

Previous studies have shown that the expression of PSMA2 was upregulated in A549 cells after infection with highly pathogenic IAV strains (Simon et al., 2015), or in primary bronchial airway epithelial cells by PR8 infection (Kroeker et al., 2013). However, in this study, I detected a significantly lower expression of PSMA2 in A549 cells after PR8 infection (Table 2). PR8 is a lab-adapted IAV strain and may induce different impact on celllar proteome than highly pathogenic IAV strains in the A549 cells and may also have a dissimilar reaction in different cells. Another study by Shahiduzzaman et al., has shown that IAV infection enhanced the activity profile of PSMA2 (Shahiduzzaman et al., 2014). However, KD of PSMA2 can cause a significant reduction in 20S proteasome activity (Figure 21D). Thus, it appears proteasomal activity is critical for IAV assembly or maturation. Further study is necessary to understand the role of 20S/26S proteosome in IAV assembly or maturation.

4.3.3 PSMA2 knockdown affects NRF2-mediated oxidative stress

Nuclear factor erythroid 2p45-related factor 2 (NRF2) is an antioxidative transcription factor. In normal conditions, it is bound with Kelch-like ECH-associated protein 1 (KEAP1) and cullin-3-based E3 ubiquitin ligase (CUL3) (Kobayashi et al., 2009). The NRF2/KEAP1 protein

complex gets ubiquitinated frequently, is degraded by the proteasome (Itoh et al., 1999) and turns off the activation of NRF2-mediated oxidative response (Figure 29II. A).

A wide range of virus infections can induce strong oxidative stress (Narayanan et al., 2014; Soucy-Faulkner et al., 2010; Strengert et al., 2014) and many of them can activate the Nrf2 pathway (Kosmider et al., 2012; J. Lee et al., 2013). Hepatitis C virus, Hepatitis B virus, and Respiratory syncytial virus can inactivate the pathway (Carvajal-Yepes et al., 2011; Komaravelli et al., 2017; Peiffer et al., 2015). Interestingly, HIV, DENV and HCMV virus induces oxidative stress to facilitate its replication (Cheng et al., 2016; Isaguliants et al., 2013; J. Lee et al., 2013; Reddy et al., 2012). However, oxidative stress during infection can activate antiviral signaling pathways (Hagen et al., 1994; Rehermann & Nascimbeni, 2005), innate immunity to inactivate the pathogen (Day & Suzuki, 2005; Kim et al., 2013; Winterbourn & Hampton, 2008). On the other hand, a high level of reactive oxygen species (ROS) can damage the cell (Schieber & Chandel, 2014). Thus, in response to ROS the cell activates the NRF-2 mediated signaling pathway, which activates the transcription of antioxidative molecules for its own protection (Kosmider et al., 2012; Lee, 2018). Therefore, an appropriate balance of oxidative response is critical for the successful completion of viral replication, preservation of cell damage or killing the pathogens (Choi & Ou, 2006; Fukuyama & Kawaoka, 2011; Hosakote et al., 2011).

IAV can induce oxidative stress, and a higher level of ROS acts on the NRF2-KEAP1 complex to activate the NRF2-mediated oxidative response pathway (Kosmider et al., 2012). NRF2 translocates to the nucleus and forms a complex with Maf and other co-activator proteins. The complex binds to the promoter of antioxidant response elements (AREs) and activates the transcription of antioxidant and cytoprotective proteins such as oxygenase-1 (HO-1), catalase (CAT), and superoxide dismutase (SOD) (Lee, 2018) (Fig. 29II. B). The antioxidant proteins translocate to the cytoplasm and reduce the ROS level to protect the cell from ROS-mediated cell injury (Lee, 2018). Interestingly, IPA analysis predicted PR8 infection significantly inactivated the NRF2-mediated oxidative response pathway (Fig. 20B1), but the proteomic data was collected at 24hpi. The oxidative response pathway activates just after virus entry, and by 24hpi the ROS level may have already been reduced by the expression of antioxidative molecules. However, a significant reduction of ROS levels (Figure 21D) and NRF-2 nuclear translocation (Figure 22A, B) indicates that NRF2-mediated oxidative response pathway was activated by the IAV infection.

IAV infection of PSMA2 KD cells causes an increase in ROS level and subsequent dissociation of the NRF2-KEAP1 complex. However, PSMA2 KD caused a significant reduction in 20S proteasome activity. Inactivation of proteasome activity may have induced an accumulation of NRF2 in the cytoplasm. Thus, it could not activate the transcription of AREs, which may have resulted in the accumulation of a higher level of intracellular ROS. However, treatment with a ROS scavenger could reverse the impact of PSMA2 KD on IAV replication (Figure 21E,F). Which clearly indicates that the accumulated ROS can inactivate the virus directly or by activation of antiviral responses (Figure 28II. C). IPA could not predict any significant activation of the NRF2-mediated oxidative response pathway by IAV infection in PSMA2 KD cells (Figure 20B3), but only PSMA2 KD caused significant activation of the pathway (Figure 20B2). By IF microscopy, I observed that NRF2 nuclear translocation was affected by PSMA2 KD (Figure 22A,B). Therefore, PSMA2 or proteasomal activity is necessary for nuclear translocation of NRF-2 and activation of the pathway, but the mechanism is still not clearly understood. PSMA2 KD caused higher ROS accumulation in the cells (Figure 21A,B), which may have pushed the pathway towards activation and was reflected in proteomic changes detected by SOMAScan. ROS plays a critical role during viral pathogenesis, as it inactivates the virus by direct killing or can induce antiviral responses. IAV requires the help of PSMA2 to activate NRF2-mediated oxidative response to escape ROS-mediated virus inactivation. Thus, I need to have a clear understanding of the role of PSMA2 in balancing the ROS-mediated antiviral response, which may aid in the development of an effective antiviral drug to combat IAV.

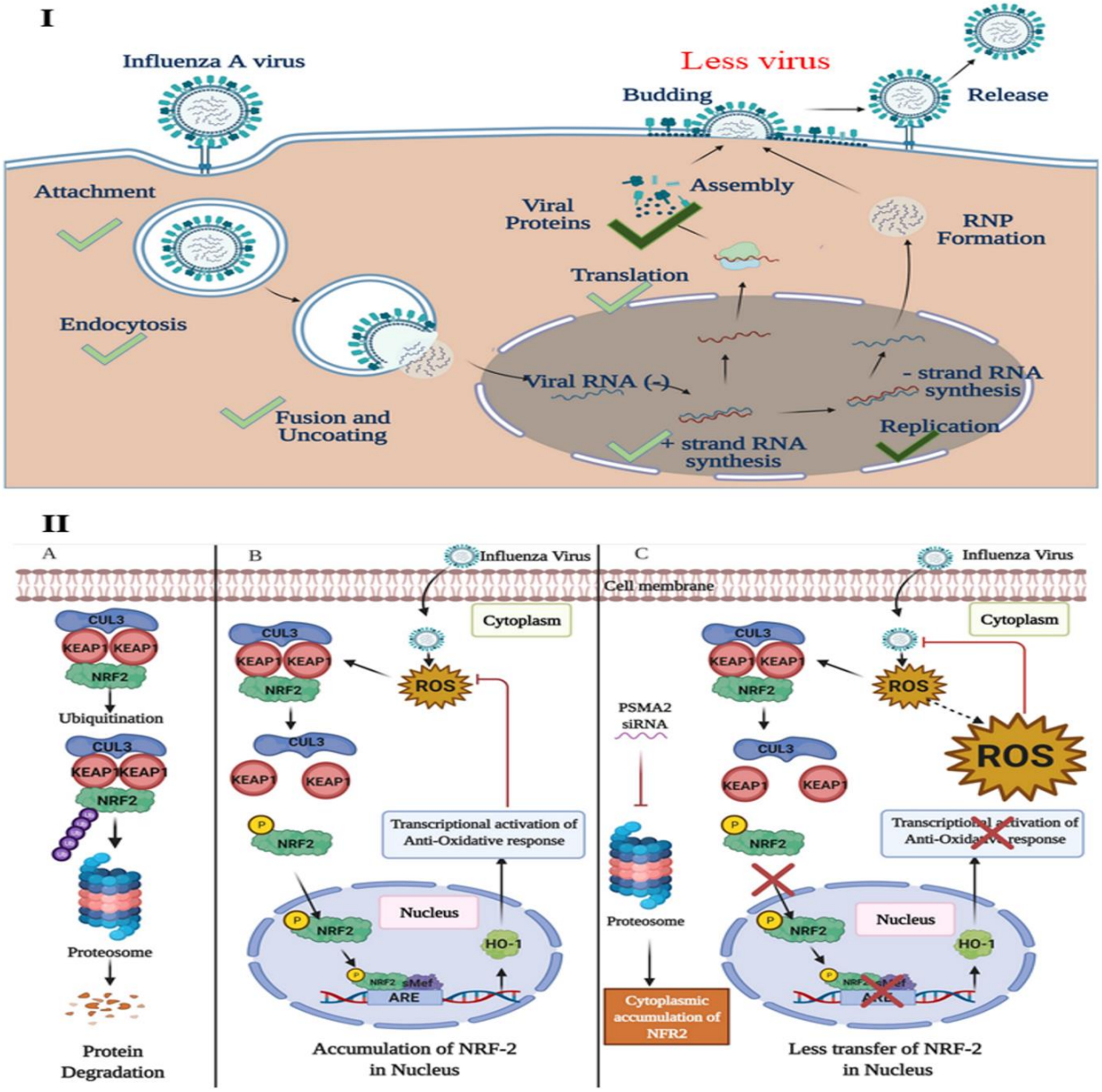


Figure 29: Proposed model showing the role of PSMA2 in IAV replication cycle and NRF2-mediated oxidative response pathway during IAV infection in human lung epithelial cells. I. The translation of viral proteins and transcription of vRNAs were not affected in PSMA2 KD cells during IAV infection. This indicates that earlier steps in the IAV replication cycle (i.e., attachment, entry, nuclear transport, and mRNA synthesis) were unaffected. Significantly fewer progeny viruses were detected in the supernatant of PSMA2 KD cells compared to the control. Furthermore, although viral protein expression was not affected by PSMA2 KD, higher intracellular intensities of NP proteins suggest that PSMA2 is involved in a maturation step of IAV replication. **II.A**. Normally in cells, NRF2 is located in the cytoplasm in a complex form bound with KEAP1 and CUL3. NRF2-KEAP1 complex gets recycled by ubiquitination and frequent degradation by the proteasome. **B.** Viral infection and any other stress condition cause increase of the cellular ROS concentration. The ROS causes the NRF2-KEAP1 complex to dissociate. Then NRF-2 gets

phosphorylated and translocate into the nucleus. In the nucleus, it works as a transcription activator and activates expression of antioxidant proteins. The antioxidant proteins translocate to the cytoplasm and reduce ROS levels to protect the cell from ROS-mediated cell injury. **C.** IAV infection of PSMA2 KD cells causes an increase in ROS levels and subsequent dissociation of the NRF2-KEAP1 complex. But PSMA2 KD causes a significant reduction in 20S proteasome activity. Inactivation of proteasome activity may cause NRF2 accumulation in the cytoplasm and is required for nuclear translocation of the protein. Thus, the transcriptional activation of antioxidant response may not be activated, which may result in a higher level of ROS accumulation in the cells. The ROS may act on the virus and inactivate it. The IAV replication cycle was adapted from (Herold et al., 2015) and modified by https://biorender.com.

4.4 CLIC1 is a critical host cellular protein for the replication of IAV.

CLIC1 is one of the most studied proteins in the Chloride Intracellular Channel (CLIC) family. It exists in two forms: soluble form or a membrane-integrated ion channel (Tulk et al., 2000; Valenzuela et al., 1997). It expresses in a range of cell types and is most prevalent in the skeletal muscle and heart cells (Valenzuela et al., 1997). Overexpression of CLIC1 is associated with different types of cancer cells (Gururaja Rao et al., 2020). As a chloride channel, CLIC1 can transform cells by increasing cell proliferation, migration, and invasiveness (Peretti et al., 2015). Interestingly, Merkel cell polyomavirus (an oncogenic virus) can also induce cell transformation with the help of CLIC1 (Stakaityte et al., 2018). Knockdown of CLIC1 protein has shown a significant reduction of West Nile virus (Krishnan et al., 2008) and vaccinia virus (Sivan et al., 2013). CLIC1 was also highly expressed in A549 cells after infection with high pathogenic IAV (Simon et al., 2015) and siRNA knockdown screening showed a significant reduction of IAV replication after knockdown of CLIC1 expression (Figure 8). The results were further confirmed, and I observed CLIC1 KD impacts the late stage of the replication cycle (Figure 23A).

Overall, the results indicate that CLIC1 might be a critical host factor for IAV replication. Interestingly, the translation of viral proteins was not affected in CLIC1 KD cells during IAV infection (Figure 25A,C,D). This indicates that earlier steps in IAV replication cycle e.g., Attachment, Endocytosis, Fusion, Nuclear transport, and mRNA synthesis, should also be unaffected. A higher amount of vRNAs were detected in the CLIC1 KD cells after PR8 infection (Figure 25E), but a significantly lower number of progeny virus was detected in the supernatant of CLIC1 KD cells compared to control. This suggests that CLIC1 is required for RNP formation or transportation of viral RNAs to the viral particle or final assembly of the virus particle (Figure. 30). Further research is required to fully comprehend the role of CLIC1 in the specific step of IAV

replication. Treatment with 5-nitro-2-(3-phenylpropyl-amino) benzoic acid (NPPB), a chloride channel inhibitor, also significantly reduced PR8 and N-Cal replication in A549 cell (Figure 24). However, in MRC5 cells, lower doses of NPPB did not suppress IAV. It is possible that the lower concentration is ineffective against the IAV or that the effect is cell line-specific. A549 is a cancer cell line, whereas MRC5 is a primary lung cell. The previous study has shown that NPPB can suppress the cancer cell line. Further study is necessary to evaluate the effect of NPPB in other cell lines.

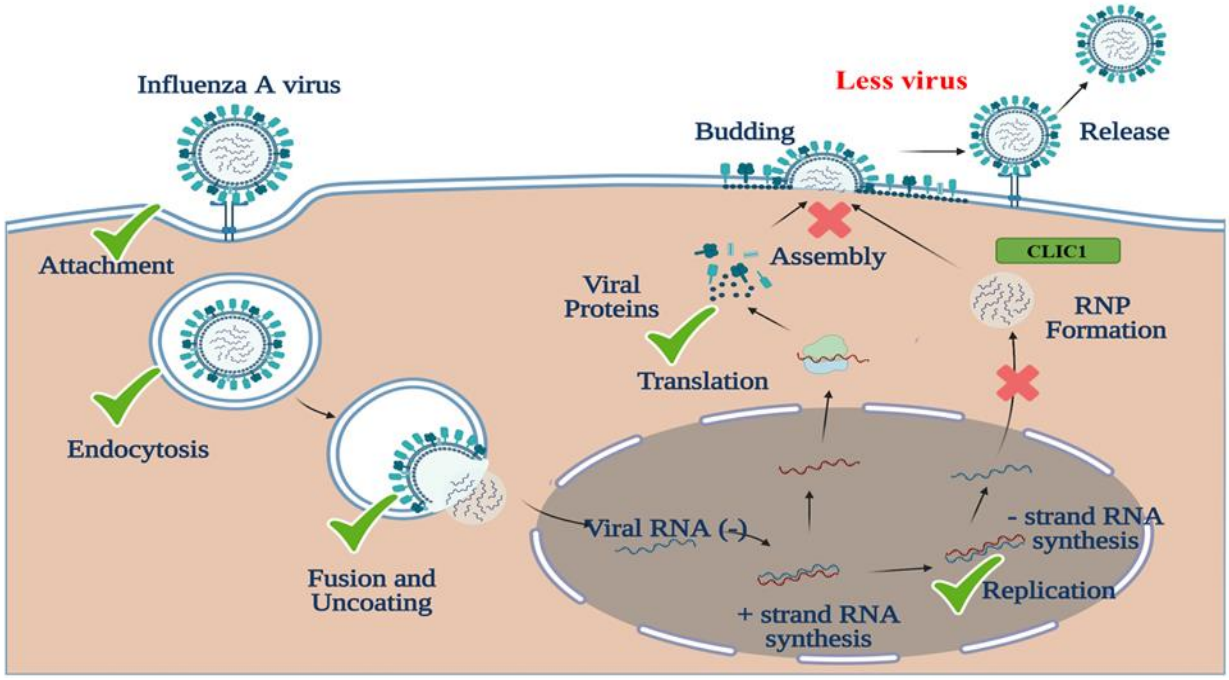


Figure 30. The proposed model represents the contribution of CLIC1 in the IAV replication cycle. The replication step of IAV was unaffected by CLIC1 KD, as demonstrated by the green tick mark. Whereas the red cross denoted the IAV step that might be impaired by CLIC1 KD. The IAV replication cycle was adapted from (Herold et al., 2015) and modified by https://biorender.com.

4.5 HSPA5 protein required for maturation of IAV during replication.

All nucleated cells in humans express the HSPA5 gene. However, nasal epithelial cells have a high level of HSPA5 expression (Cunnea et al., 2003). HSPA5 (also known as BiP) is a chaperone involved in protein folding and maintaining protein quality in the lumen of the endoplasmic reticulum. The HSPA5 interacts with an Endoplasmic Reticulum (ER)-resident protein, called ERdj5, to direct the proper folding of nascent peptides and proteolysis of the

misfolded proteins (Cuevas et al., 2017; Dana et al., 1990; Evensen et al., 2013; Oka et al., 2013). It also acts as a major inhibitor of unfolded protein response (UPR) (Ng et al., 1992; Oikawa et al., 2009), does proteolytic modification of several proteins and transports the secretory proteins from across ER after translational regulators (Macario & De Macario, 2007). HSPA5 may also be involved in cell proliferation and apoptosis (Kang et al., 2015). Recent studies have shown that HSAP5 can physically interact with Zika virus E protein and is a possible host factor for the virus replication (Khongwichit et al., 2021; Turpin et al., 2020). HSPA5 is also a critical protein for entry and replication of Japanese Encephalitis virus (Nain et al., 2017), dengue virus (Jindadamrongwech et al., 2004) and coxsackievirus A9 (Triantafilou et al., 2002). Interestingly, HSPA5 has a binding domain for SARS-CoV-2 spike (Elfiky, 2020) protein and is required for virus entry into the host cells (Carlos et al., 2021). Treatment with epigallocatechin-3-gallate (EGCG), an inhibitor of HSPA5 (a polyphenol from green tea), reduces alpha (HCoV-229E), beta-coronavirus (HCoV-OC43 and SARS-CoV-2) replication in Rhabdomyosarcoma (RD) cells (Carlos et al., 2021; Jang et al., 2021).

In HSPA5 KD cells, a significantly low level of NS1 protein was detected at the early stage of viral replication (Figure 28C). Interestingly, the NP proteins were expressed significantly higher in the later stage of the IAV replication cycle in HSPA5 KD cells (Figure 28D). I also observed higher abundance of NP proteins inside the HSPA5 KD cells by IF microscopy (Figure 28F). The transcription of vRNAs was not affected in HSPA5 KD cells during IAV infection (Figure 28E). This indicates that earlier steps in the IAV replication cycle, e.g., attachment, entry, endocytosis, fusion, nuclear transport, and mRNA synthesis, should also be unaffected. However, I have detected a significantly less number of progeny viruses in the supernatant of HSPA5 KD cells compared to the control. One of the main functions of HSPA5 is folding the protein into appropriate structures. The HSPA5 may be necessary for the proper folding of NP and NS1 protein in IAV replication. The cellular proteasome could have degraded the misfolded/unfolded NS1 proteins, and the NP protein could not correctly assemble into the maturated viral particles (Figure 31). Further investigation is necessary to clearly understand the role of HSPA5 in viral protein folding and maturation.

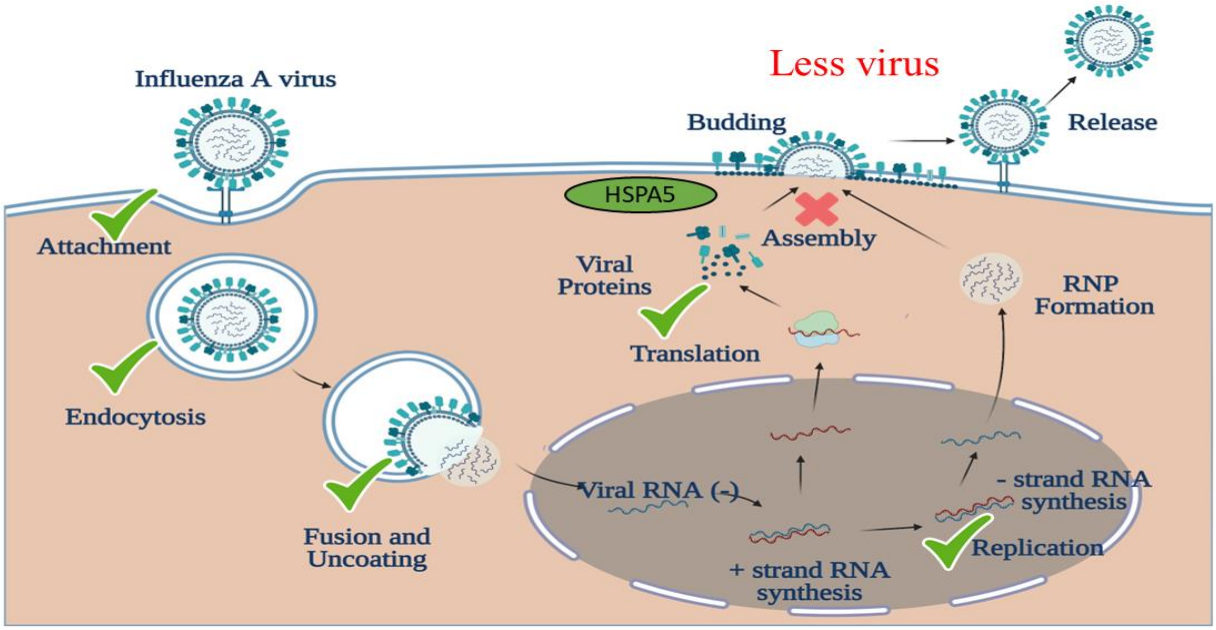


Figure 31: The proposed model represents the contribution of HSPA5 in the IAV replication cycle. The replication step of IAV was unaffected by HSPA5 KD, as demonstrated by the green tick mark. In contrast, the red cross denoted the IAV step that might be impaired by HSPA5 KD. The IAV replication cycle was adapted from (Herold et al., 2015) and modified by https://biorender.com.

4.6 Summary

Interestingly, all three FN-1 interacting proteins investigated in this study are required for the terminal stages of IAV replication. A prior study found that FN-1 is required for IAV-HA binding to sialic acid and entry into the host cell (Leung et al., 2012). Protein-protein interaction analysis using the STRING database revealed that FN-1 interacts directly with CLIC1 and HSPA5, as well as indirectly with PSMA2 (Appendix Figure. 2). The results suggest that the functional activity of PSMA2, CLIC1, and HSPA5 in IAV replication is not necessarily dependent on the fibronectin interaction. Unlike SARS-CoV-2, coxsackievirus A9, or dengue viruses, the entry or attachment of IAV was not affected by HSPA5 (Figure 32). However, in HSPA5 KD cells, the NP proteins are accumulated, indicating HSPA5 is necessary for appropriate folding of viral protein/proteins and their assembly in the virion. HSPA5 may also play an important role in the appropriate folding of IAV-NS1 protein. In the absence of HSPA5, the misfolded or unfolded IAV-NS1 may have been degraded by the cellular proteasome. CLIC1 interacts directly with FN-1 and facilitates the uptake of CTL1-fibronectin co-aggregates into the cells (Knowles et al., 2012). It

suggests that IAV can bind to FN-1 aggregates and be internalized with the help of CLIC1. However, in this study, I did not find any effect of CLIC1 KD on IAV attachment or entry into the infected cells. The higher level of vRNAs in the CLIC1 KD cells suggests that CLIC1 might be involved in the vRNP formation or transport to the assembly sites. HSPA5 interacts with PSMA2 directly and indirectly with CLIC1 through FN-1. More research is needed to determine if these proteins operate together or independently.

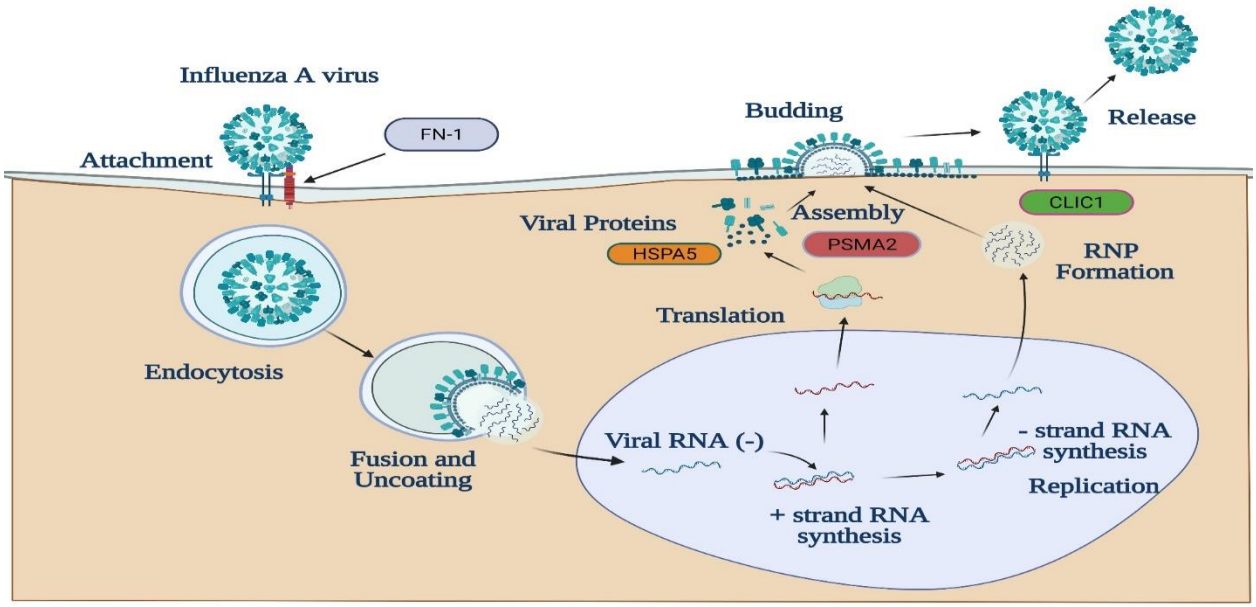


Figure 32. The function of fibronectin (FN-1) interacting proteins (PSMA2, CLIC1, and HSPA5) in the IAV replication cycle. HSPA5 is probably involved in the viral protein folding or assembly. Whereas PSMA2 in the assembly or maturation of the virus. However, CLIC1 is necessary for viral RNP formation or transport to the progeny virion.

4.7 Study Limitations.

One of the main limitations of this study is that I used A549 cells and the PR8 strain of IAV. PR8 is a mouse-adapted laboratory strain of IAV. However, many studies have used PR8, to investigate the infection process and pathophysiology of IAV replication since it is less hazardous and can be worked within a BSL-2 facility. Despite the fact that A549 is a human lung epithelial cancer cell line, I utilized it in this study because it is susceptible to IAV infection and easy to maintain for many passages. I evaluated the proteomic dysregulation in IAV-infected cells and the impact of PSMA2 KD on the host proteome during viral replication using SomaScan methods. SomaScan can detect around 1300 pre-selected proteins from a single sample, though it cannot detect all. A proteomic analysis using mass spectrometry can give us more data for in-depth

investigation. PSMA2, CLIC1 and HSPA5 have been already identified as fibronectin interacting proteins by a previous study (Simon et al., 2015), but my focus was on determining their involvement in IAV replication. As a result, the physical interaction of FN-1 with these proteins was not investigated in this study.

4.8 Conclusions

I have identified PSMA2, CLIC1 and HSPA5 are three critical host dependency factors for IAV replication. In addition, two drugs, EGCG and NPPB have demonstrated potent antiviral properties against IAV. PSMA2 is a crucial component of the proteasome. Recent studies have recommended using proteasome inhibitors as the antiviral therapy against COVID-19 and HIV treatment (Chandel et al., 2020; Ghosh et al., 2016; Sagawa et al., 2020). The proteasome inhibitors could also be a potential drug against IAV replication and require further investigation. This study also identified that CLIC1 is involved in the terminal steps (most probably vRNP transport to the virion, virion assembly, or maturation) of IAV replication. HSPA5 could be a potential host factor for IAV viral protein folding and virion assembly.

Interestingly, CLIC1 and HSPA5 inhibitors have showed potential antiviral efficacy against multiple strains of IAV. In the future, these drugs will need to be studied in animal model. On the other hand, PSMA2, CLIC1, and HSPA5 proteins, can directly interact with hundreds of cellular proteins and involved in several signaling pathways. Unwanted side effects may occur when an antiviral drug targets a host cellular protein. Thus, further study is needed to clearly understand the roles of PSMA2, CLIC1, and HSPA5 in IAV replication, as well as the effect of protein knockdown. A clear knowledge of the mechanism might aid in the development of an antiviral drug that targets a specific interaction required by IAV, hence eliminating undesired side effects.

However, all three proteins were found to have a role in the replication of other viruses; but not all viruses utilize the same protein for the same purpose. Furthermore, each protein in the host cell is engaged in several cellular functions. As a result, developing an antiviral drug targeting a host cellular protein is very challenging. Despite the discovery of hundreds of proteins crucial for IAV replication, there is no antiviral drug targeting host proteins that can completely inhibit

viral replication. On the other hand, identifying a host cellular protein that is a crucial factor for a wide range of viruses opens the possibility to developing a broad-spectrum antiviral drug to battle emerging viral pathogens and overcome viral escape through frequent mutations. So, further research is needed to understand the role of host proteins in viral replication and get prepared to prevent the devastation of next viral pandemic.

4.9 Future Directions

Initially, I optimized the experimental conditions for knocking down PSMA2, CLIC1, and HSPA5 expression in A549 cells using siRNA. During this process, I found that serum-reduced media (Opti-MEM) can affect cell survival and protein expression in A549 cells. However, I only evaluated the effect of Opti-MEM media on the expression of PSMA2, CLIC1, and HSPA5 protein. Still, the impact of serum starvation on cellular proteins or cellular signaling pathways is not clearly understood. On the other hand, I only checked the impact of PSMA2, CLIC1 and HSPA5 on cell viability and cell morphology. A proteomic analysis of serum-starved cells or PSMA2, CLIC1, and HSPA5 knockdown cells using mass spectrometry might help us clearly comprehend the impacts on cell physiology and signaling pathways.

Then, I investigated the involvement of three FN-1 interacting proteins (PSMA2, CLIC1, and HSPA5) in IAV replication that were overexpressed in IAV-infected cells, and siRNA knockdown of these proteins significantly reduced viral titer. However, knocking down a few other FN-1 interacting proteins (CLF1, CD81, and MCM7) resulted in a >3-fold increase in viral multiplication (Figure 8). Further investigation is necessary to determine the importance of these proteins in IAV replication.

I found that IAV uses PSMA2 for downregulation of the NRF-2-mediated oxidative stress response. However, several aspects of the process remain unclear, such as how PSMA2 KD limits NRF-2 translocation into the nucleus and what influence PSMA2 KD has on downstream molecule expression in the NRF-2 medicated oxidative response. More research is needed to fully comprehend the mechanism.

In this study, I have investigated how CLIC1 and HSPA5 knockdown affect IAV replication (transcription, translation, and viral proteins localization). Further investigation is required to identify the role of CLIC1 and HSPA5 in the precise step/steps of the IAV replication cycle. In the future, I hope to do a proteomic study to determine the roles of CLIC1 and HSPA5 in the cellular signaling pathways that are required for IAV replication. To clearly understand the mechanism, the effect of PSMA2, CLIC1, and HSPA5 over-expression on IAV replication should be investigated. More study is required to understand the therapeutic value of these proteins as a target for the development of antiviral against IAV.

More study is needed to explore the effect on IAV replication in PSMA2, CLIC1, and HSPA5 knockout (KO) cell lines or mouse models to understand the therapeutic significance of these proteins as a target for IAV antiviral development.

However, I discovered that the NPPB and EGCG drugs are effective antivirals against IAV. More research is needed to determine the efficacy in different cell lines and influenza A and B virus strains. If these drugs consistently inhibit IAV strains in different cell lines, they should be tested in mice and nonhuman primates.

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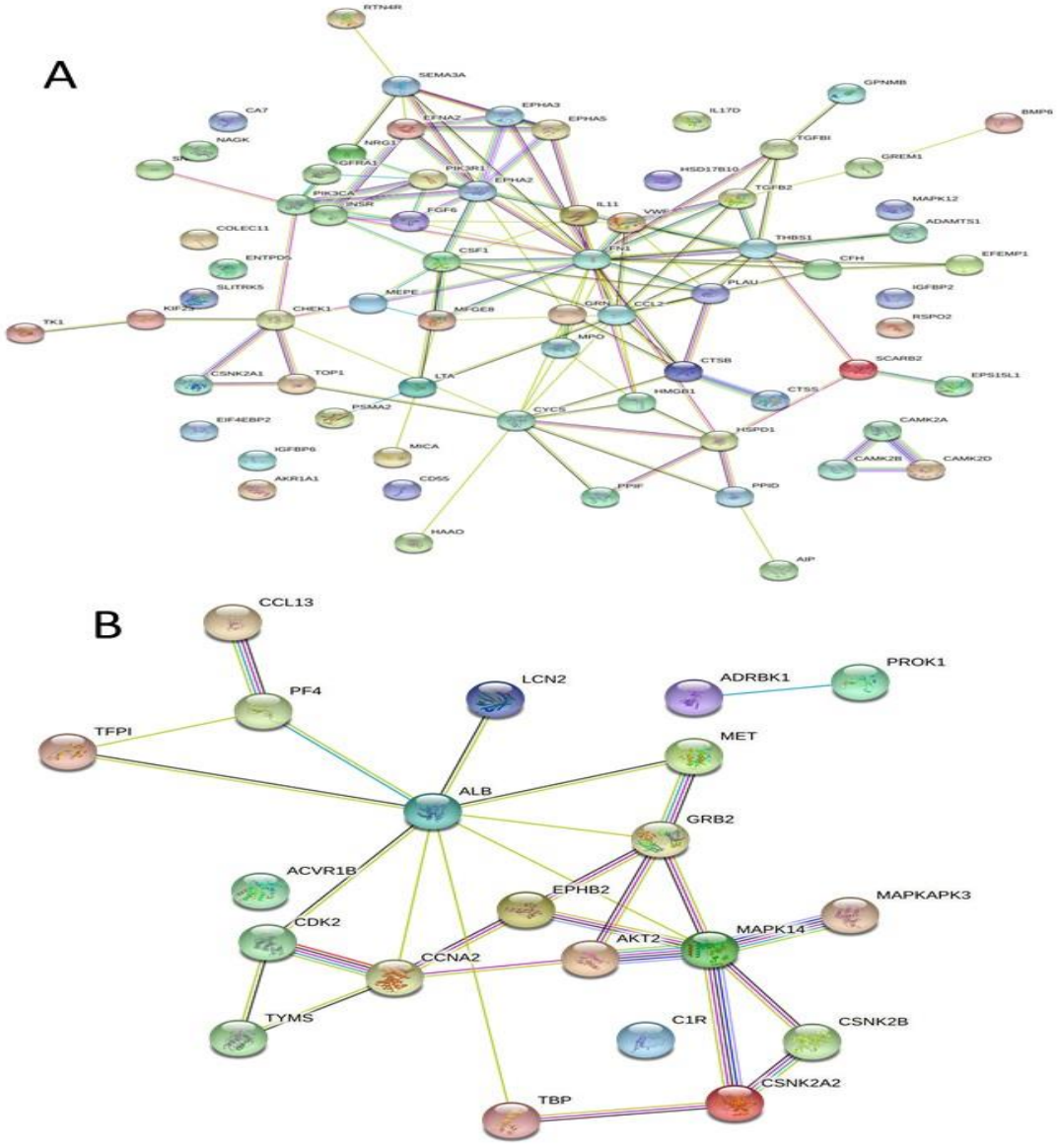
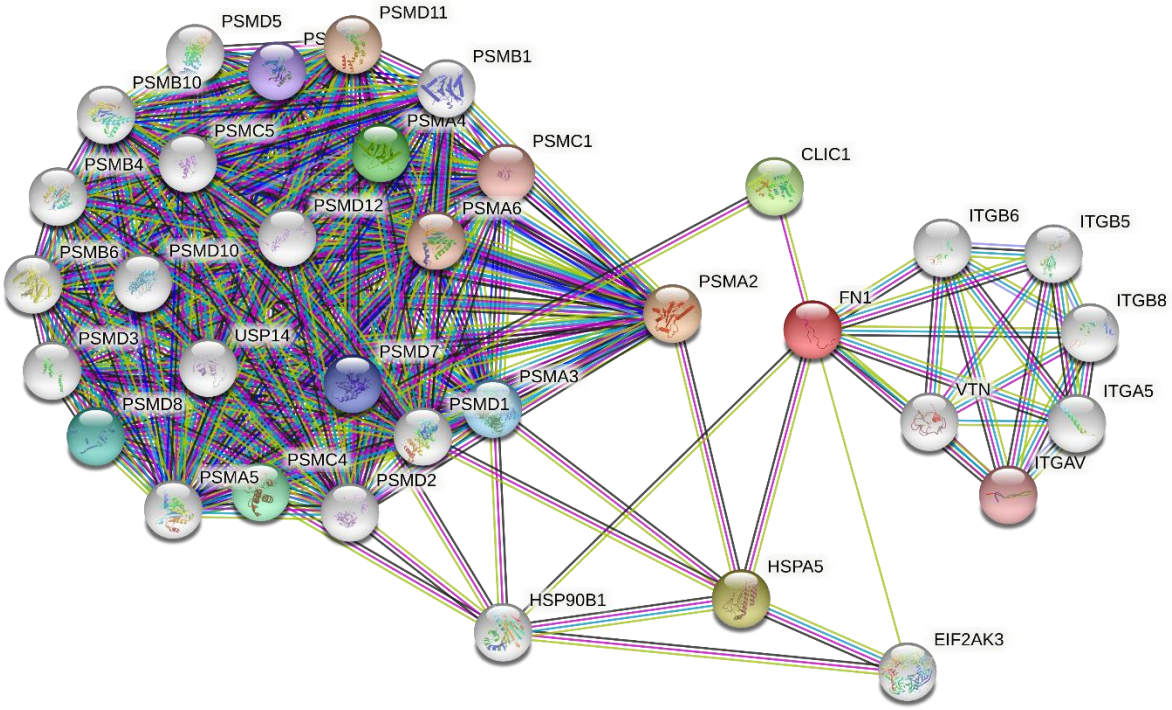
APPENDIX:



Appendix Figure 1. A. Direct and indirect interactions of the proteins dysregulated in A549 cells after PR8 infected but did not significantly affected during PR8 infection in PSMA2 KD cells. B. Direct and indirect interactions of the proteins dysregulated during PR8 infection in PSMA2 KD cells but didn't significantly affected in A549 cells after PR8 infected .



Appendix Figure 2. Direct and indirect interactions of PSMA2, CLIC1, and HSPA5 with fibronectin (FN1). Fibronectin directly interacts with CLIC1 and HSPA5. It interacts with PSMA2 by an indirect interaction via HSPA5. CLIC1 can also interact with HSAP5 via an indirect interaction through FN-1.

Appendix Table 1.

Appendix Table 1. Disease and functions dysregulated by PSMA2 KD, PR8 infection and PSMA2 KD+PR8 infection.				
	Diseases and Bio Functions	**PSMA2 KD +PR8**	**PSMA2 KD**	**PR8**
1	Differentiation of nervous system	-2.733	-0.381	-2.814
2	Phosphorylation of L-amino acid	N/A	1.038	-2.574
3	Differentiation of neurons	-2.146	-0.404	-2.543
4	Invasion of cells	-1.312	1.997	-2.482
5	Shock Response	-1.475	N/A	-2.388
6	Cell proliferation of breast cell lines	-0.388	N/A	-2.359
7	Proliferation of lung cancer cell lines	-1.749	N/A	-2.325
8	Outgrowth of neurites	-2.16	1.277	-2.202
9	Septic shock	-1.186	N/A	-2.177
10	Neovascularization of organ	N/A	N/A	-2.157
11	Proliferation of neuronal cells	-2.049	1.631	-2.126
12	Binding of blood cells	N/A	-0.135	-2.117
13	Adhesion of immune cells	-0.67	N/A	-2.101
14	Growth of malignant tumor	-1.822	1.871	-2.09
15	Growth of neurites	-1.971	1.449	-2.065
16	Cell movement	-2.259	2.539	-1.99
17	Phosphorylation of L-tyrosine	N/A	N/A	-1.977
18	Proliferation of epithelial cells	-0.06	2.16	-1.933
19	Cell proliferation of breast cancer cell lines	-1.904	2.979	-1.885
20	Cell viability of tumor cell lines	-0.824	3.639	-1.784
21	Cell viability	-1.775	2.665	-1.763
22	Cell death of cerebral cortex cells	-1.265	-1.968	-1.714
23	Hematopoiesis of mononuclear leukocytes	-2.651	-0.457	-1.615
24	Cell proliferation of tumor cell lines	-0.831	2.94	-1.598
25	Migration of cells	-2.332	2.289	-1.563
26	Sprouting	-2.622	N/A	-1.552
27	Leukopoiesis	-2.41	0.294	-1.506
28	Differentiation of mononuclear leukocytes	-2.401	N/A	-1.458
29	Growth of embryo	-2.288	0.674	-1.415
30	Development of genitourinary system	-2.102	N/A	-1.37
31	Tubulation of cells	-1.455	2.177	-1.37
32	Differentiation of phagocytes	-2.416	N/A	-1.36
33	Growth of epithelial tissue	0.436	2.777	-1.256
34	Growth of carcinoma	-2.233	N/A	-1.189
35	Growth of tumor	-0.854	2.35	-1.009
36	Advanced malignant solid tumor	-1.732	2.418	-0.97
37	Metastatic solid tumor	-1.732	2.418	-0.97
38	Visceral metastasis	-1.192	2.207	-0.888
39	Advanced extracranial solid tumor	-1.067	2.2	-0.824
40	Growth of organism	-0.905	2.005	-0.733
41	Cell movement of tumor cell lines	-0.327	3.043	-0.731

42	Colony formation of cells	N/A	2.127	-0.684
43	Advanced malignant tumor	-2.332	3.063	-0.567
44	Secondary tumor	-2.332	3.063	-0.567
45	Migration of tumor cell lines	-0.228	2.478	-0.168
46	Extracranial solid tumor	N/A	2.039	-0.054
47	Necrosis	0.139	-2.143	0.066
48	Neoplasia of cells	-1.453	2.608	0.096
49	Cell death of tumor cell lines	-1.019	-2.43	0.146
50	Migration of embryonic cell lines	-0.551	2.915	0.149
51	Hemostasis	1.798	1.976	0.589
52	Proliferation of endothelial cells	0.592	2.296	0.59
53	Apoptosis of tumor cell lines	-0.738	-2.842	0.688
54	Cell movement of fibroblast cell lines	-0.895	2.923	0.72
55	Proliferation of muscle cells	2.754	-0.365	0.749
56	Development of endothelial tissue	0.496	2.023	0.835
57	Apoptosis	0.392	-2.075	0.842
58	Activation of blood platelets	2	N/A	1.342
59	Coagulation	2.753	N/A	1.886
60	Apoptosis of carcinoma cell lines	N/A	-0.661	2.109
61	Bleeding	N/A	-0.782	2.139
62	Organismal death	4.247	-2.422	5.526
63	Branching of cells	-2.404	1.662	N/A
64	Differentiation of antigen presenting cells	-2.226	N/A	N/A
65	Metastatic potential	-2.219	N/A	N/A
66	Differentiation of myeloid leukocytes	-2.209	N/A	N/A
67	Differentiation of macrophages	-1.964	N/A	N/A
68	Differentiation of embryonic tissue	-1.414	2.245	N/A
69	Cell movement of embryonic cell lines	-1.117	2.355	N/A
70	Migration of epithelial cell lines	-0.551	2.607	N/A
71	Thrombus	0.86	1.969	N/A
72	Coagulation of blood	2.376	N/A	N/A
73	Formation of dendrites	N/A	-2.183	N/A
74	Function of neurons	N/A	1.982	N/A
75	Replication of RNA virus	N/A	2.024	N/A
76	Catabolism of protein	N/A	2.145	N/A
77	Differentiation of stromal cells	N/A	2.159	N/A
78	Cell viability of brain cells	N/A	2.183	N/A
79	Replication of Influenza A virus	N/A	2.407	N/A

Red= activated, Blue= inhibited, N/A= Z Score undetermined by IPA

Appendix Table 2.

Appendix Table 2. Canonical Pathways significantly dysregulated by PSMA2 KD, PR8 infection and PSMA2 KD+PR8 infection.				
Serial no.	**Canonical Pathways**	**PSMA2 KD+ PR8 (Z score)**	**PSMA2 KD (Z score)**	**PR8 (Z score)**
1	Cyclins and Cell Cycle Regulation	N/A	-2	N/A
2	Role of NFAT in Regulation of the Immune Response	N/A	0.447	-2.449
3	Opioid Signaling Pathway	N/A	0.816	-2.828
4	EIF2 Signaling	N/A	1	-2.449
5	Paxillin Signaling	N/A	1	-2.449
6	GDNF Family Ligand-Receptor Interactions	N/A	1	-2.236
7	cAMP-mediated signaling	N/A	1	-2
8	Synaptic Long Term Potentiation	N/A	1	-2
9	Dopamine-DARPP32 Feedback in cAMP Signaling	N/A	1	-2
10	GNRH Signaling	N/A	1.342	-2.236
11	G Beta Gamma Signaling	N/A	1.342	-2
12	Type I Diabetes Mellitus Signaling	N/A	1.342	-2
13	fMLP Signaling in Neutrophils	N/A	2	-2.646
14	NGF Signaling	N/A	2	-2.449
15	PKCθ Signaling in T Lymphocytes	N/A	2	-2.236
16	ErbB4 Signaling	N/A	2	-2.236
17	Regulation of eIF4 and p70S6K Signaling	N/A	2	-2
18	PAK Signaling	N/A	2	-2
19	FcγRIIB Signaling in B Lymphocytes	N/A	2	-2
20	PI3K Signaling in B Lymphocytes	N/A	2	-1.89
21	Gαi Signaling	N/A	2	N/A
22	Ephrin Receptor Signaling	N/A	2	N/A
23	Apelin Liver Signaling Pathway	N/A	2	N/A
24	Actin Cytoskeleton Signaling	N/A	2	N/A
25	Cholecystokinin/Gastrin-mediated Signaling	N/A	2.236	-2.236
26	Neurotrophin/TRK Signaling	N/A	2.236	N/A
27	Phospholipase C Signaling	N/A	2.449	-2.449
28	NRF2-mediated Oxidative Stress Response	N/A	2.449	-2
29	Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	N/A	N/A	-2.449
30	Neuropathic Pain Signaling In Dorsal Horn Neurons	N/A	N/A	-2.449
31	Gα12/13 Signaling	N/A	N/A	-2.449
32	Estrogen-Dependent Breast Cancer Signaling	N/A	N/A	-2.449
33	CNTF Signaling	N/A	N/A	-2.449
34	UVC-Induced MAPK Signaling	N/A	N/A	-2.236
35	Aldosterone Signaling in Epithelial Cells	N/A	N/A	-2.236
36	Antiproliferative Role of Somatostatin Receptor 2	N/A	N/A	-2.236
37	Lymphotoxin β Receptor Signaling	N/A	N/A	-2.236
38	Th1 Pathway	N/A	N/A	-2.236

39	IL-9 Signaling	N/A	N/A	-2
40	IL-22 Signaling	N/A	N/A	-2
41	Signaling by Rho Family GTPases	N/A	N/A	-2
42	Calcium-induced T Lymphocyte Apoptosis	N/A	N/A	-2
43	Melanoma Signaling	N/A	N/A	-2
44	Telomerase Signaling	N/A	N/A	-2
45	Inhibition of Angiogenesis by TSP1	N/A	N/A	-2
46	Relaxin Signaling	N/A	N/A	-2
47	UVA-Induced MAPK Signaling	N/A	N/A	-2
48	IL-2 Signaling	N/A	N/A	-2
49	Endometrial Cancer Signaling	N/A	N/A	-2
50	SAPK/JNK Signaling	N/A	N/A	-2
51	Corticotropin Releasing Hormone Signaling	N/A	N/A	-2
52	Non-Small Cell Lung Cancer Signaling	N/A	N/A	-2
53	LXR/RXR Activation	N/A	N/A	2
54	PTEN Signaling	2.236	-1.265	2.121
55	Small Cell Lung Cancer Signaling	-1	-1	-2
56	Glioblastoma Multiforme Signaling	-1	0	-2.449
57	CD28 Signaling in T Helper Cells	-1	1.633	-2.121
58	STAT3 Pathway	-1	N/A	-2.333
59	Acute Phase Response Signaling	-1.134	2.121	-1.897
60	B Cell Receptor Signaling	-1.342	1.265	-2.111
61	Growth Hormone Signaling	-1.342	2	-2.121
62	Gαq Signaling	-1.342	2.449	-2.828
63	IL-6 Signaling	-1.508	0.333	-2.138
64	Renin-Angiotensin Signaling	-1.633	1.342	-2.53
65	IL-7 Signaling Pathway	-1.89	-0.378	-2.646
66	HMGB1 Signaling	-1.89	1	-2.496
67	HGF Signaling	-1.89	1.633	-2.828
68	IL-3 Signaling	-1.89	2.449	-2.714
69	IL-8 Signaling	-1.89	N/A	-2.333
70	Dendritic Cell Maturation	-1.89	N/A	-2.333
71	Adrenomedullin signaling pathway	-2	0.707	-2.828
72	Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency	-2	1	-2.449
73	CREB Signaling in Neurons	-2	1	-2.449
74	p70S6K Signaling	-2	1.342	-2.646
75	ERK/MAPK Signaling	-2	1.633	-2.646
76	Huntington's Disease Signaling	-2	1.633	-2.121
77	ErbB Signaling	-2	1.89	-2.828
78	RANK Signaling in Osteoclasts	-2	2	-3
79	Endothelin-1 Signaling	-2	2	-2.828
80	LPS-stimulated MAPK Signaling	-2	2	-2.646
81	Thrombopoietin Signaling	-2	2	-2.449
82	Leukocyte Extravasation Signaling	-2	2.236	-3
83	Prolactin Signaling	-2	2.236	-2.449
84	3-phosphoinositide Biosynthesis	-2	N/A	-2.828
85	Apelin Endothelial Signaling Pathway	-2	N/A	-2.828

86	mTOR Signaling	-2	N/A	-2.828
87	Superpathway of Inositol Phosphate Compounds	-2	N/A	-2.828
88	UVB-Induced MAPK Signaling	-2	N/A	-2.828
89	CXCR4 Signaling	-2	N/A	-2.828
90	CCR3 Signaling in Eosinophils	-2	N/A	-2.646
91	Macropinocytosis Signaling	-2	N/A	-2.449
92	GM-CSF Signaling	-2	N/A	-2.449
93	P2Y Purigenic Receptor Signaling Pathway	-2	N/A	-2.449
94	PEDF Signaling	-2	N/A	-2.449
95	p38 MAPK Signaling	-2	N/A	-2.236
96	Acute Myeloid Leukemia Signaling	-2	N/A	-2.236
97	SPINK1 General Cancer Pathway	-2	N/A	-2.236
98	Glioma Signaling	-2	N/A	-2.121
99	Renal Cell Carcinoma Signaling	-2	N/A	-2
100	Amyotrophic Lateral Sclerosis Signaling	-2	N/A	-1
101	FGF Signaling	-2.121	2.236	-2.53
102	14-3-3-mediated Signaling	-2.236	0	-2.828
103	Endocannabinoid Developing Neuron Pathway	-2.236	0.816	-2.828
104	Cardiac Hypertrophy Signaling	-2.236	1.134	-3.162
105	AMPK Signaling	-2.236	1.342	-2.828
106	Type II Diabetes Mellitus Signaling	-2.236	1.342	-2.828
107	Role of NFAT in Cardiac Hypertrophy	-2.236	1.667	-3.207
108	CD40 Signaling	-2.236	2	-2.828
109	Fc Epsilon RI Signaling	-2.236	2.236	-2.828
110	NF-κB Activation by Viruses	-2.236	2.236	-2.449
111	PDGF Signaling	-2.236	2.828	-2.333
112	Apelin Cardiomyocyte Signaling Pathway	-2.236	N/A	-2.828
113	Mouse Embryonic Stem Cell Pluripotency	-2.236	N/A	-2.828
114	VEGF Family Ligand-Receptor Interactions	-2.236	N/A	-2.449
115	Ovarian Cancer Signaling	-2.236	N/A	-2.236
116	FAT10 Cancer Signaling Pathway	-2.236	N/A	-2.236
117	Leptin Signaling in Obesity	-2.236	N/A	-2.236
118	IL-17A Signaling in Airway Cells	-2.236	N/A	-2.121
119	eNOS Signaling	-2.449	1	-2.449
120	NF-κB Signaling	-2.449	1.414	-3.317
121	Tec Kinase Signaling	-2.449	1.89	-3.162
122	FLT3 Signaling in Hematopoietic Progenitor Cells	-2.449	2	-3
123	Thrombin Signaling	-2.449	2.236	-3
124	IGF-1 Signaling	-2.449	2.53	-2.828
125	Nitric Oxide Signaling in the Cardiovascular System	-2.449	N/A	-2.449
126	Colorectal Cancer Metastasis Signaling	-2.646	0	-3
127	GP6 Signaling Pathway	-2.646	0.447	-3
128	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	-2.646	1	-2.53
129	Pancreatic Adenocarcinoma Signaling	-2.646	1.342	-2.449
130	EGF Signaling	-2.646	2.646	-3.162

Red= activated, Blue= inhibited, N/A= Z Score undetermined by IPA

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1. **Rashid, M. U**., Gao, A., & Coombs, K. M. (2022). Influenza A virus uses PSMA2 for downregulation of NRF2-mediated oxidative stress response. *Journal of virology*, jvi-01990.

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