

**QUANTITATIVE PHOSPHOPROTEOME
PROFILING OF CIRCULATING INFLUENZA VIRUS
STRAINS IN MUMBAI REGION**

**THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY (SCIENCE) IN
MICROBIOLOGY**

**SUBMITTED BY
MS. SINGH PINKY RAVINDRA KIRAN**

**UNDER THE GUIDANCE OF
DR. SHASHIKANT P. VAIDYA**

**DEPARTMENT OF CLINICAL PATHOLOGY
HAFFKINE INSTITUTE FOR TRAINING RESEARCH & TESTING
MUMBAI – 400 012**

JULY 2023

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TO THE UNIVERSITY OF MUMBAI**

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STATEMENT BY THE CANDIDATE

As required by the University of Mumbai ordinance 770, I wish to state that the embodied in the thesis titled **“QUANTITATIVE PHOSPHOPROTEOME PROFILING OF CIRCULATING INFLUENZA VIRUS STRAINS IN MUMBAI REGION”** forms my own contribution to the research work carried out under the guidance of Dr. Shashikant P. Vaidya at Department of Clinical Pathology, Haffkine Institute for Training, Research & Testing, Mumbai – 400012. This work has not been submitted for any degree of this or any other university. Whenever references have been made to previous works of others, it has been clearly indicated as such and included in the Bibliography.

Signature of candidate



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Signature of the Guide



Dr. Shashikant P. Vaidya

CERTIFICATE FROM GUIDE

This is to certify that **SINGH PINKY RAVINDRA KIRAN** has duly completed this thesis for the Ph.D. (Science) Degree of the University of Mumbai and this thesis entitled **“QUANTITATIVE PHOSPHOPROTEOME PROFILING OF CIRCULATING INFLUENZA VIRUS STRAINS IN MUMBAI REGION”** is up to the standards both respect to its content and literacy presentation for being referred to an examiner.

I further certify that the entire work has been done by the candidate under my guidance and that no part of it has been submitted for any degree or diploma of any University.

Signature of the Guide



Dr. Shashikant P. Vaidya

CERTIFICATE FROM CO-GUIDE

This is to certify that **SINGH PINKY RAVINDRA KIRAN** has duly completed this thesis for the Ph.D. (Science) Degree of the University of Mumbai and this thesis entitled **“QUANTITATIVE PHOSPHOPROTEOME PROFILING OF CIRCULATING INFLUENZA VIRUS STRAINS IN MUMBAI REGION”** is up to the standards both respect to its content and literacy presentation for being referred to an examiner.

I further certify that the entire work has been done by the candidate under my guidance and that no part of it has been submitted for any degree or diploma of any University.



Signature of the Co-Guide

Dr. Sandeepan R. Mukherjee

"The secret of getting ahead is getting started." — Mark Twain

Thank you late Dr. Sweta Kothari, Ma'am for getting my journey started....

And Dr. Shashikant P. Vaidya, Sir for completing it....

**Dedicated to
My Family
&
Friends**

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
A549	Adenocarcinomic human alveolar basal epithelial cells
ACN	Acetonitrile
AGE	Agarose Gel Electrophoresis
ANOVA	Analysis of variance
APS	Ammonium persulfate
ARDS	Acute respiratory distress syndrome
ATP	Adenosine tri phosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine Serum Albumin
BSC	Biosafety cabinet
BSL	Biosafety Level
BSL-2	Bio-Safety level-2
Cds	Complete coding sequence
COPD	Chronic obstructive pulmonary disease
CPE	Cytopathic effect
cRNA	complementary RNA
CT	Cycle threshold
CTLs	Cellular lymphocytes

Abbreviation	Meaning
DCs	Dendritic cells
DDI	Double deionized water
DHB	2,5-dihydroxybenzoic acid
DMEM	Dulbecco's Minimal Essential Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
F12K	Kaighn's Modification of Ham's F-12 Medium
FA	Formic acid
FBS	Fetal bovine serum
Fe-NTA	Ferric nitrilotriacetate
FITC	Fluorescein iso-thiocyanate
HA	Hemagglutinin
HA	Hemagglutination Assay
HEF	Hemagglutinin-esterase fusion
hpi	Hour post infection
IAA	Iododacetamide
IAV	Influenza A viruses and
IBV	Influenza B virus

Abbreviation	Meaning
ICTV	International Committee on Taxonomy of viruses
IFITM	Interferon-induced transmembrane proteins
IFN	Interferon
IFN- γ	Interferon gamma
IFN- α	Interferon alpha
IFN- β	Interferon beta
IL	Interleukin
ILI	Influenza-like illness
IMAC	Immobilised metal affinity chromatography
ISAV	Infectious salmon anemia virus
iTRAQ	Isobaric tags for relative and absolute quantification
Kb	Kilobase
KDa	Kilo Dalton
LC- MS	Liquid chromatography mass spectrometry
M	Matrix protein
m/z	Mass-to-charge ratio
M1	Matrix protein 1
M2	Matrix protein 2
MCP	Monocyte chemotactic protein
MDCK	Madin Darby Canine Kidney cell line

Abbreviation	Meaning
MEM	Minimal Essential Medium
MHC	Major histocompatibility complex
min	Minute
MIP	Macrophage Inflammatory Proteins
ml	Millilitre
mM	Milimolar
MOI	Multiplicity Of Infection
MP	Maximum Parsimony
mRNA	messenger RNA
MS	Mass spectrometry
N	Nucleoprotein
NA	neuraminidase
NCBI	National Centre For Biotechnology Information
NCCS	National Centre for Cell Sciences
NK	Natural killer
nLC	Nano – liquid chromatography
NOS2	Nitric oxide synthase 2
NP	Nucleoprotein
NS	Non-structural protein
NS	Not significant

Abbreviation	Meaning
NS1	Non-structural protein 1
NS2	Non-structural protein 2
OD	Optical Density
PA	Polymerase acidic protein
PAGE	Polyacrylamide gel electrophoresis
PB1	Polymerase basic protein 1
PB2	Polymerase basic protein 2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PRRs	Pattern recognition receptors
PSM	Peptide spectrum match
PTM	Post-translational modification
q RT PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
q- TOF	Quadrupole time of _____
qRT PCR	Real-time reverse transcription polymerase chain reaction
RBCs	Red blood cells
RG	Resolving gel
RNA	Ribonucleic acid
RNP	Ribonucleoprotien
RP- HPLC	Reverse phase high performance liquid chromatography

Abbreviation	Meaning
RT	Room Temperature
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
s	Seconds
SDS	Sodium dodecyl sulphate
SDS PAGE	Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
SILAC	Stable Isotope Labeling by/with Amino acids in Cell culture
ssRNA	Single-stranded RNA
TAE	Tris-acetate-EDTA
TCID ₅₀	50% Tissue Culture Infective Dose
TEABC	Triethylammonium bicarbonate
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TiO ₂	Titanium dioxide
TNF	Tumor necrosis factor
TNF- α	Tumor necrosis factor alpha
TOF	Time of flight
TPCK	Tosyl phenylalanyl chloromethyl ketone
UV	Ultraviolet
V	Volts

Abbreviation	Meaning
VGM	Virus growth medium
vRNA	viral RNA
WHO	World Health Organization
µg	Microgram
µl	Microlitre
µM	Micromolar

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CHAPTER 1

INTRODUCTION

1. INTRODUCTION

A serious threat to human progress and existence is posed by infectious illnesses. Worldwide, infectious diseases continue to exist and spread among people, leaving them disabled and dead. People bear a disproportionately large portion of the burden of infectious disease as a result of persistent infections that frequently recur as new or emerging epidemics and unforeseen pandemics that greatly raise the global morbidity load. As antibiotic and antiviral resistance grows, the morbidity and mortality linked to these resurfacing and developing infectious illnesses rises. Despite the availability of vaccines, several infectious diseases continue to be a major cause of death. (1)

The influenza virus, which belongs to the Orthomyxoviridae family, is a highly contagious respiratory pathogen. (2) Despite being well-known for its annual seasonal epidemics, influenza A virus strains of zoonotic origin can potentially trigger unforeseen pandemic outbreaks on a global scale. These outbreaks, which occur every 10 to 50 years, are distinguished by the introduction of an antigenically new strain of influenza A virus. As humans lack pre-existing immunity to these novel strains, the infection's severity and mortality rate can increase considerably. Globally, severe influenza infection is a leading cause of illness and mortality. Contrary to popular belief, influenza infection is frequently linked to several clinical diseases that impact organ systems other than the respiratory tract, according to clinical findings. (3,4)

Records documenting outbreaks of respiratory diseases resembling influenza, both in the form of epidemics and pandemics, can be traced back over four centuries. The widespread and rapid dissemination of pandemic influenza is a more recent occurrence, likely linked to population growth and the expansion of transportation systems that facilitate the global transmission of new viral strains. Throughout history, animals have likely played a significant role in the occurrence of influenza epidemics, including modern pandemics. There have been simultaneous reports of respiratory disease outbreaks in horses and humans during the 18th and 19th centuries. It has recently been claimed that swine and birds have a significant role in the onset of influenza pandemics. (5,6)

In contrast, influenza pandemics are uncommon events, but because of their unfamiliarity and potential for catastrophic effects, they are viewed as more dangerous. Even a century later, there is still a great deal of anxiety due to the knowledge of the staggering number of deaths brought on by the 1918 influenza epidemic. Although estimates of the death toll have varied

widely, more recent study that took into account data from low-income nations like India has raised the figures globally. (7) The 1918 influenza pandemic claimed the lives of an estimated 50 million people worldwide, or about 3% of the world's population at the time, according to a widely recognised estimate today. (8) Influenza pandemics have occurred throughout history, dating back to as early as 1510 when a flu originating from Africa spread rapidly across Europe. Another significant pandemic emerged in Asia in the 1580s, subsequently spreading to Africa, Europe, and America. In 1688, England, Ireland, and Virginia were hit by another devastating outbreak. Over the years, the virus continued to mutate, causing widespread illness and affecting nearly every family. (6)

Table 1: Influenza pandemics throughout history (9)

Name	Years	Subtype	Estimated deaths
Global Influenza Pandemic (Russian flu)	1889-1890	H3N8 or H2N2	1 million
Spanish flu	1918-1920	H1N1	50 million
Asian flu	1957-1958	H2N2	2 million
Hong Kong flu	1968-1969	H3N2	1 million
Swine flu	2009-2010	H1N1 pdm09	575,400

Vaccination and antiviral drugs are the principal methods for preventing the transmission of human influenza. Currently, antiviral drugs available for treating influenza A virus infections target viral proteins. Despite the availability of various treatment options, there is currently no definitive universal solution for influenza, and the threat of pandemics remains ongoing. This underscores the need for a deeper comprehension of the diverse pathophysiological mechanisms linked to influenza infection, as well as the exploration of uncharted mechanisms that hold prognostic and therapeutic potential. However, because influenza viruses mutate rapidly, they can easily develop resistance to antiviral drugs that target viral proteins. To combat this issue, it is important to identify novel drugs that target virus-host interactions, which are essential for influenza virus infection and replication. By inhibiting these interactions, it may be feasible to reduce the incidence of drug-resistant mutants and mitigate the adverse health effects of influenza viruses. (10–13)

It is necessary to gain a deeper understanding of the underlying mechanisms underlying influenza infection. This includes investigating unexplored mechanisms with potential prognostic and therapeutic applications. It is generally acknowledged that immunological and genetic factors of the organism play a role in the evolution of influenza viruses in both humans and animals. The immune system is a well-balanced system that protects the body from various invading pathogens. The immune system of the host employs innate, adaptive, and complement responses in order to eradicate viruses and viral particles. In addition, the immune system is equipped with effective sensors that detect invading pathogens and coordinate responses to eliminate multiple viral copies. Influenza is a self-limiting infection that can be effectively fought off by activating the body's innate immunological response, which is the first line of defence against disease. (14,15)

Many of the observed clinical and pathological manifestations may be attributable to the early cytokines produced at the infection site, according to the evidence. Influenza viruses have been found to activate certain cytokines and chemokines, such as TNF- α , INF- β , IFN- γ , MIG (Monokine Induced by IFN- γ), IP-10 (Interferon- γ -Inducible Protein), MIP-1 (Macrophage Inflammatory Protein), MCP-1 (Monocyte Chemoattractant Protein), RANTES (Regulated on Activation Normal T-cell Expressed and Secreted), and IL-8, in both animals and humans. (16,17)

Interferons (IFNs) are a specialised class of cytokines that can trigger antiviral, antigrowth, and immunomodulatory responses. (15) Isaacs and Lindenmann discovered them in 1957 as substances secreted by virus-infected cells that inhibit further infection of neighbouring cells. The activity of IFNs entails the stimulation of IFN-stimulated gene (ISG) transcription, which actively suppresses critical viral propagation processes, ranging from viral entry and uncoating to maturation and release. ISGs encode specific antiviral molecules that can modulate IFN signalling either positively or negatively. Numerous interferons have been approved as therapeutic agents, and substantial progress has been made in comprehending their function and antiviral application. ISGs instigate signalling pathways that induce interferon production, which in turn activates other signalling pathways, resulting in an innate immune response against the virus. Interferons also stimulate the expression of the IFIT and IFITM families. The RIG-I-like receptor family (RLRs) is another closely related family that contains the retinoic acid-inducible gene (RIG-I). Various interferons, including IFN- (interferon alpha), IFITM proteins (including IFITM-1, IFITM-2, and IFITM-3), RIG-I, and MHC-1 (major histocompatibility complex I), have been shown to be activated by influenza viruses. (18,19)

The response of the host to pathogenesis is a dynamic and intricate process. In conjunction with proteomics and gene expression, phosphoproteomics can provide system-level information about the immune response, which is essential for a comprehensive and better understanding of disease mechanisms. (20) When viruses infect host cells, they interact with the proteins within the host to take control of the cell's functions. This is done through a process called phosphorylation, where the viruses interfere with the regulatory networks within the cell. As a result, the viruses can replicate more effectively while inhibiting the normal cellular processes. The process of adding a phosphate group to a target protein such as serine (S), threonine (T), and tyrosine (Y) to substrate through protein kinases, known as phosphorylation, is a common posttranslational modification. This study seeks to use quantitative phosphoproteomic analysis to identify alterations in biological processes within a human lung cell line (A549) during an infection with the different influenza viruses. (21–23)

CHAPTER 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE:

Influenza viruses, which are members of the family Orthomyxoviridae, are RNA viruses with envelopes. They possess a segmented genome and are distinguished by single-stranded RNA with a negative sense. The International Committee on the Taxonomy of Viruses (ICTV) officially designated and endorsed the term "influenza viruses" in the 1960s. (24) This name was chosen to emphasise their capacity to bind to mucus membranes and distinguish them from the Paramyxoviridae family of enveloped negative-strand RNA viruses. There are four genera within the Orthomyxoviridae family: type A, type B, type C, and Thogotovirus. Influenza virus types A, B, and C can be distinguished based on the antigenic distinctions between their matrix (M) and nucleoprotein (NP) proteins. The antigenic properties of influenza viruses' hemagglutinin (HA) and neuraminidase (NA) glycoproteins determine their subtypes. Influenza A and B viruses possess unique HA and NA glycoproteins, whereas influenza C viruses lack NA and contain a hemagglutinin-esterase fusion (HEF) protein. In contrast, Thogoto virus contains a single glycoprotein unrelated to any known influenza virus protein. (2,25,26)

Within influenza A viruses, further differentiation is based on the antigenicity of their HA and NA molecules. There are 18 known HA subtypes (H1, H2, etc.) and 11 NA subtypes (N1, N2, etc.) identified genetically. Among these subtypes, only three HA subtypes (H1, H2, H3) and two NA subtypes (N1, N2) have caused human epidemics and are responsible for sustained person-to-person transmission. Additional distinguishing characteristics of influenza A, B, and C viruses include the range of host species they infect. (27) Many bird species are susceptible to infection by influenza A viruses, in addition to humans and other mammals including horses and pigs. However, influenza C viruses have been discovered from both humans and swine in China, while influenza B viruses only infect primates. In addition, there is a lot more variation in the amino acid sequences of influenza A virus surface glycoproteins than there is in influenza B viral surface glycoproteins. The influenza C virus, on the other hand, only has one glycoprotein, yet it serves multiple purposes. (26,28)

2.1. Types of influenza viruses and their characteristics

Table 2: Types of Influenza Virus and their Characteristics

Types of Influenza	Description about the types
Influenza A	a. Influenza A is classified based on the glycoproteins present on the surface of the virus envelope namely Haemagglutinin (HA) and Neuraminidase (NA). b. Till today 18 Haemagglutinin and 11 Neuraminidase sub-types have been identified. c. Among these, only 3 types of Haemagglutinin (H1, H2 and H3) and 2 types of Neuraminidases (N1 and N2) have been recognized to cause epidemic disease in humans. d. Influenza A consist of eight negative-sense viral RNA segments as its genome. e. Aquatic birds are the primary source of Influenza A, but also circulate among other animals such as pigs, cattle, etc.
Influenza B	a. Influenza B is classified based on the lineages and strains. b. The genome of Influenza B comprises of eight negative-sense viral RNA segment c. Influenza B belongs to one of the two lineages either Yamagata and Victoria d. Mostly affects humans and children are more Prominently affected from Influenza B.
Influenza C	a. Influenza C is classified on the basis of the single glycoprotein present on its surface b. HEF (hemagglutinin- esterase- fusion) is the glycoprotein for the identification of Influenza C. c. HEF corresponds functionally to the HA and NA of the Influenza A and B viruses. d. Influenza C comprises of seven negative-sense viral RNA segments as its genome. e. Influenza C affects all individuals of all ages but it seems to cause

Influenza D	a. Influenza D is related to Influenza C b. It is also identified on the basis of HEF glycoprotein. c. Similar to Influenza C, the genome of Influenza C comprises of seven negative-sense viral RNA segments. d. They primarily affect cattle and pigs but are not known to cause illness or infection in humans.
-------------	---

2.2. Taxonomy and Classification: (29)

Species: *Alphainfluenzavirus influenzae*

Realm: *Riboviria*

Kingdom: *Orthornavirae*

Phylum: *Negarnaviricota*

Subphylum: *Polyploviricotina*

Class: *Insthoviricetes*

Order: *Articulavirales*

Family: *Orthomyxoviridae*

Genus: *Alphainfluenzavirus*

Species: *Alphainfluenzavirus influenzae*

Species: *Betainfluenzavirus influenzae*

Realm: *Riboviria*

Kingdom: *Orthornavirae*

Phylum: *Negarnaviricota*

Subphylum: *Polyploviricotina*

Class: *Insthoviricetes*

Order: *Articulavirales*

Family: *Orthomyxoviridae*

Genus: *Betainfluenzavirus*

Species: *Betainfluenzavirus influenzae*

2.3. Nomenclature:

In February 1980, the World Health Organization (WHO) released a memorandum outlining the revised system of nomenclature for influenza viruses, replacing the

previous system in use since 1971. This revised system became universally adopted following the publication of the memorandum. (30) Influenza viruses are divided into three categories based on their antigenic differences: A, B, and C, whereas Influenza A viruses are further subdivided into numerous subtypes. (25)

The complete nomenclature for each newly isolated influenza virus includes the virus type, the host of origin (if non-human), the geographical location of isolation, the strain number, the year of isolation, and, in the case of influenza A viruses, the hemagglutinin (HA) and neuraminidase (NA) subtypes. The antigenic description of the HA and NA subtypes is provided within parentheses. For instance, if an influenza A virus is isolated from a mallard duck in Memphis, Tennessee, in 1995, with a strain number of 123 and an H3N8 subtype, it would be designated as A/mallard/Memphis/123/95 (H3N8). (26)

In the standard nomenclature for influenza viruses isolated from humans, the naming convention includes the virus type, the location of isolation, the isolate number, the year of isolation, and, for influenza A viruses, the hemagglutinin (HA) and neuraminidase (NA) subtypes. For instance, if a human influenza A virus is isolated from Panama in 1999, with the isolate number 2007, an HA subtype 3, and an NA subtype 2, it would be designated as A/Panama/2007/1999(H3N2). As for influenza B and C viruses, no antigenic subtypes have been identified thus far. (31)

2.4. History of Influenza Virus:

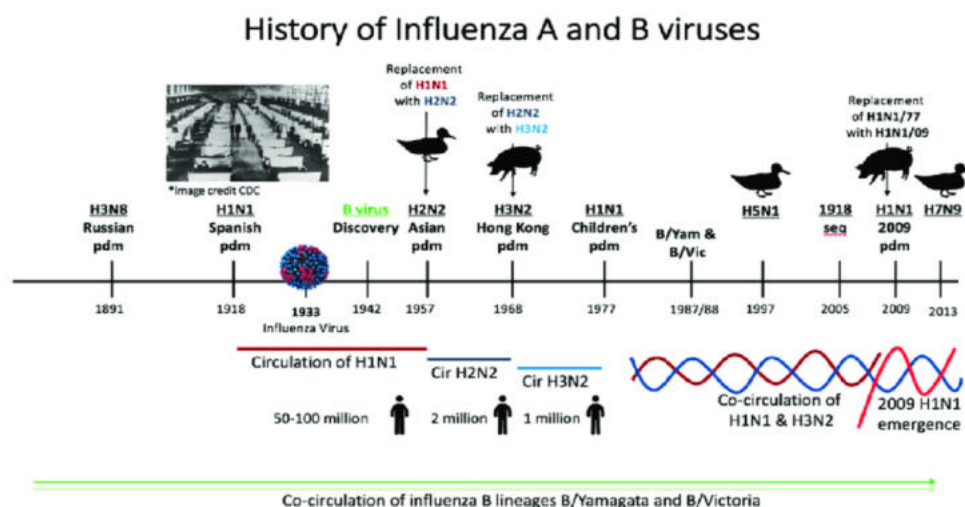


Figure 1: History of Influenza Virus (32).

A timeline of the human influenza virus's history since the 1890s. The distribution of influenza virus types A and B since 1890 is depicted on a timeline. There have been

six alleged pandemics throughout this time. Only archival medical records exist for the Russian pandemic of 1891, which occurred before the influenza virus was identified. Seroarcheology claims that an H3N8 virus was to blame for this outbreak. The Spanish Influenza Pandemic, also known as 1918, occurred. After lung samples from pandemic victims were sequenced in 2005, the H1N1 virus that had caused the pandemic was finally discovered. According to estimates, this virus was responsible for 50–100 million fatalities. H1N1 viruses were in circulation up until 1957, when the Asian pandemic caused H1N1 to be replaced by an H2N2 pandemic virus. During the 1968 pandemic in Hong Kong, the H3N2 virus took the place of the H2N2 virus. H1N1 first reappeared in 1977, and H1N1 and H3N2 have subsequently co-circulated. The seasonal H1N1 virus 'lineage' that had been in circulation since 1977 was replaced in 2009 by an H1N1 strain with swine origins. Avian virus spillover incidents also result in human infection in addition to the seasonal influenza viruses that are in circulation. For the influenza A viruses H5N1 and H7N9, major avian viral spillover occurrences happened in 1997 and 2013, respectively. Additionally, influenza B viruses have been co-circulating in people throughout this time.(32)

2.5. Virion structure:

The core of influenza viruses consists of segmented, negative-sense RNA and is encased in a lipid envelope. These viruses exhibit pleomorphic morphology, appearing either spherical or filamentous, or a combination of both. The spheres form measure around 100 nm in diameter, while the longest filaments can stretch to 300 nm. When influenza A and B viruses undergo limited passages in tissue culture or eggs, they tend to have a more filamentous shape than spherical particles. However, isolates that have undergone extensive laboratory passages consist predominantly of spherical virions measuring 80-120 nm in diameter. Under an electron microscope, it is challenging to distinguish between the morphologies of influenza A and B virions. On the other hand, influenza C virions can be differentiated by the organization of the hemagglutinin-esterase fusion (HEF) glycoprotein spike, which forms hexagonal arrays on the virion surface. (26,31)

Influenza A and B viruses possess two prominent glycoproteins, hemagglutinin (HA) and neuraminidase (NA), embedded in the virion surface membrane. The outer surface of these viruses is covered with approximately 500 spikes that radiate outward, ranging in length from 10 to 14 nm. These spikes consist of rod-shaped HA spikes and

mushroom-shaped slender stalks of NA spikes. In influenza A virions, the ratio of HA to NA spikes is approximately four to one, prominently protruding from the host-derived lipid membrane. The HA and NA spikes are not easily distinguishable under an electron microscope unless they are removed from the surface using a protease or when the virions are treated with detergent. (31)

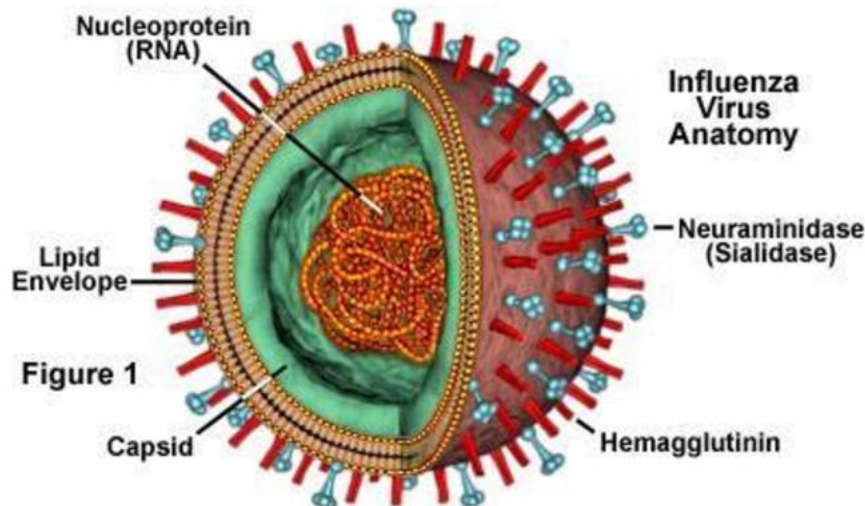


Figure 2: Structure of influenza virus (33)

In addition to HA and NA, influenza A viruses contain another integral membrane protein called the M2 ion channel protein, which is present in low quantities within the virions. Influenza viruses A and B have similarities in their structures, but influenza C has a more diverged structure. The structure comprises of:

1. Surface glycoprotein's: Hemagglutinin & Neuraminidase
2. The polymerase-associated proteins: PB2, PBI, PA
3. 8 Single stranded, negative sense RNA segments
4. The Nucleocapsid Protein
5. Non-structural Protein M2 and Membrane Protein M1
6. Non-structural proteins: NS1 and NS2 Proteins

The viral matrix protein M1 underlies the lipid bilayer and is the most abundant protein found in the virions. The lipid envelope of influenza viruses is strengthened and given rigidity by a protein shell. Within the virion, the viral RNAs are located, and they are loosely encapsulated by the nucleoprotein (NP). In influenza A and B viruses, the

nucleoprotein wraps around the eight genomic segments. These genomic segments contain the virus's genetic material, which is responsible for coding one or two proteins. Polymerase complexes composed of three proteins: PB1, PB2, and PA are found at the terminals of nucleocapsids. These proteins create a heterotrimeric influenza RNA polymerase after binding to the viral RNA. In contrast, the NP protein functions as a non-specific single-stranded RNA binding protein. It interacts with the viral RNA at 20 nucleotide intervals along its whole length. Additionally, there are two other proteins produced by alternate splicing: NS1 and NS2. (25,34)

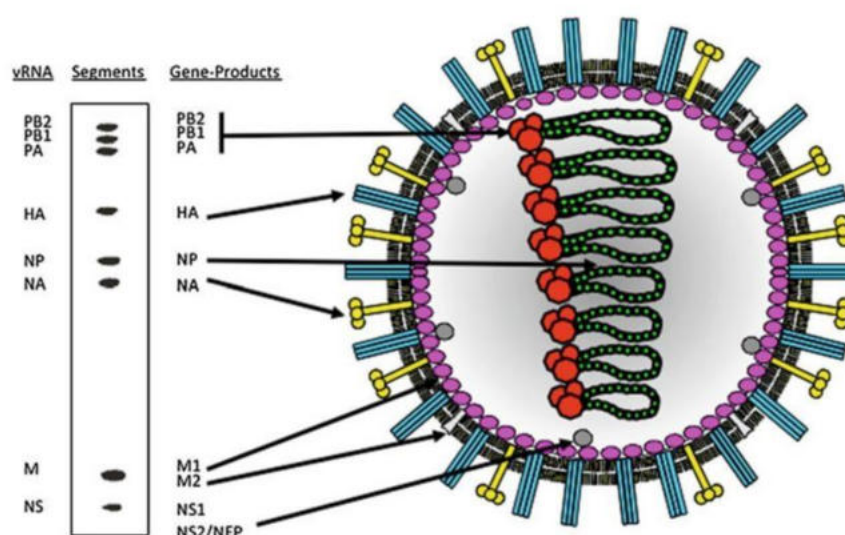


Figure 3: Cartoon structure of the influenza virus A viral fragment. (35)

Table 3: Function of influenza proteins (35)

Segment	vRNA (bases)	Protein	AA	Functions
1	2,341	PB2	759	Recognizing and binding to the 5' cap structures of host cell mRNAs, the cap-binding component of viral RdRp acts as a transcription primer for viral mRNA production.
2	2,341	PB1	757	The central polymerase domain of viral RdRp is responsible for elongating primed nascent viral mRNA and serves as an elongation protein for template RNA and vRNA synthesis.

Segment	vRNA (bases)	Protein	AA	Functions
		PB1-F2	87–91	Pro-apoptotic activity
3	2,233	PA	716	Displays protease activity. (Component of the viral RdRp with endonuclease activity involved in cap-snatching)
4	1,778	HA	566	Binds to sialic acid receptors and promotes viral entry by membrane fusion. Plays a crucial role as a major surface antigen for attaching virions to host cell receptors. (Surface glycoprotein involved in receptor binding and membrane fusion)
5	1,565	NP	498	Binds to and encapsulates viral RNA, facilitating its transport within the cell nucleus and cytoplasm. (Nucleoprotein responsible for encapsidating viral genomic and antigenomic RNA)
6	1,413	NA	454	Functions as an enzyme that cleaves sialic acid from H and N molecules, as well as glycoproteins. Essential for virus penetration. (Surface glycoprotein with Neuraminidase activity involved in receptor destruction)
7	1,027	M1	252	Plays a vital role in viral assembly by recruiting viral components to the assembly site at the cell membrane. (Matrix protein involved in assembly)
		M2	97	The membrane-spanning domain functions as a signal for transportation to the cell surface. It exhibits ion channel activity, allowing the passage of protons into the interior of the virus. This proton influx facilitates the dissociation of the RNP

Segment	vRNA (bases)	Protein	AA	Functions
				(ribonucleoprotein) complex from other viral components, leading to the complete uncoating of the virus. This ion channel activity also plays a role in protecting the conformation of the HA (hemagglutinin) protein.
8	890	NS1	230	It suppresses the immune response of the host, restricting the production of interferons (IFNs). It also modulates the replication of viral RNA, synthesis of viral proteins, and overall functioning of the host cell. It regulates the activity of the viral RdRp, acts as an interferon antagonist, enhances the translation of viral mRNA, and inhibits processes such as pre-mRNA splicing, cellular mRNA polyadenylation, and PKR (Protein Kinase R) activity.
		NEP	121	It aids in the transportation of recently synthesized RNPs (ribonucleoproteins) from the nucleus to the cytoplasm, thereby enhancing the speed of virus production. This protein acts as a nuclear export factor, facilitating the movement of viral components to the appropriate cellular location for efficient virus replication.

2.6. Influenza A vs. influenza B virus:

There are significant differences between influenza A and influenza B viruses, despite the fact that both types of viruses share certain similarities in appearance, virion structure, and the roles of proteins. The influenza A virus is capable of naturally infecting a broad variety of avian species, as well as humans and other mammalian

species, such as pigs and horses. On the other hand, influenza B viruses can only typically infect human beings and not other animals. The two forms of virus have significantly distinct surface glycoproteins, which is one of the key differences between them. When compared to its counterpart in influenza B viruses, the surface glycoprotein known as hemagglutinin (HA) found on influenza A viruses displays a significantly higher degree of variety in terms of the amino acid sequences that it contains. At first, the HA protein is synthesised as a precursor molecule. Subsequently, serine proteases break the precursor molecule apart into two functional proteins, which are designated HA1 and HA2. The particular amino acid sequence that is present at the cleavage site is what defines how the HA protein is broken down by cellular proteases. (28)

In the interaction with sialic acid, a terminal structure present on glycoproteins and glycolipids, both neuraminidase (NA) and HA play critical roles. Particularly NA is important in the last stages of infection. It eliminates sialic acids from cell-surface receptors as well as freshly synthesised HA and NA proteins on developing virions that were sialylated during the glycosylation process in the host cell. NA prevents virion aggregation by cleaving sialic acids and prevents the virus from rebinding to a dying host cell via HA. This makes viral progeny to be released more easily and more likely to spread to new target cells. Instead of starting an infection, main role of viral HA's is the attachment during the infection has been largely limited in aiding exit of virion progeny from the infected cells. (36)

2.7. Antigenic variation:

The outbreaks of influenza occur due the evolutions or changes that occur in the genome of the virus. The main changes that are seen in influenza virus is at the antigens of the virus. These changes occur majorly due to genetic re-assortment. Both substantial and continuous changes are seen to occur in the HA protein. Antigenic shift and antigenic drift are the two mechanisms by which influenza virus evolve quickly. The emergence of novel antigenic phenotypes is necessary for Influenza A and B virus survival in the nature. (37)

2.8. Antigenic Drift:

Antigenic drift occurs due to small changes or mutations in the glycoproteins HA and NA of the influenza virus. These point changes occur each time when the virus

replicates. These antigenic glycoproteins are the source through which the virus causes pathogenicity in the host and so the antibodies are produced against these antigens. So whenever similar antigens attack the host antibodies are released against them as a way of protection. These point mutations that occur during antigenic drift may lead to inefficiency of the human body to recognize the mutated antigens and so antibodies will not be released against them and can neutralize the antigenically different virus. This is the reason why people are infected multiple times with influenza virus.

2.9. Antigenic shift:

Periodic pandemics are caused by the antigenic shifts. These shifts are caused when one or both of the antigens are altered. These alterations are different from mutations. Three mechanisms are used to explain antigenic shift and the developments in influenza virus pandemic

1. Genetic recombination between avian virus strains and human virus strains are currently in circulation. When two different influenza viruses of different origin infect a cell at the same time, genetic reassortment occurs. The 1957 and 1968 strains had evolved from genetic re-assortment, according to the genetic and biochemical analyses. The H, N and PB1 gene segments of the 1957 H2N2 pandemic strains came from an avian virus, whereas the remaining 5 come from the previous human H1N1 strain. (38)
2. Direct avian/mammalian to human virus transmission: The spread of swine flu H1N1 virus in 1918 and 1976 Fort Dix Epidemics are an example of human-human transmission. The spread of H5N1 and H9N2 are examples of direct transmission from birds to humans in Hong Kong.
3. Virus recycling: Mechanism of emergence of virus at dormant stage or concealed in animal or bird reservoirs. For example, the 1977 H1N1 virus was revived from its frozen state and then reintroduced to people. (39)

2.10. Life cycle of influenza virus:

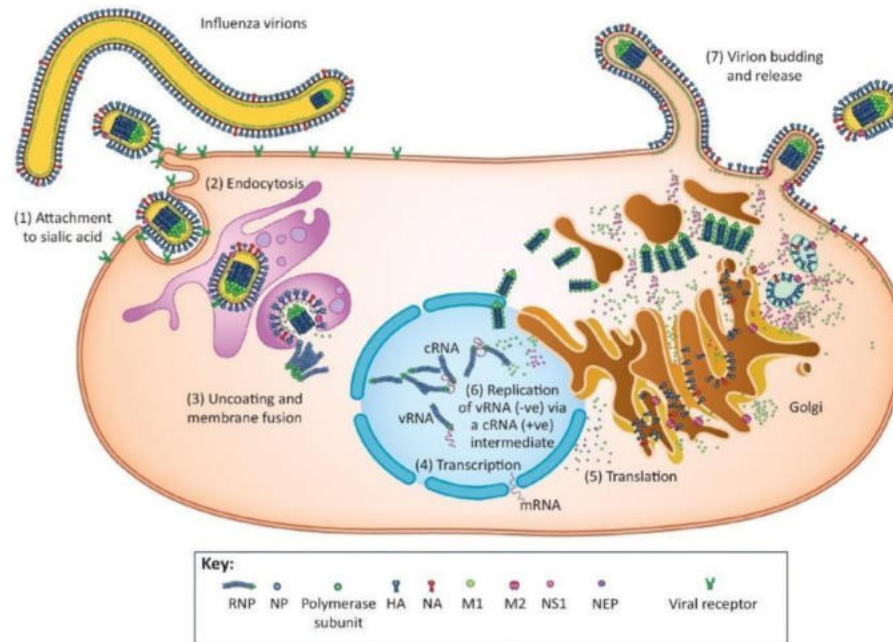


Figure 4: Life Cycle of influenza virus (40)

The life cycle of the influenza virus can be categorized into several distinct stages:

- 1. Host cell entry:** The virus enters the host cell.
- 2. Nuclear entry of viral RNAs:** The viral RNA molecules are transported into the nucleus.
- 3. Virus replication and transcription:** The virus undergoes replication and transcription of its genetic material.
- 4. Translation:** The viral proteins are synthesized.
- 5. Export of viral ribonucleoproteins (vRNPs) from the nucleus:** The vRNPs are transported out of the nucleus.
- 6. Assembly and budding at the plasma membrane of the host cell:** The viral components assemble and the virus buds from the host cell's plasma membrane.

- 7. Post-translational processing:** The newly formed viral proteins undergo modifications and maturation processes.

2.10.1. Entry into the host cell:

When an influenza virus enters a host cell, it begins the process of replicating itself in that cell. Spikes are generated on the viral lipid membrane by a surface protein known as HA. These spikes have the ability to recognise certain receptor polysaccharides on the epithelial cells of the host. When it comes to human viruses, HA binds to sialic acid that has galactose attached to it via (2,6) connections. On the other hand, viruses that infect birds and horses are able to recognise (2,3) links, whereas viruses that infect pigs are capable of recognising either type of linkage. These connections can be seen in high numbers within the epithelial cells that line the respiratory system. Clathrin-mediated endocytosis is the major method that influenza A viruses (IAVs) use to internalise host cells; however, additional pathways, such as clathrin-independent endocytosis and macropinocytosis, have also been identified. [C]lathrin-mediated endocytosis is the primary process that IAVs utilise to internalise host cells. During the process of internalisation, a number of cellular factors, including as the small GTPases Rab5 and Rab7 and the interferon-inducible transmembrane (IFITM) proteins IFITM1, IFITM2, and IFITM3, play a part in the regulation of the process. Other cellular factors include Rab5 and Rab7. (41,42)

The virus enters the host cell by a process called receptor-mediated endocytosis after it has first attached to its receptor. The presence of a low pH environment within the endosome is what ultimately causes the fusion of the viral membrane with the endosomal membrane. This process takes place in the space in between the endocytic vesicle and the lysosome. Because the viral HA functions as a host protease, it is able to cleave itself, which leads to the development of a fusion peptide that is referred to as area 12. This fusion peptide is inserted into the endosomal membrane, which ultimately results in the joining of the viral membrane and the endosomal membrane. In addition to making fusion easier to accomplish, the acidic environment of the endosome is responsible for activating the M2 ion channel. As a result of the opening of the M2 ion channel, the nucleus of the virus becomes more acidic, which causes the disruption of

protein-protein connections and the release of the viral ribonucleoprotein (vRNP) complex from the viral matrix protein (M1). The vRNP complex is then disassembled and released into the cytoplasm of the host cell, where it can continue to facilitate viral replication.

2.10.2. Entry of vRNAs into the nucleus:

After being released into the cytoplasm, the viral ribonucleoprotein (vRNP) complex must then enter the cell nucleus. It is in the cell nucleus that viral transcription and replication take place. Each of the viral proteins that make up the vRNP complex, NP, PA, PB1, and PB2, have their own unique nuclear localization signals (NLSs). These nuclear localization signals (NLSs) allow the viral proteins to engage with the nuclear import machinery of the cell, which enables the viral proteins to be transported into the nucleus.

2.10.3. Virus replication and transcription:

The influenza virus genome consists of negative sense, eight single-stranded RNA segments that are coated with nucleocapsid protein and viral RNA polymerase. These segments are negatively charged and serve as non-coding strands. In order to produce viral RNAs, they must be copied into complementary positive strands, known as coding strands. The process of transcription takes place in the nucleus, requiring the entry of these RNA segments through nuclear pores.

During transcription, the PB2 protein plays a crucial role. It initiates the process by binding to the 5' cap of the host mRNA transcript. Unlike replication, transcription does not require a primer. Instead, the viral RNA-dependent RNA polymerase (RdRp) internally initiates RNA synthesis on the viral RNA. The viral mRNAs have a 5' methylated cap and a poly(A) tail, similar to cellular mRNAs. However, the 5' caps of viral mRNAs are actually "snatched" from the host mRNA through a mechanism known as "cap-snatching." The viral RdRp, which consists of PB1, PB2, and PA proteins, is involved in this process. PA, with its endonuclease activity, snatches the 5' cap along with 10-13 nucleotides from the host mRNA, which serves as a primer for the synthesis of viral mRNA. PB1 plays an important role in viral RNA synthesis. Transcription continues

until polyadenylation signals indicate the end of viral RNA. The poly(A) tail contributes to the stability of viral mRNA. Splicing of two influenza A virus (IAV) mRNAs leads to the production of M1 and M2 proteins or the interferon antagonist (NS1) and nuclear export (NEP/NS2) proteins. Host machinery is primarily responsible for splicing, and NS1 regulates this process by binding to various components, including U6 short nuclear RNAs and UAP56. Interestingly, despite relying on the host cell's splicing machinery, influenza prevents the host cell from utilizing its own splicing machinery for processing host cell mRNAs.

2.10.4. Translation:

The translation of viral mRNA is dependent on various host components, including eIF4A, eIF4E, and eIF4G. These host components interact with viral mRNAs and polymerase complexes, respectively. An infection with the influenza A virus (IAV) causes a disruption in the process of protein synthesis in the host cell, which then causes viral mRNA translation to take precedence. This choice is reinforced by components that interact specifically with complexes of viral mRNA and polymerase. During IAV infection, newly synthesized nuclear mRNAs may be depleted due to cap-snatching activity. This results in the rapid degradation of mRNAs before they can be exported from the nucleus and translated. The viral polymerase interacts with the C-terminal domain of the biggest subunit of cellular DNA-dependent RNA polymerase II (Pol II), whereas the NS1 protein interacts with cellular poly(A)-binding protein II (PABII) and cleavage and polyadenylation specificity factor (CPSF) proteins. These interactions, when taken as a whole, lead to the inhibition of mRNA synthesis in the host.

Following cytoplasmic synthesis, the viral polymerase subunit proteins and nucleoprotein (NP) are transported into the nucleus through nuclear localization signals. This allows them to catalyze viral RNA replication and transcription within the nucleus. Additionally, the M1, NEP/NS2, and NS1 proteins are also transported into the nucleus to perform their respective functions. M1 and NEP/NS2 are involved in exporting viral ribonucleoprotein (vRNP) complexes from the nucleus, while NS1 is responsible for processing and exporting both cellular and viral proteins.

2.10.5. Export of vRNPs from the nucleus:

The export of vRNPs from the nucleus occurs exclusively for the negative-sense vRNPs and follows the CRM1-dependent pathway through nuclear pores. The nucleoprotein (NP) directly interacts with CRM1, although it does not exhibit GTP hydrolysis activity. The matrix protein 1 (M1) interacts with vRNPs through its C-terminal end, while the N-terminal portion of M1 contains a nuclear localization signal (NLS) responsible for vRNP import. The N-terminal region of M1 also binds to the nuclear export protein (NEP), masking the NLS.

Within the nucleus, vRNPs associate with Rce1 for export, and this process is facilitated by Crm1, a cellular export factor. Additionally, the Y box binding protein 1 (YB-1) binds to vRNPs, promoting their nucleocytoplasmic export and facilitating interaction with microtubules for transport to the plasma membrane. In the endoplasmic reticulum, viral proteins such as hemagglutinin (HA), neuraminidase (NA), and M2 undergo glycosylation (in the case of HA and NA) or palmitoylation (in the case of HA and M2). The transport of virus proteins to the cell membrane requires the involvement of microtubule-organizing centers (MTOCs), microtubules, and various host components including members of the coatamer I (COPI) protein family, a Rab GTPase (Rab11A), and the HIV Rev-binding protein (HRB). These factors contribute to the efficient transport of virus proteins to the cell membrane.

2.10.6. Assembly and budding at the host cells plasma membrane:

The influenza virus goes through the process of assembling viral particles and then leaves the host cell after the vRNPs have left the nucleus and exited the cell. It is an enveloped virus, which means that it uses the plasma membrane of the host cell in order to generate viral particles, which are then released to infect cells that are in close proximity to the host cell. The majority of viral particles emerge from the apical side of polarised cells during the budding process. Hemagglutinin (HA) and neuraminidase (NA), two of the most important viral proteins, are known to connect with lipid rafts on the plasma membrane. Lipid rafts are areas of the membrane that are rich in sphingolipids and cholesterol.. The assembly of the ribonucleoprotein complex in viral RNAs requires specific packaging signals unique to each viral segment. The deletion and mutational

analysis of the M2 protein's tail are critical in the process of viral particle formation.

The matrix protein 1 (M1) plays a crucial role in the final stages of viral particle assembly by facilitating the closure and budding of the particle. The incorporation of vRNPs into the newly assembling particle is mediated by the cytoplasmic tail of the M2 protein. In order for the influenza virus to bud, the M2 protein is involved in membrane scission and the release of the viral particle, while cellular ATPase is required. The viral neuraminidase (NA) protein enzymatically removes sialic acids from host cells, promoting the release of the virus and preventing virion aggregation. Overall, these processes contribute to the formation and release of influenza virus particles from the host cell, enabling the virus to spread and infect other cells.

2.10.7. Post-translational processing:

Several post-translational modifications take place in proteins of the influenza A virus (IAV), including glycosylation of HA and NA proteins, palmitoylation (S-acylation) of HA and M2 proteins, and SUMOylation of M1, NS1, NP, PB1, and NEP/NS2 proteins. Additionally, nuclear import and export processes can impact the phosphorylation of M1 and NP proteins. These phosphorylation events are catalyzed by protein kinase C (PKC), cyclin-dependent kinases (CDKs), and extracellular signal-regulated kinases (ERKs), leading to the phosphorylation of NS1. (43,44)

2.11. Clinical manifestations

The majority of individuals infected with influenza experience illness, although up to half of adult infections may go unnoticed (subclinical) depending on the specific strain and pre-existing immunity. Infection can manifest in various ways, such as the common cold, pharyngitis, tracheobronchitis, or bronchiolitis in infants.

Influenza syndrome is a contagious respiratory illness characterized by several clinical symptoms. Common symptoms of classic influenza include fever, malaise, headache, myalgia (muscle pain), cough, and sore throat. Fever can be high and may persist or come and go depending on the use of antipyretic medications. Mild conjunctivitis and a clear nasal discharge are also frequently

observed. Fever and systemic symptoms typically subside after 3 to 5 days, but respiratory symptoms such as dry cough, substernal burning, nasal congestion, and mild neutropenia may persist, worsening the respiratory complaints. Recovery from influenza is usually slow, with cough and malaise lasting anywhere from two to four weeks. Even seemingly mild influenza cases can lead to small airway dysfunction, which may have long-term implications for gas exchange and pulmonary mechanics, contributing to fatigue and reduced exercise tolerance during the recovery period. Smokers and allergic patients tend to experience more severe influenza symptoms and a prolonged recovery period. Infections with the H3N2 subtype of influenza are associated with a higher frequency of lower respiratory symptoms, abnormalities in pulmonary function, and visits to healthcare providers compared to infections with the H1N1 subtype.

Influenza can manifest differently in children, and various syndromes may be observed. While some symptoms are common across different age groups, infants and young children may experience non-specific febrile illness or respiratory conditions such as croup, bronchiolitis, or bronchitis, which can be challenging to distinguish from illnesses caused by other respiratory viruses like respiratory syncytial virus or parainfluenza viruses. Gastrointestinal symptoms such as nausea, vomiting, diarrhea, and abdominal discomfort are also common in children. Febrile convulsions often occur as the initial symptom in a significant number of children.

In the context of a known outbreak, influenza is often diagnosed based on clinical symptoms and epidemiological factors. However, diagnosing influenza can be challenging in certain situations due to the similarity of acute respiratory symptoms with other viral diseases such as respiratory syncytial virus, parainfluenza virus, and adenovirus. Moreover, elderly and debilitated influenza patients may not exhibit typical systemic symptoms. Key symptoms for diagnosis include fatigue, lethargy, disorientation, decreased activity level, unexplained cough, and fever. Complications may arise, such as bacterial pneumonia or exacerbations of underlying conditions like asthma, chronic obstructive pulmonary disease (COPD), or congestive heart failure. (45)

2.12. Pathogenesis

The fundamental mechanism underlying the pathophysiology of influenza involves the inflammation and dysfunction of the lungs resulting from direct viral infection of the respiratory epithelium. This is further compounded by the immune responses triggered to contain the spread of the virus, which contribute to lung inflammation. While this inflammation can potentially affect other organs and lead to multiorgan failure, such complications typically arise following severe respiratory distress and lung damage. Moreover, influenza virus infection has been linked to cardiac complications, including an increased risk of myocardial disease in the weeks following the infection. However, the precise mechanisms responsible for these cardiac issues are not completely understood, except for a general inflammatory response. (46)

2.12.1. Viral Factors in Pathogenesis and Transmission

Receptor Specificity: The question of whether altering the viral specificity for host cell receptors, specifically sialic acids, could lead to the emergence of a pandemic influenza strain remains a significant puzzle in influenza research. The surface glycoprotein hemagglutinin plays a crucial role in determining the host range as it selectively attaches to specific sialic acid residues on host cells. Notably, specific amino acid variations in hemagglutinin have been identified as significant factors influencing the specificity and pathogenicity of sialic acid receptors. In human influenza virus isolates, hemagglutinin exhibits a preference for binding to 2,6-linked sialic acids, whereas avian influenza virus isolates show a preference for 2,3-linked sialic acids. Interestingly, different host species, including humans, have varying distributions of sialic acid receptors in their respiratory tracts. It is important to note that besides the binding to 2,3-linked and 2,6-linked sialic acids, other factors may also contribute to receptor specificity. However, the biological significance of receptor binding in viral entry, replication, spread, tissue tropism, and transmission is still not fully understood and requires further investigation. (31)

Replication Efficiency: The replication efficiency of the influenza virus is closely linked to its pathogenicity. In animal models, certain amino acid sequences found in the polymerase genes have been shown to confer lethal

properties to the virus. One well-known marker of pathogenicity is the presence of lysine at position 627 in the PB2 subunit protein of the polymerase complex. Identifying the specific residues in the polymerase that contribute to lethality is crucial in order to elucidate the underlying mechanism that governs replication efficiency. Gaining a deeper understanding of how receptor specificity and polymerase-driven replication impact the pathogenicity and transmission of influenza virus will provide valuable insights into host adaptation, pandemic potential, and the development of antiviral medications. (26)

Transmissibility: Factors beyond viral DNA can impact the transmissibility of viruses. One common mode of transmission is through droplets produced during coughing or sneezing. However, research has shown that in the case of the H3N2 influenza virus, aerosol transmission can occur in a guinea pig model of human illness, and this type of transmission is facilitated by lower humidity and temperature conditions. These findings provide insights into the periodicity of human influenza outbreaks. It is noteworthy that the H3N2 influenza virus can be transmitted between guinea pigs without the need for coughing or sneezing, which differs from the transmission pattern observed in ferrets. (47)

Coinfection: The role of influenza virus and bacterial coinfections in disease development is an area of great interest in research. Historical evidence suggests that a significant number of fatalities during the 1918 Spanish flu pandemic were attributed to secondary bacterial pneumonia. However, our comprehension of the complex interactions between multiple pathogens and their combined impact on the infected host remains limited. (48)

2.12.2. Host Factors in Pathogenicity

1. The presence of specific receptors on the surface of host cells.
2. The availability of necessary host cell enzymes for viral entry and replication.
3. The immunocompetence of the individual host.
4. The presence of specific immunity against particular viral epitopes in the host and the target population.

5. The ability of the immune system to effectively control viral replication while minimizing harmful inflammation and collateral damage to the host.
(49)

Table 4: Host and viral mechanisms of influenza-associated pathogenesis (50)

Direct viral induced pathology	Innate immune responses	Adaptive immune responses
Epithelial cell death (apoptosis and necrosis)	Local and systemic cytokine production	Exuberant T cell responses (CD4 and CD8) Excess cytokine production
Alveolar compromise	Innate immune cellular infiltration (neutrophils, inflammatory monocytes)	Immune-cell mediated epithelial denudation
Denudation of the airways	Extracellular matrix degradation	Amplification of inflammation, local & systemic cytokine production

2.13. Immunology:

Following infection, the influenza virus triggers an acute response in the host. This response kicks off a chain of immunological events, which in turn activates a number of different immune system components. Innate immunity is responsible for the initial response, which includes the production of cytokines such as IFN- α /B, the recruitment of neutrophil granulocytes and natural killer cells, and the activation of other cells. These occurrences are factors that lead to the quick onset of clinical symptoms. The involuntary immune response is extremely important because it sets the stage for the adaptive immunological response that will follow. Two of its most important functions are the regulation of viral replication and antigen load, and the activation of antigen-specific lymphocytes by the induction of co-stimulatory molecules on innate immune cells during the course of their contacts with the virus. However, the influenza virus has developed mechanisms to evade and counteract the innate immune response. One of these mechanisms is the expression of the non-structural

protein 1 (NS1) gene, which is believed to sequester viral dsRNA, preventing its recognition by cellular sensors that would otherwise trigger the release of IFN α /B. In addition, the influenza virus has developed mechanisms to evade and counteract the adaptive immune response.

Even though it takes a few days for the adaptive immune response to reach its full level of effectiveness, it plays a significant part in the process of confining the virus and, finally, eliminating it. In addition to this, it creates a memory response, which provides long-term resistance against reinfection with the same virus strain. However, this protection is typically only afforded against certain subtypes or strains of influenza, and it is extremely uncommon for there to be any sort of cross-protection between subtypes. Both systemic and localised antibody production (also known as humoral immunity) and cytotoxic T cell responses (also known as cellular immunity) take place in response to an infection with influenza. Both of these immune responses are essential for healing from acute illness and preventing reinfection.

It is important to note that infections only provide limited protection against the various subtypes or forms of influenza, and that cross-protection within a subtype is not typically seen. This information is important to keep in mind. Together, the production of antibodies and the activation of cytotoxic T cells, two components of the immune response, play an essential part in the process of healing from acute infection and giving resistance against reinfection. These immune responses are necessary in order to battle influenza; however, the mechanics of protection are complex and can change based on the particular virus subtype or strain. (51)

2.14. Innate Immunity

2.14.1. Pathogen Recognition Receptor of Influenza Virus

Innate immunity is essential for both the control of viral infections and the beginning of the adaptive immune response. The influenza A virus can be recognised by the pathogen recognition receptors (PRRs) found in innate immunity cells. These PRRs include RIG-I, TLR3, TLR7, and NOD-like receptors. RIG-I is able to identify the virus by looking for single-stranded RNAs, while TLR7 looks for the virus in specialised cells. The generation of

pro-inflammatory cytokines and type I interferons is brought about by the activation of these mechanisms. The signalling of TLR7 activates both IRF7 and NF- κ B, whereas the signalling of RIG-I activates IRF3 and NF- κ B. Although the significance of TLR8 in influenza virus infection is not completely understood at this time, it is expressed in human monocytes and macrophages and is involved in the generation of IL-12. (52)

Recent studies have illuminated the significance of cytoplasmic complexes called inflammasomes in detecting influenza viruses. The inflammasome complex activates caspase-1, leading to the generation of pro-IL-1 and IL-18. Key components of the cytoplasmic inflammasome complex include NLRP1, NLRP3, and NLRC4. Activation of these pathways induces antiviral responses, neutrophil activation, macrophage recruitment, and dendritic cell (DC) maturation. As a result, type I interferons (IFN- α/β), type II interferon (IFN- γ), and type III interferons (IFN- λ) are produced. The presence of type I IFN triggers antiviral signaling cascades, involving the phosphorylation of tyrosine kinase 2 (Tyk2) and Janus kinase 1 (Jak1), followed by the phosphorylation of signal transducers and activators of transcription (STAT) 1 and STAT2. This leads to the formation of an antiviral state within the cell via the transcription factor complex ISGF3, comprising phosphorylated STAT1, STAT2, and IRF9. IFN- γ , the primary type II interferon, initiates a potent adaptive and memory cytotoxic T cell response against the influenza virus. Type III IFN, or IFN- λ , contributes to the prevention of influenza A infection in the lungs. Studies indicate that plasmacytoid dendritic cells (pDCs) produce a higher amount of IFN- λ than monocyte-derived DCs following influenza A infection, suggesting that pDCs are the primary cells responsible for IFN- λ production. (53,54)

2.14.2. Macrophages, Natural Killer Cells and Dendritic Cells

During the early stages of influenza virus infection, alveolar macrophages and monocytes are activated, triggering a pro-inflammatory response characterized by the release of TNF- α and IL-6. These cells play a crucial role in preventing the spread of infection by engaging in opsonophagocytosis of influenza virus particles and phagocytosis of apoptotic infected cells. Additionally, they influence the response of the adaptive immune system. Natural killer (NK) cells,

a subset of cytotoxic lymphocytes, contribute to clearing the influenza virus infection through two distinct mechanisms. Firstly, they recognize HA proteins by binding to sialylated NKp44 and NKp46 receptors, allowing them to identify infected cells. Secondly, NK cells induce cell death by interacting with the Fc region of antibodies attached to influenza virus-infected cells via their CD16 receptor, leading to the release of the virus from infected cells. Dendritic cells (DCs), expert antigen-presenting cells, play a critical role in bridging the innate and adaptive immune responses.

Dendritic cells (DCs) are responsible for transmitting viral antigens to naive and memory T cells, which is the first step in the adaptive immune response. In the context of an illness caused by the influenza virus, DCs perform ongoing surveillance of the lungs for the presence of foreign substances and diseases. During an infection with the influenza virus, DCs acquire antigens through one of two different pathways. Direct infection of DCs is the initial step in the process, which leads to the degradation of viral proteins into smaller peptides by proteasomes in the cytoplasm. These peptides are subsequently transported to the endoplasmic reticulum (ER), where they are loaded onto MHC class I molecules. These MHC class I/peptide complexes are recognised by cytotoxic T lymphocytes (CTLs) that are specific to a particular virus. The phagocytosis of virus particles or apoptotic epithelial cells is the second method that DCs utilise to acquire antigens. The binding of degraded viral proteins to MHC class II molecules on the cell surface enables identification of the virus by CD4⁺ T helper cells, which in turn causes the proliferation of B cells and their subsequent maturation into plasma cells that produce antibodies. DCs are able to present epitopes to CD8⁺ T lymphocytes through a process known as cross-presentation, which describes the interaction between the two types of lymphocytes. (55–57)

2.15. Adaptive Immunity

2.15.1. Humoral Immunity

The humoral immune system is responsible for the production of antibodies that fight different influenza antigens. It is critical to have an understanding of the host's antibody response in order to accurately forecast the severity of a disease

and produce safe and effective vaccinations. Among these antibodies, HA-specific antibodies are particularly significant because they inhibit the virus' ability to attach itself to host cells, hence preventing illness and rendering the virus harmless. To further assist in the process of phagocytosis, HA-specific antibodies have the ability to bind to infected cells that express Fc receptors on their surface. Antibodies that target the conserved stem region of HA are able to inhibit the replication of a variety of influenza virus subtypes; however, it is not yet known whether or not these antibodies can stimulate an immune response against all variants of HA. By inhibiting NA enzymatic activity and promoting antibody-dependent cell-mediated cytotoxicity (ADCC), NA antibodies contribute to the suppression of viral transmission.

Due to the low presence of the M2 protein in infected cells, antibodies that are specific to the M2 protein are produced in a less significant amount during natural infection. Although the precise protective mechanism is not completely understood, there is a possibility that NP-specific antibodies play a role in the influenza virus infection. These antibodies have the ability to trigger the lysis of infected cells via the complement pathway. However, antigenic drift and surface antigen shift can reduce the efficiency of antibodies' ability to bind to HA and NA, which can result in reinfection. However, heterotypic antibodies that have cross-reactivity to a variety of viral antigens can offer a significant degree of immunological protection. Although serum anti-HA antibodies are essential for effective influenza protection, it is preferable to have a diverse range of immunological responses in order to achieve the highest possible level of overall immunity.

Infections such as influenza frequently enter the body through the mucosal tissues, and the principal antibodies responsible for protecting the mucosal lining are IgA, with some contribution coming from IgM. These antibodies are absolutely necessary for neutralising infections, preventing the entry of pathogens, and suppressing the multiplication of viruses. The HA and NA proteins of the influenza virus are specially targeted by neutralising antibodies, in particular those of the IgA isotype. throughout primary immune reactions, organised lymphoid tissues are responsible for the initiation of immune

responses; at this stage, IgM antibodies predominate, but this shifts to IgG antibodies throughout subsequent immune responses. There appears to be a considerable relationship between the early influenza A-specific IgM response and the production of IgG antibodies because a strong early IgM response is required for a strong future IgG antibody response. Despite the fact that antibodies have a finite lifespan, antibody-secreting cells (ASCs) that exist for a long period ensure that antibody titers are maintained throughout a person's entire life. Certain cytokines, including as TACI, BLyS, and APRIL, play essential roles in the development of adequate humoral immunity and in the provision of protection against secondary viral infections. Targeting TACI on innate immune cells and B cells in order to boost the survival of antiviral ASCs can result in better maintenance of antibody titers and increased protection. (58)

2.15.2. Cellular Immunity

CD4⁺ and CD8⁺ T lymphocytes are the first cells of the immune system to become active in response to an infection with the influenza virus. Helper T cells that are CD4⁺, including the Th1 and Th2 subsets, recognise virus-specific peptides when they are presented by antigen-presenting cells through MHC class II. This results in the production of co-stimulatory molecules. Controlling the cellular immune response to an influenza infection also involves the participation of Th17 cells and regulatory T cells, often known as Tregs. Producing IL-6, which suppresses the function of Treg cells, is one of the ways that Th17 cells contribute to T helper responses. After an infection, Tregs inhibit inflammation by releasing IL-35, an anti-inflammatory protein that regulates CD8⁺ T cell and T helper cell responses. Tregs also regulate the responses of T cells. It is important to note that IL-35 levels rise during recurrent pneumococcal pneumonia that follows an influenza illness.

Targeting infected cells is possible for cytolytic CD4⁺ T cells, which are largely T helper (Th) cells. T helper cells can be subdivided into Th1 and Th2 subgroups according to the patterns of cytokine production they produce. While Th1 cells emit interferon alpha and interleukin 2, which predominantly contribute to cellular immune responses, Th2 cells release interleukin 4 and interleukin 13, which primarily enhances B-cell immune responses. The cell-mediated immune

responses of genetically altered mice that lacked the IFN- gene were comparable to those of wild-type controls, despite the fact that these mice had higher levels of Th2 cytokines and influenza-specific IgG1 antibodies. During in vitro re-stimulation, the CD4 cell clones that were taken from these mice showed that they had the ability to protect against a potentially fatal influenza virus challenge by using a cytolytic mechanism. In addition, CD4⁺ T cells were able to lyse virally infected cells in animals that were deficient in class I molecules. This demonstrated that CD4⁺ T cells have the ability to compensate for defects in IFN- or CD8⁺ T cells. CD4⁺ T cells make a considerable contribution to the stimulation of antiviral B cell responses, which ultimately results in the formation of long-lived plasma cells as well as switching between antibody classes and affinity maturation. This effector role is carried out by T follicular helper (Tfh) cells, which are a unique fraction of CD4⁺ T cells. The findings of this study have significance for immunological memory as well as vaccination in experimental influenza infection.

When a person gets infected with the influenza virus, the naive CD8⁺ T lymphocytes that are found in the draining lymph nodes get activated. This happens when viral epitopes are presented on MHC class I molecules. These activated T cells then undergo the process of differentiating into cytotoxic T lymphocytes, often known as CTLs, before migrating to the site of the infection. CTLs are able to identify cells that are infected with the influenza virus and remove them via their lytic activity. This prevents the creation of new virus particles. Their ability to cause lysis is made possible by the release of granzymes and perforin, specifically GrA and GrB. Granzymes are able to penetrate infected cells thanks to the pores that are created in the cell membrane by perforin. This ultimately leads to the death of the cells. In addition, GrA is capable of inhibiting the reproduction of viruses by cleaving both viral proteins and host cell proteins that are involved in the process of protein synthesis. These actions are non-cytotoxic. Infected cells can also be induced to undergo apoptosis by CTLs thanks to the connections between Fas and FasL.

In addition, CTLs are responsible for the production of cytokines, which boost the expression of MHC molecules and hence improve antigen presentation.

After an infection, virus-specific CTLs can be discovered in the lymphoid organs, the circulation, and the site of the infection itself. There is a correlation between the production of IL-17 by T cells and dendritic cells (DCs) and the generation and maintenance of memory CTL cells. These memory CTLs have the potential to become active again in the event of a subsequent influenza virus infection. The co-stimulatory cues that memory CTLs receive during their initial differentiation phase have an effect on both the responsiveness and affinity of those cells when they are confronted with a secondary infection. The NP, M1, and PA proteins are the primary targets of cytotoxic T lymphocytes (CTLs) created in response to influenza virus infection in humans. The CTL response displays strong cross-reactivity because these proteins have a high degree of conservation; this cross-reactivity can even be observed between distinct subtypes of influenza A viruses. (59,60)

2.16. Gene expression analysis:

When infections occur, hosts activate specific immune defense systems and employ particular immune defense mechanisms. However, pathogens can manipulate host signaling pathways to promote their own survival and persistence. The ability of infectious agents to thrive and cause harm in host tissues relies on their ability to adapt to the host microenvironment. Profiling the transcriptional state of host and pathogen genes serves various purposes. Firstly, it allows for the identification of new interactions by utilizing infection models such as cell or organ cultures, which may involve unknown host factors. Secondly, by combining microarray analysis with isogenic mutants, the involvement of specific virulence factors in host recognition and subsequent immune responses can be characterized. Thirdly, the use of resistant and susceptible mutant cell lines can help identify gene clusters and mechanisms that restrict infection and facilitate healing. Therefore, analyzing the transcription patterns of both the host and pathogen genes provides a more comprehensive understanding of the intricate dynamics in this complex relationship. Transcriptional profiling of gene expression in host-pathogen interactions is still in the early stages of development, especially given the vast array of infectious agents and the diverse range of host cells that can be targeted.

2.17. Proteomics

Advancements in genomics, including genome sequencing, array technology, and mRNA expression profiling, have provided researchers with abundant data on infectious diseases. While genomics has been valuable in various research aspects, it has certain limitations when studying diseases. Genomic data primarily focuses on DNA and RNA information, but proteins are the functional molecules in cells, with mRNA serving as intermediates between genes and proteins. Hence, assessing mRNA levels in relation to stimuli, such as a disease, provides only an approximation of protein levels and may not exhibit a direct correlation with them. Moreover, some RNAs are not translated into proteins at all. Additionally, proteins can undergo posttranslational modifications, leading to different protein isoforms that can significantly affect their function. Genomic methods and transcriptional profiling cannot directly assess protein abundance or post-translational modifications. To complement genomic information and gain a better understanding of the cell's status under specific conditions, proteomics analysis is required to bridge the gap left by genomics. (61)

When examining the real alterations in gene expression profiles linked to particular situations, such as disease states or the presence of particular pathogens, proteomic analysis frequently has an advantage over DNA microarray analysis. (62) Studies examining age-related changes in the liver provide two instances illustrating the value of proteomics. In one study, Keppler et al. looked at the livers of older rats and discovered that cathepsin B activity was four times higher and cathepsin B protein level was two times higher than in younger rats. (63) Surprisingly, Northern blot examination of cathepsin B mRNA showed no difference in steady-state levels between elderly and young rat livers. This discrepancy between mRNA and protein levels highlights a key drawback of estimating protein abundance exclusively from mRNA levels.

Another example is the discovery made by Helenius and colleagues, who observed that the age-related increase in nuclear factor-kappa-B (NF- κ B)-binding activity in rat liver coincided with an increase in the levels of p52 and p65 components. (64) Multiple bands belonging to each protein were identified by Western blot analysis, demonstrating the role of post-translational changes in the regulation of those proteins. Transcription analysis alone would not have

revealed the existence of several isoforms of a single peptide. A more detailed picture with increased sensitivity provided by proteomics-based methods enable the identification of various isoforms as well as the determination or prediction of particular post-translational changes, which are frequently essential for comprehending protein function.

Procedures of proteomics and the tools utilized for these procedures are continuously undergoing development, evolving, offering a wide range of strategies for protein analysis. The specific sample preparation steps for proteomic analysis can vary greatly depending on the experiment's objectives, such as enhancing solubilization or reducing sample complexity prior to protein separation. For instance, pretreating serum samples often involves removing albumin and immunoglobulins. When comparing two samples, a differential labeling technique can be employed prior to gel-based separation. One such method is two-dimensional differential in-gel electrophoresis (2D-DIGE), where samples are labeled with different fluorescent dyes emitting at different wavelengths.

Two-dimensional gel electrophoresis, often known as 2DE, has been the method of choice for performing protein separation for the purpose of proteome analysis for many years. This method separates proteins based on their isoelectric point as well as their size. Methods that use liquid chromatography, often known as LC, are frequently utilised for the separation of proteins. A more advanced technique known as multidimensional protein identification technology (MudPIT) makes use of different kinds of liquid chromatography (LC) to separate complicated mixtures of peptides, which makes it possible to do extensive proteome analysis. After the proteins have been separated, a number of different mass spectrometry (MS) procedures are utilised in order to develop peak lists predicated on peptide time-of-flight (TOF) observations. In order to identify proteins, these peak lists are subsequently compared to databases that contain information regarding the values of recognised peptides. MS techniques that have been around for a while include electrospray ionisation (ESI), matrix-assisted laser desorption and ionisation (MALDI), and others. Ionisation of liquid samples is accomplished through the use of ESI, which is also conveniently combined with a variety of procedures for protein separation. On

the other hand, MALDI is able to perform analyses on a wide variety of large-mass proteins with molecular weights reaching up into the hundreds of kilobatons. (65)

It is vital to deploy high-throughput technologies that offer comprehensive insights into the molecular activities occurring during host-pathogen interactions in order to properly comprehend the complex interactions that occur between influenza A viruses and the hosts that they infect. These technologies should be able to provide this information. The incorporation of proteomics into influenza virology has brought about a sea change in the way researchers approach the study of virus entrance, replication, and the host's immune response to viral infection. These processes are extremely dependent on the dynamic control of viral and host proteins. These proteins are able to undergo alterations at either the translational or posttranslational level. (66–68)

2.18. Posttranslational Modifications Are Key to Cellular Signal Transduction

Posttranslational modification (PTM) refers to the chemical modifications that occur on proteins following translation, resulting in alterations to their interactions, stability, and subcellular localization. These modifications involve enzymatic processes and are extremely dynamic; they play essential roles in a variety of cellular functions. Mass spectrometry is frequently used to detect PTMs by analysing the changes in peptide masses, and it can provide specific information regarding the amino acid sites at which modifications occur. (69)

Post-translational modifications (PTMs) such as phosphorylation, ubiquitination, glycosylation, methylation, acetylation, and acylation are among those that have received a significant amount of research. Utilising mass spectrometry, the field of proteomics makes it possible to conduct qualitative and quantitative analyses of a wide variety of modification sites found inside proteins that are important in the regulation of biological processes. Within the realm of influenza virology, post-translational modification (PTM) proteomics has been a significant contributor to the discovery of the dynamic regulation of viral and host proteins. This regulation can occur as a result of modifications including as phosphorylation, ubiquitination, SUMOylation, neddylation, ISGylation, glycosylation, palmitoylation, methylation, and acetylation.

Protein-modifying processes are mediated by host systems and have a significant impact on the activities of viral proteins, despite the fact that influenza viruses do not include protein-modifying enzymes. (70,71)

Cell lysis and protein isolation are often two of the first steps in the process of doing a proteomic analysis. At this key stage, inhibitors are introduced in order to prevent the degradation of proteins in a non-specific manner and to maintain the post-translational modification (PTM) status that is desired. These inhibitors work by blocking enzymes such as phosphatases and deacetylases. Trypsin, a protease that is regularly employed, was selected because it has a high level of specificity when it comes to cleaving peptide bonds. This results in the generation of peptides that are ideally suited for detection and fragmentation using mass spectrometry (MS). After that, the peptides are separated by employing nanoflow liquid chromatography (LC), which is then immediately attached to the MS system (LC-MS) so that they may be analysed. The overall efficiency of the measurements as well as their ability to be reproduced are both strongly impacted by the quality of the separation stage. The most effective chromatographic separation will result in the unique elution of peptides, which will in turn lead to enhanced signal intensities, correct peptide identification, and accurate protein quantification. In laboratories that study proteomics, cutting-edge LC technology, such as nanoflow ultrahigh-performance liquid chromatography (UHPLC), is frequently used to achieve quick and sensitive separations. This enables researchers to analyse small sample quantities in a relatively short amount of time. (72,73)

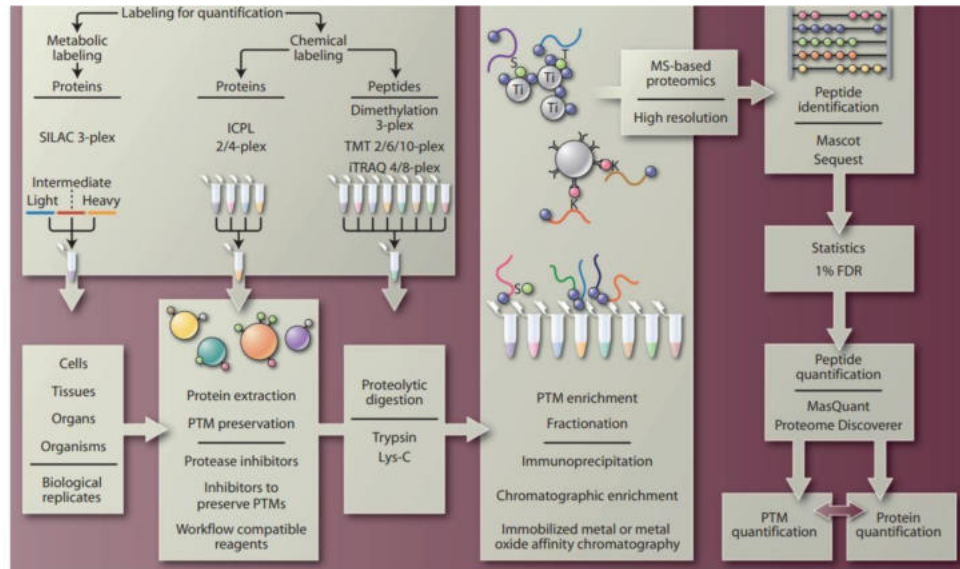


Figure 5: Work-flow for analyzing PTMs along with common enrichment methods for these PTMs (74)

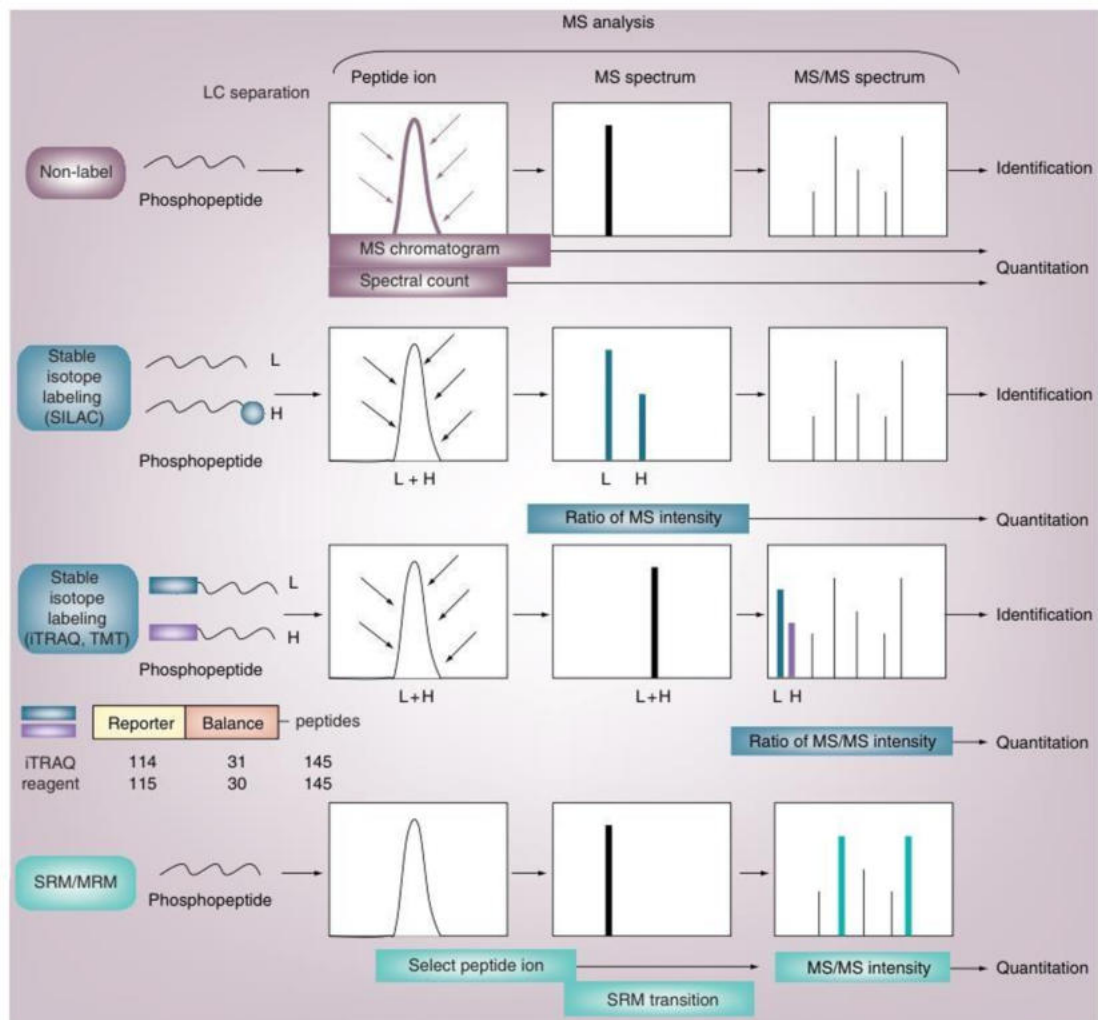


Figure 6: Quantification methods for liquid chromatography-tandem mass spectrometry analysis (75)

2.18.1. Phosphorylation

Protein phosphorylation, a type of post-translational modification (PTM) that has received a great deal of attention from researchers, is an essential component in the control of virtually all biological activities, including signal transmission, protein synthesis, and the regulation of transcription. Phosphate groups are moved from molecules such as adenosine triphosphate (ATP) or guanosine triphosphate (GTP) to particular locations on substrate proteins during this process. Within cells, a great number of proteins are subjected to the constant and dynamic phosphorylation and dephosphorylation at specific locations via complicated signalling networks, which are closely regulated. Protein kinases are responsible for the addition of phosphate groups to a protein through the process of phosphorylation, whereas protein phosphatases are responsible for the removal of phosphate groups through the process of dephosphorylation. Protein kinases, which are transmitted by over 500 genes in the human genome, are indispensable components of signal transduction pathways, highlighting the significance and complexity of phosphorylation in cellular processes. Notably, dysregulation of protein kinase-mediated signalling pathways has been linked to cancer, autoimmune diseases, metabolic disorders, and pathogenic infections. (76–78)

In order to obtain a comprehensive understanding of complex signalling networks, it is essential to develop analytical methods that can globally identify phosphorylated proteins and characterise their phosphorylation sites under various biological conditions. In addition, a thorough and therapeutically pertinent understanding of the cellular functions of individual protein kinases would benefit from a comprehensive understanding of their *in vivo* substrates. Nevertheless, the limited abundance of most signalling proteins, the low stoichiometry of phosphorylation events, and the dynamic regulation of phosphoproteins pose significant obstacles to these analytical strategies. Recent developments in diverse proteomic technologies have made possible a more sensitive, exhaustive, and quantitative analysis of protein phosphorylation in cells and tissues. The development of mass spectrometry (MS) technology has

enabled proteome-level analysis of protein phosphorylation, presenting an extraordinary opportunity to identify novel biomarkers and therapeutic targets. (71)

Numerous studies document the proliferation of phosphoproteomic research in the field of influenza biology and its extensive documentation. Hutchinson et al. conducted a comprehensive analysis of phosphorylation patterns within the proteomes of numerous influenza A viruses (IAVs) and an influenza B virus (IBV) in an early study of note. (79) This investigation uncovered an extraordinary 39 phosphorylation sites, which were present in nearly all virally encoded proteins. These results indicate that cellular kinases play a crucial role in the viral life cycle. In addition, a number of other studies have concentrated on profiling phosphorylation sites within specific viral components, such as NP and M1. These findings collectively provide a valuable foundation for investigating the functional significance of phosphorylation events in influenza biology, ultimately leading to the development of innovative antiviral treatment strategies. (80,81)

In addition to investigating phosphorylation modifications within influenza virus particles, scientists have also characterised the phosphoproteome of infected host cells. Diverse studies have utilised mass spectrometry-based phosphoproteomics techniques to investigate the phosphorylation events occurring in infected host cells. Weber et al. conducted an investigation using deep sequencing of the phosphoproteome in IAV-infected mouse lung cells (MLE-15). (82) The researchers combined immobilised metal affinity chromatography (IMAC) with antibodies specific to phosphotyrosine-containing peptides in order to achieve extensive coverage of phosphorylated proteins. This allowed them to accomplish their goal of identifying all phosphorylated proteins. This comprehensive technique made it possible to identify a large number of phosphorylated proteins, such as protein kinases and phosphatases, that showed signs of activation or deactivation in response to viral infection. These findings shed important light on the signalling network that is controlled by phosphorylation in host cells that have been infected with influenza A virus (IAV).

Similarly, Soderholm et al. investigated cellular pathways affected by IAV infection in human macrophages using a mass spectrometry-based phosphoproteomics method. (83) Before IMAC enrichment, they utilised robust cation exchange for fractionation. Their research identified a large number of human phosphoproteins whose phosphorylation patterns were altered by virus infection. The significance of cyclin-dependent kinase in viral replication and antiviral response was demonstrated by functional investigations.

In a separate study, Dapat et al. focussed on the phosphoproteome of a human lung cell line (A549) infected with an influenza A virus (A/H1N1) pandemic strain from 2009. (84) Phosphopeptides were enriched with a combination of titania and octadecylsilica (ODS) columns, then labelled with iTRAQ. The analysis identified 78 distinct phosphorylation modifications in infected A549 cells, highlighting the significance of RNA splicing for the efficient replication of IAV RNA.

Yanguéz et al. conducted a novel investigation into the dynamics of the phosphoproteome in A549 human lung epithelial cells at 5 and 15 minutes after IAV infection. (85) They utilised a quantitative proteomics technique based on SILAC coupled with Ti-IMAC and TiO₂ chromatography. This method revealed more than 200 distinct phosphorylations in host proteins. Notably, they identified kinases responsible for IAV infection, including G protein-coupled receptor kinases (GRKs), through bioinformatics analysis and functional validation experiments, demonstrating the high sensitivity of their method in detecting subtle changes in the phosphoproteome.

2.18.2. Ubiquitination and Ubiquitin-Like Modification

Substrate proteins undergo ubiquitination, a type of posttranslational modification (PTM), when a polypeptide called ubiquitin, which comprises 76 amino acids, is covalently attached to them. This linkage is formed via an isopeptide bond between a lysine residue on the substrate protein and a carboxyl group on the C-terminus of ubiquitin. (86) In eukaryotic species, the process of ubiquitination, which is analogous to the process of phosphorylation, governs a number of cellular functions, including as the breakdown of proteins, the transport of molecules, and the control of transcription. In addition to ubiquitin,

a number of other ubiquitin-like modifiers, such as SUMO (small ubiquitin-related modifier), NEDD8 (neural precursor cell-expressed developmentally downregulated-8), and ISG15 (interferon-stimulated gene 15 kDa protein), have been found. These ubiquitin-like modifications go via a process that is analogous to ubiquitination, which includes the participation of several enzymes in their respective reactions. The comprehensive study of ubiquitination modifications, much like the study of phosphorylation, focuses primarily on two aspects: a) the specific enrichment of ubiquitinated or ubiquitin-like proteins and peptides; and b) the identification of modification sites along the primary structure of the modified protein. Both aspects are essential to understanding how ubiquitination modifications work. (87)

2.18.3. Acetylation

The process of enzymatically adding acetyl groups to the N-terminal amino group and the amino group of the lysine residues in the substrate protein is known as protein acetylation. According to a large body of data, the enzymes lysine acetyltransferases (KATs) and lysine deacetylases (KDACs) play a major role in the regulation of lysine acetylation, which is thought to be highly regulated. (88) By removing the positive charge from the -amino group, the acetylation of lysine residues in the substrate protein modifies the protein's shape and affects its function. More than 2000 lysine-acetylated human proteins have been discovered, although histones and a few nucleoproteins have received substantial study. (74) On the other hand, N-terminal acetylation, which results in enhanced hydrophobicity of the N-terminus, happens during protein translation and is catalysed irreversibly by N-terminal acetyltransferases (NATs). Protein acetylation has a profound impact on how viruses interact with their host cells because it is involved in so many biological processes. (89)

2.18.4. Glycosylation

One of the most prevalent protein post-translational modifications (PTMs) is glycosylation, which entails the covalent bonding of oligosaccharide chains to polypeptide chains. The glycosylation process takes place on about half of the proteins in the human proteome. Glycosyltransferases identify particular recognition sequences and transfer the initial monosaccharide to the matching

site to start the process. The glycan chain is then incrementally extended by other glycosyltransferases. (90)

Numerous biological functions, such as cell adhesion, subcellular recognition, and cell communication, depend heavily on protein glycosylation. N-linked glycosylation and O-linked glycosylation are the two primary kinds of glycosylation in cells. With the development of proteomics, techniques based on mass spectrometry (MS) have become popular for examining glycopeptides and glycosylation sites at the proteome level. Using lectins to enhance glycoproteins before LC-MS/MS analysis is a useful technique. The glycans can be released from glycopeptides using peptide-N-glycosidase F for N-linked glycans or β -elimination for O-linked glycans. Following the removal of the glycan, the changes in peptide mass units are measured by mass spectrometry and are related to the type of glycosylation (N-linked or O-linked). The locations of glycosylation can be identified from MS/MS data. Although glycosylation can now be quickly and accurately detected, considerable work still has to be done to completely characterise this sort of alteration, especially when it comes to positional isomers of complex glycans. (91)

2.18.5. S-Acylation

The process of covalently attaching fatty acyl moieties, often the 16-carbon palmitoyl moiety, to particular cysteine residues in proteins is known as protein palmitoylation, also known as protein S-acylation. (92) This change is reversible in nature and is catalysed and removed by the enzymes palmitoyl acyl transferases and acyl protein thioesterases, respectively. Characterization of protein palmitoylation is difficult due to its dynamic and reversible nature. Analytical techniques that can profile palmitoylated proteins at the proteome level are required. The "palmitate-centric" approaches and the "cysteine-centric" approaches are two widely used techniques for identifying palmitoylation.

2.18.6. METHYLATION

Protein methylation is a prevalent type of post-translational modification (PTM) that takes place as a result of the transfer of a methyl group from S-adenosyl-L-methionine to substrate proteins by methyltransferase enzymes. This process

takes place after the proteins have been translated. This change has an effect on a number of biological processes, such as the trafficking of proteins, the repair of DNA, the processing of RNA, and the turnover of proteins. Lysine and arginine residues are the most likely candidates for protein methylation; however, other residues, such as aspartic acid, glutamic acid, histidine, cysteine, serine, and threonine, can also be methylated to a lesser extent. (93)

Protein methylation profiling at the proteome level is still in its infancy as compared to other PTMs that have been widely explored. The methodological development of analysing arginine methylation has recently advanced thanks to the use of numerous proteases, stable isotope labelling, and multidimensional liquid chromatography separation. (94,95) These methods allow for a more thorough and secure analysis of proteome-scale methylation. The creation of new analytical techniques with greater specificity and sensitivity is still required, though. These developments will help us comprehend how protein methylation controls different biological activities.

2.19. Conclusion

Mass spectrometry (MS) has completely changed the field of biomolecular research, notably in terms of protein post-translational modifications (PTMs). Due to its great sensitivity, adaptability for high-throughput analysis, and capacity to identify specific PTM sites in peptide sequences, MS has become a potent tool for PTM investigation. Different proteomic approaches based on MS have been developed for the investigation of the biological roles of influenza virus PTMs. Despite tremendous improvements in the study of the relationship between influenza and its host utilising proteomics technology, there are still a number of areas of this subject that need more research.

3. RATIONALE:

Understanding the host-pathogen interaction during influenza infections requires genomes and proteomics investigations. Genomics reveals host susceptibility and virus strain diversity. It identifies host variables that affect susceptibility, resistance, and immunity. Proteomics studies protein functions. Analysing the proteome of infected host cells reveals protein expression alterations, post-translational modifications, and viral-host protein interactions. This identifies immunological signalling molecules, antiviral proteins, and viral replication or pathogenesis components involved in the host response to influenza infection. Phosphorylation, a vital cell regulation process, alters protein structure, activating, deactivating, or modifying them. Thus, identifying these phosphorylated proteins requires understanding cell signalling mechanisms after viral infection. This will help to create new antiviral medicines and understand the disease's cellular processes. Kinases, phosphatases, and phosphor-binding proteins control these phosphoproteins. The structure and dynamics of these networks must be understood to properly comprehend cellular activities and illnesses. Phosphoproteomic profiling can reveal whether proteins or pathways are activated or changed by phosphorylation. This integrative approach can identify critical host factors, signalling pathways, and protein-protein interactions in influenza infection. It can also help find therapeutic targets, improve antiviral medications, and mitigate influenza outbreaks. Genomic and proteomics research are excellent tools for understanding influenza host-pathogen interactions.

CHAPTER 3

AIM AND OBJECTIVES

Aim:

Quantitative phosphoproteome profiling of circulating influenza virus strains in Mumbai region.

Objectives:

1. To establish infection of circulating influenza virus strains in A549 i.e. human lung carcinoma, epithelial cell lines.
2. Primer designing for target genes to study the immune response during the infection.
3. Gene expression analysis of the target genes at different time points post infections using SYBR green qRT-PCR chemistry.
4. To analyse proteomic profile of A549 cells infected with influenza viruses at different time points using quantitative proteomics approach
5. To analyse phosphoproteomic profile of A549 cells infected with influenza viruses using quantitative proteomics approach i.e. iTRAQ.
6. To compare proteome and phosphoproteomic profile of circulating influenza viruses.
7. Statistical and data analysis of gene expression; and omics data.

CHAPTER 4

MATERIALS AND

METHOD

4. MATERIAL AND METHODS:

4.1. REVIVAL OF A549 CELLS

4.1.1. Materials

- a. Cryovial containing Adenocarcinomic human alveolar basal epithelial cells (A549) frozen cells was purchased from National Centre for Cell Sciences (NCCS)
- b. Kaighn's Modification of Ham's F-12 Medium (F12K) (Gibco)
- c. Eagle's minimal essential medium (MEM) (Gibco)
- d. Fetal bovine serum (FBS) (Gibco)
- e. Cell culture vessel (Tissue-culture treated T-25 flask) (Corning)
- f. Disposable, sterile 15 ml centrifuge tubes (Tarson)
- g. Water bath at a temperature of 37°C
- h. Miscellaneous (Cotton swabs dipped in 70% alcohol, Plastic bag for discard (HiMedia), Gloves)
- i. 5% CO₂ incubator (37°C) (Eppendorf)

4.1.2. Protocol

- a. The cryovials containing the frozen A549 cells were removed from liquid nitrogen storage and immediately placed into a 37°C water bath.
- b. The cells were thawed by gently swirling the vial in the 37°C water bath.
- c. The vial are thoroughly swabbed 70% alcohol before opening in Biosafety cabinet level-2.
- d. Pre-warmed 20% complete F12K growth medium was transferred into the conical centrifuge tube containing the thawed A549 cells.
- e. The cells were then centrifuged at 1500 rpm for 10 minutes at 25°C.
- f. After the centrifugation, visibility of the cell pellet was checked. The supernatant was carefully decanted without disturbing the cell pellet.
- g. The cells were gently re-suspended in 20% complete F12K growth medium, and transferred into the tissue culture treated flask and incubated in 5% CO₂ incubator at 37°C for 24 hours.

NOTE: Before starting off with the work, all the reagents were kept at 37 °C for 30 minutes and simultaneously Bio-Safety Cabinet UV was kept ON for 30 minutes.

4.2. PASSAGING THE CULTURED CELLS

4.2.1. Materials

- a. T-25 flask with A549 cells, confluency around 80 - 90%
- b. Kaighn's Modification of Ham's F-12 Medium (F12K) (Gibco)
- c. Fetal bovine serum (FBS) (Gibco)
- d. 1X - Phosphate-buffered saline, pH 7.0- 7.2 (PBS) (Gibco)
- e. Pre-warmed 0.25% Trypsin – EDTA (Gibco)
- f. Cell culture vessel (6 or 12 well plate) (Corning)
- g. Disposable, sterile 15 ml centrifuge tubes (Tarson)
- h. Miscellaneous (Cotton swabs dipped in 70% alcohol, Plastic bag for discard (HiMedia), gloves)
- a. 5% CO₂ incubator (37°C) (Eppendorf)

4.2.2. Protocol

- a. The complete growth medium contained in the T-25 flask was aseptically decanted.
- b. By gently rocking the flask, the cells were washed with 1 mL PBS. This was followed by decanting the PBS without disturbing the monolayer.
- c. Add 1 ml of pre-warmed Trypsin-EDTA and the flask was placed in the CO₂ incubator (5% CO₂ at 37°C) until the cells detached.
- d. To check for detachment, the cells were observed using an inverted microscope. (If cells are < 90% detached, incubate for a few more minutes. Check for dissociation every 30 seconds. You may also tap the vessel to enhance cell detachment).
- e. Once more than 90% of cells had detached, an equal volume of 10% complete F12K medium or Fetal Bovine Serum (FBS) was IMMEDIATELY added to inactivate Trypsin-EDTA.
- f. The contents of the T-25 flask were emptied into a sterile conical centrifuge tube. Followed by centrifugation at 1500 rpm for 10 minutes at 25°C. The supernatant carefully decanted without disturbing the pellet. The pellet is re-suspended in a 1 ml of pre-warmed complete F12K growth medium and 10 ul is used to determine the cell count.
- g. The cell suspension is transferred into new cell culture flask and incubated in 5% CO₂ incubator at 37°C for 24 hours

NOTE: Before starting off with the work, all the reagents were kept at 37 °C for 30 minutes and simultaneously Bio-Safety Cabinet UV was kept ON for 30 minutes.

4.3. FREEZING

4.3.1. Materials

- a. T-25 flask with A549 cells, confluency around 80 - 90%
- b. Kaighn's Modification of Ham's F-12 Medium (F12K) (Gibco)
- c. Fetal bovine serum (FBS) (Gibco)
- d. Cryoprotectant - DMSO (Dimethyl sulphoxide)
- e. Pre-chilled Freezing Mixture (MEM: FBS: DMSO = 6:3:1), (2 to 8°C)
- f. 1X - Phosphate-buffered saline, pH 7.0- 7.2 (PBS) (Gibco)
- g. Pre-warmed 0.25% Trypsin – EDTA (Gibco)
- h. Cryogenic vials (Tarson)
- i. Disposable, sterile 15 ml centrifuge tubes (Tarson)
- j. Miscellaneous (Cotton swabs dipped in 70% alcohol, Plastic bag for discard (HiMedia), gloves)
- b. 5% CO₂ incubator (37°C) (Eppendorf)

4.3.2. Protocol

- a. Freezing mixture was prepared by adding Culture media, FBS and DMSO in the ratio 6:3:1 and stored at 2 to 8°C.
- b. The confluent A549 cells were detached by warm trypsinization. Once detached from the tissue culture flask, the cells were re-suspended in complete F12K growth medium.
- c. The cells were then centrifuged at 15,000 rpm for 10 minutes. The supernatant aseptically was decanted without disturbing the pellet.
- d. The total numbers of cells and percent viability were determined using a hemocytometer. Depending on the desired viable cell density, the required volume of freezing medium was calculated.
- e. The cell pellet was re-suspended in a pre-chilled (2 to 8°C) freezing mixture.
- f. Cryogenic vials were used for dispensing aliquots of cell suspension and storage.
- g. The cryogenic vials containing the cell suspension were placed in an isopropyl chamber where the temperature decreases approximately 1°C per minute.
- h. The frozen cells are first stored at –80°C overnight and later, the cryovials are transferred to liquid nitrogen storage in the gas phase at –196°C.

CAUTION: DMSO is hazardous. Thus it should be properly handled, stored, and disposed.

NOTE: Before starting off with the work, all the reagents were kept at 37 °C for 30 minutes and simultaneously Bio-Safety Cabinet UV was kept ON for 30 minutes.

4.4. INFLUENZA VIRUS PROPAGATION/INFECTION

For the experiments Influenza A Virus - pandemic H1N1, pH1N1 (clinical isolate), H3N2 (clinical isolate) and influenza B (clinical isolate) were used. Strains of influenza virus was propagated on MDCK cells and then acclimatized to A549 cells. Virus titer was measured using tissue culture infective dose (TCID₅₀) titration. The Spearman Karber formula was used to calculate TCID₅₀.

Calculations:

$$\log ID_{50} = \log (\text{highest dilution giving 100\% CPE}) + 0.5$$

-total number of test units showing CPE

Number of test units per dilution.

The virus stocks with MOI (Multiplicity of Infection) of 1 were used for infection. Confluent A549 cells were subjected to virus infection. (It must be noted that an overgrown monolayer is less susceptible to virus infection. Also, serum in the growth medium inhibits viral infection and must therefore be removed by PBS wash.)

4.4.1. Materials

- a. Influenza virus strains - pH1N1, H3N2 and Influenza B (Clinical isolates)
- b. Kaighn's Modification of Ham's F-12 Medium (F12K) (Gibco)
- c. Fetal bovine serum (FBS) (Gibco)
- d. 1X - Phosphate-buffered saline, pH 7.0- 7.2 (PBS) (Gibco)
- e. Virus growth medium (VGM) - 2 µg/ml TPCK (Tosyl phenylalanyl chloromethyl ketone) trypsin + 50 units/ml Nystatin + 0.2 mg/ml Gentamicin in F12K medium
- f. 6 well plates seeded with A549 cell line with 100% confluency
- g. Disposable, sterile 15 ml centrifuge tubes (Tarson)
- h. Miscellaneous (Cotton swabs dipped in 70% alcohol, Plastic bag for discard (HiMedia), gloves)
- i. 5% CO₂ incubator (37°C) (Eppendorf)

4.4.2. Protocol

- a. The complete growth medium in the 6-well plates was aseptically decanted without disturbing the monolayer. The monolayer was wash twice with 1X PBS.
- b. The vial of influenza virus stock was carefully thawed.
- c. The thawed virus was added to each well (except the cell control).
- d. Plates were incubated for an hour in a CO₂ incubator at 37°C.
- e. After incubation, virus growth medium supplemented with TPCK was added to each well and 2% complete F12K growth medium was added in cell culture control.
- f. Plates were incubated for 72 hours in a CO₂ incubator at 37°C to allow infection.
- g. After incubation, the virus was harvested in sterile 15 ml centrifuge tubes and Hemagglutination assay was performed to check the viral titer.
- h. Aliquots of the suitable viral titers were stored by dispensing in well-labeled cryogenic tubes.
- i. The cryogenic vials containing the cell suspension were placed in an isopropyl chamber where the temperature decreases approximately 1°C per minute, followed by storage at -80°C freezer.

4.5. HEMAGGLUTINATION ASSAY

4.5.1. Materials

- a. K2EDTA tube (BD Vacutainer)
- b. Guinea pig/ Chick blood
- c. 1X - Phosphate-buffered saline, pH 7.0- 7.2 (PBS) (Gibco)
- d. 96-well, v-bottom plate (Corning)
- e. Disposable, sterile 15 ml centrifuge tubes (Tarson)
- f. Influenza virus stock (strain pH1N1, H3N2 and Influenza B)
- g. Miscellaneous (Cotton swabs dipped in 70% alcohol, Plastic bag for discard (HiMedia), Gloves)
- h. 5% CO₂ incubator (37°C) (Eppendorf)

4.5.2. Protocol

i. Standardization of Red Blood Cells (RBC)

- a. Chick blood was collected in a K2EDTA (ethylenediamine tetra acetic acid) tube. (EDTA is a chelating agent and acts as anticoagulant which will bind and inhibit calcium ions which will block the coagulation cascade – thereby inhibiting clot formation)

- b. The whole blood was gently transferred in a 15 ml falcon tube and centrifuged at 1500 rpm for 10 minutes (acceleration-1 and break-1) at 25°C.
- c. The supernatant was discarded i.e., plasma and buffy coat layer using a clean pipette.
- d. The red cells were re-suspended in PBS (pH 7.0-7.2) with the aid of a micropipette, and the tube gently inverted to mix the contents.
- e. Followed by centrifugation for 10 minutes at 1500 rpm and the supernatant was discarded making sure not to disturb the RBC pellet.
- f. The 4th and 5th steps were repeated 3 to 4 times until the supernatant was clear.
- g. The clear supernatant was discarded and the RBCs were diluted with PBS to make a 10% RBC solution which was stored in the refrigerator at 2 to 4°C.

ii. Hemagglutination Assay

- a. 50 µl of PBS was pipetted into wells from 2 to 12 across in a 96-well V-bottom plate.
- b. 100 µl of the influenza virus stock was added into the first column of the 96-well plate, as a result this column was the most concentrated of all.
- c. A two-fold dilution series was performed across the 96-well plate by transferring 50 µl between wells, while performing dilution, the viral culture was mixed by gently re-suspending twice or thrice using a micropipette. The resultant 50 µl from the last well was discarded.
- d. 50 µl washed 0.75% RBCs were added to all wells. The plate was tapped gently to mix.
- e. An incubation period of 45 minutes at 25°C was given.
- f. The plate was observed for visible agglutination and viral titer of the sample was recorded. The last well showing complete agglutination is recorded as the viral titer.
- g. Virus stocks with desired HA titers were stored in cryogenic vials at -80°C.

4.6. Immunofluorescence Assay

4.6.1. Materials

- a. 1X - Phosphate-buffered saline, pH 7.0- 7.2 (PBS) (Gibco)
- b. Fluorescent dye-labelled antibodies – Influenza A and B (Thermo scientific)
- c. 4% paraformaldehyde (Sigma)
- d. 100% ice-cold methanol (Merck)

- e. 0.1% Triton (Merck)
- f. Bovine Serum Albumin - BSA (Sigma)
- g. 4',6-Diamidino-2-Phenylindole, Dihydrochloride - DAPI (Thermo scientific)
- h. 96-well plate seeded with A549 cells (Corning)
- i. Disposable, sterile 15 ml centrifuge tubes (Tarson)
- j. Influenza virus stock (strain pH1N1, H3N2 and Influenza B)
- k. Miscellaneous (Cotton swabs dipped in 70% alcohol, Plastic bag for discard (HiMedia), Gloves)
- l. 5% CO₂ incubator (37°C) (Eppendorf)

4.6.2. Protocol

- a. Seed 1.5×10^2 cells per well of a 96 well plate. Incubate the plates at 37°C in 5% CO₂.
- b. 24 - 48 hours post cell seeding, remove the cell culture medium and infected the cells with different strains of influenza.
- c. After infection, add virus growth medium to the wells, discard the VGM and rinse the cells thrice using 1X PBS.
- d. **Fixation:** After washing, the cells were fixed using 50 µl of 4% paraformaldehyde for 10 min at 37°C.
- e. Remove the paraformaldehyde solution from all the wells and wash the cells thrice with 1X PBS.
- f. Again, fix cells using 50 µl of 100% ice-cold methanol for 5 min at -20°C.
- g. Remove the methanol and wash the cells three times with 1X PBS.
- h. **Permeabilization:** After fixation, add 50 µl of 0.1% Triton X-100 made in 1X PBS and incubate the plate containing the cells at room temperature for 15 min.
- i. After incubation, remove the Triton-X-100 solution and wash the cells three times with 1X PBS.
- j. **Blocking:** Add 50 µl of 2% BSA made in 1X PBS in all the wells and incubate the cells at room temperature for 60 min. (Alternately, the cells can be incubated overnight in 2% BSA or 1X PBS before proceeding to immunostaining.)
- k. **Immunostaining:** Add 20 µl of fluorescent dye-labelled antibody diluted in 0.1% BSA along with a counterstain for the nucleus. DAPI was added to the wells in 0.1% BSA and incubate for 45 min at room temperature protected from light to the cells.

1. The antibody solution along with DAPI was removed and cells were washed three times with 1X PBS and the plates were observed under Cytation 7.

4.7. PRIMER DESIGNING

4.7.1. Tool used: NCBI Primer BLAST

4.7.2. Protocol

- a. The gene ID of the target gene was obtained from nucleotide GenBank (mRNA cds is preferred)
- b. Go to NCBI Primer BLAST submission form; paste the gene ID in the query box.
- c. Standardize and set the primer parameters as per requirement
- d. Select the product size (Min 80, Max 200)
- e. Select Max Tm difference [1]
- f. Select Database [nr]
- g. Add organism [*Homo Sapiens*]
- h. Select “Show results in new window”
- i. Click on “Get Primers”

4.7.3. List of primers

The primers were synthesized from Eurofins Genomics India Pvt Ltd.

Table 5: List of primers

Sr. No.	Gene	Type	Sequence	Amplicon size (bp)
	IFN- γ	Forward	TGAATGTCCAACGCAAAGCA	91
		Reverse	TGACTCCTTGTTGCTTCCC	
	IL-10	Forward	TGATGCCCCAAGCTGAGAAC	152
		Reverse	ATTCTTCACCTGCTCCACGG	
	IL-6	Forward	TGCAATAACCACCCCTGACC	163

Sr. No.	Gene	Type	Sequence	Amplicon size (bp)
		Reverse	GTGCCCATGCTACATTTGCC	
	β ACTIN	Forward	CCACCATGTACCCTGGCATT	189
		Reverse	CGGACTCGTCATACTCCTGC	
	TNF-A	Forward	CTCCCTCAGCAAGGACAGC	142
		Reverse	GAGCCGTGGGTCAGTATGTG	
	CCL5	Forward	TCATTGCTACTGCCCTCTGC	167
		Reverse	TCGGGTGACAAAGACGACTG	
	IFN-A	Forward	AGTCACCCATCTCAGCAAGC	122
		Reverse	GAGATCACAGCCCAGAGAGC	
	IL-8	Forward	CTCCAAACCTTTCCACCCCA	154
		Reverse	TTCTCCACAACCCTCTGCAC	
	IFITM-2	Forward	AGGACCCACACCACTAACA	84
		Reverse	AAAGAGGGGCTGAGGGCTAA	
	IFITM-3	Forward	TGCTGATCTTCCAGGCCTAT	101
		Reverse	TGCTGATCTTCCAGGCCTAT	

Sr. No.	Gene	Type	Sequence	Amplicon size (bp)
	RIG-1	Forward	CATGGCTTGGCAGGAAGTCT	136
		Reverse	CATGGCTTGGCAGGAAGTCT	
	MHC- 1	Forward	TTCTCAGGGAAAGCAGAAGTCC	199
		Reverse	CCTACACATCACAAACAGCCAC	

4.8. RNA EXTRACTION

4.8.1. Method used: Silica based spin-column for RNA extraction.

4.8.2. Materials

- Genes 2 me SpinRNA Viral RNA Extraction Kit
- Sample (6 well plates infected with pH1N1)
- AVL lysis buffer
- Absolute ethanol
- CW wash buffer
- AVE extraction buffer
- Micro centrifuge tubes (Tarson)
- Miscellaneous (gloves, micropipette, centrifuge, laminar flow hood)

4.8.3. Protocol

- The aim was to extract total cellular RNA using spin-columns (Genes 2 me SpinRNA Viral RNA Extraction Kit).
- The VGM was decanted from each well of the culture vessel, followed by giving a PBS wash.
- To each well 450 µl of AVL lysis buffer was added, aspirated vigorously using a micropipette.
- The entire 450 µl volume contained in the Eppendorf was transferred to the spin-column. The column was centrifuged at 10,000 rpm for 1 minute.

- e. The supernatant present in the collection tube of the spin-column was discarded and 500 µl of wash buffer was added. Followed by centrifugation at 10,000 rpm for 1 minute. The supernatant in the collection tube was discarded and the washing step was repeated.
- f. After a second wash, the column was given a dry-spin at 13,000 rpm for 3 minutes.
- g. Then, the collection tube was discarded and the column was added to an empty Eppendorf, 60 µl elution buffer i.e., AVE carefully was added over the filter of the spin-column. Followed by centrifugation at 13,000 rpm for 1 minute.
- h. The spin-column was discarded and the eluted RNA was stored at -80°C in well labeled micro centrifuge tubes.

4.9. QUANTIFYING GENE EXPRESSION USING q RT-PCR

4.9.1. Materials

- a. Lyophilized primers (Eurofins Genomics India Pvt Ltd)
- b. RNase free water
- c. PCR kit
- d. 0.2 ml PCR tube
- e. PCR instrument (QuantStudios 5 Real-Time PCR System)
- f. Miscellaneous (mini centrifuge, micropipette, gloves, cotton swabs soaked in 70% ethanol)

4.9.2. Protocol

- a. The lyophilized primers were reconstituted by dissolving in an appropriate volume of RNasefree water, this results in 100 pmol/µl solution. Let it equilibrate in a refrigerator at 2-8°C for about 12-24 hours.
- b. Forward and reverse primers were diluted to 10 µM.
- c. A different q RT-PCR master mix was prepared for each primer pair. It is crucial to prepare a sufficient mix for the samples as well as controls (calculate an extra 10% to allow for pipetting error).
- d. 8 µl of PCR master mix was dispensed into each tube and 2 µl of template (i.e., extracted RNA) was added in sterile 0.2 ml PCR tube to make the total volume 10µl.
- e. The desired template file with optimal parameters was set up in the quantitative PCR instrument (QuantStudios 5 Real-Time PCR System) before initiating the run.

- f. The tubes were placed into the instrument and designated properly before commencing the run.
- g. The fold change was analyzed and the data was interpreted after the run is completed.

4.9.3. q RT- PCR Master Mixture

Table 6: Components of PCR master mix

Reagents	Role
Template RNA	The target RNA that needs to be amplified
Primers- forward & reverse	Serve as complementary regions to the 5' & 3' end of the target RNA respectively
Reverse transcriptase	Enzyme to produce cDNA from template RNA strand.
dNTPs	Building blocks from which the new DNA strand is synthesized.
PCR buffer	Provides minimal optimum conditions for enzyme activity
ROX	It is a reference dye that does not take part in the PCR reaction but allows normalization of non-specific signals. Thus, provides a baseline in PCR reactions

4.9.4. Composition of PCR Master mix for q RT-PCR

Table 7: Composition of q RT-PCR master mix

Reagents	Volume 1X μ l @ 0.4 μ M primer conc.	Volume 1X μ l @ 0.6 μ M primer conc.	Volume 1X μ l @ 0.1 μ M primer conc.
TBG buffer	5	5	5
Enzyme	0.4	0.4	0.4
Forward primer	0.4	0.6	0.1
Reverse primer	0.4	0.6	0.1
Nuclease free water	2.6	2.2	3.2

ROX	0.2	0.2	0.2
Template	1	1	1

Optimized parameters used in the Real-Time PCR system, on the software program, as specified by the manufacturer.

4.9.5. q RT-PCR Cycle conditions

	Hold stage		PCR stage			Melt curve stage		
Temp (°C)	50	95	95	53	72*	95	60	95
Time (sec)	180	300	10	30	45	15	60	1
Cycles	1		40			Dissociation*		

* OPTICS ON

4.10. CALCULATION OF FOLD CHANGE

- In the final stage, melt curve analysis was carried out.
- Cycle threshold (CT) values of samples satisfying the melt curve analysis were taken up and further processed. The relative quantification of the change in gene expression patterns of individual genes with respect to the housekeeping gene β -Actin was calculated using 2-ddct method.
- The CT values obtained through individual gene assays were exported to a Microsoft Excel sheet and calculations were done.

The formulas used to determine gene expression is as follow:

$$\text{DDCT} = (\text{CT gene of interest} - \text{CT internal control})$$

$$\text{Fold change} = 2^{-\text{DDCT}}$$

$$2^{-\text{DDCT}} = [(\text{CT gene of interest} - \text{CT internal control}) \text{ Sample A} - (\text{CT gene of interest} - \text{CT internal control}) \text{ Sample B}]$$

4.11. STATISTICAL ANALYSIS

- The gene expression of each cytokine gene was expressed as fold change in the infected cells with respect to their respective time point uninfected controls and the fold change between the infected cells at 24 hrs post infection with respect to 4 hrs post infection.
- The triplicate fold change values were subjected to t-test. Graphs were plotted out and assessed using Graph Pad Prism Software (Graph Pad Inc. v. 9)

4.12. AGAROSE GEL ELECTROPHORESIS

4.12.1. Materials

- a. 50X TAE buffer (Thermo Fisher)
- b. Agarose powder (Thermo Fisher)
- c. Ethidium bromide (EtBr) (Thermo Fisher)
- d. Molecular Ladder (GeneRuler 1 kb DNA Ladder - Thermo Fisher)
- e. Sample (Amplified PCR product)
- f. Gel box
- g. Power pack
- h. Loading dye (Invitrogen 6X DNA Loading dye - Thermo Fisher)
- i. Miscellaneous (well combs, beaker, measuring cylinder, microwave)
- j. Gel Documentation system (BioRad ChemiDoc XRS+)

4.12.2. Protocol

- **1% Agarose gel preparation**

- a. 1 g of agarose was weighed.
- b. The agarose powder was mixed with 100 ml of 1X TAE buffer in a glass beaker.
- c. The solution was heated in a microwave for 2-3 minutes, swirling in between until the agarose was completely dissolved.
- d. The agarose was cooled up to 50°C for about 5 minutes.
- e. EtBr was added to a final concentration of approximately 0.2-0.5 µg/ml.
- f. The agarose was poured into the gel tray and the comb was placed. The gel was allowed to solidify at room temperature.
- g. Loading buffer was added to each sample. (2 µl + 2 µl)
- h. Once the gel is solidified, it was placed into the gel box (electrophoresis unit).
- i. Fill the gel box with 1X TAE buffer until the gel was covered.
- j. 4 µl of the molecular weight ladder was added to the first well followed by the samples into the additional wells.
- k. The gel was run at 100 V until the dye line was approximately 75-80% of the way down the gel.
- l. The power was turned OFF, electrodes were disconnected from the power source, and then the gel was carefully removed from the gel box.
- m. The bands were visualized using the Bio-Rad gel documentation system

4.13. SAMPLE PREPARATION:

- In-solution trypsin digestion

4.13.1. Materials

- Halt TM Protease and Phosphatase Inhibitor Cocktail (100X) – (Thermo Scientific)
- Triethyl ammonium bicarbonate - (Thermo Scientific)
- MiliQ water

- Composition of 2% SDS lysis buffer

2% SDS

1 mM sodium orthovanadate	}	or Protease and Phosphatase Inhibitor Cocktail (1X)
2.5 mM sodium pyrophosphate		
1 mM beta-glycerophosphate		
50 mM triethyl ammonium bicarbonate		

These reagents can be mixed and make up the volume in miliQ water for the required volume of lysis buffer.

4.13.2. Protocol

- The cell samples infected with different strains of influenza virus at different time points were subjected for cell lysis by incubating with 500 µl of 2% SDS lysis buffer.
- Later, the samples were subjected to repeated sonication on ice (three times with 10-sec pulse and 30% amplitude each).
- The sonicated samples then will be heated at 90°C in a dry bath for 5 minutes.
- The lysates are then allowed to cool to room temperature and centrifuge at 12,000 rpm for 10 minutes.
- The supernatant was collected and protein concentration was estimated using the BCA assay and was also confirmed visually resolving on a 10% SDS-polyacrylamide gel electrophoresis (PAGE) gel.

4.14. PROTEIN ESTIMATION BY BCA ASSAY AND SDS-PAGE

The protein concentration of lysates is estimated using a Bicinchoninic acid assay (BCA) protein estimation kit following the vendor's protocol, and equal amounts of protein from each sample were further used for downstream processing. Once the quantity is estimated, the protein extract was subjected to SDS-PAGE to measure the amount of protein and quality check.

4.14.1. BCA Protein Assay

- **Preparation of the BCA working reagent (WR)**

- a. To create a working reagent (WR), mix 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A: B). For the above example, combine 50 ml of Reagent A with 1 ml of Reagent B.
- b. Prepare an adequate volume of working reagent (WR) based on the number of samples to be tested. The reagent remains stable for several days, when stored in a sealed container at room temperature (RT).

- **Protocol**

- c. Pipette 20 μ L of each standard or unknown sample replicate into a microplate well (working range = 20 – 2000 μ g/ml)
- d. Add 200 μ L of the WR to each well and mix the reagent in the plate thoroughly using a plate shaker for 30 seconds.
- e. Cover plate with an aluminum foil and incubate at 37°C for 30 minutes.
- f. After incubation, measure the absorbance at 560 nm on a plate reader.
- g. Prepare a standard curve by plotting the OD obtained at 560 nm measurement for each BSA standard vs. its concentration in μ g/ml.
- h. Use the standard curve to determine the protein concentration of each unknown sample.

4.14.2. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE):

4.14.3. Procedure:

A) Casting the Gel

- a. The glass plates and spacers were cleaned and wiped with 70% Isopropyl Alcohol followed by DDI H₂O to avoid contamination.
- b. Glass plates and spacers were assembled in gel casting apparatus.

- c. **Preparation for the Resolving gel:** The components for the resolving gel were mixed as mentioned in the table below.

Resolving Gel	10%	
	1 gel	2 gels
DDI H ₂ O	1.6 ml	3.2 ml
1.5 M Tris-HCl, pH 8.8 (RG Buffer)	1.3 ml	2.6 ml
40% Acrylamide Stock	1.0 ml	2.0 ml
20 % SDS	100 µl	200 µl
30% Ammonium Persulfate	10 µl	20 µl
TEMED	4 µl	8 µl

Note: Always add Ammonium Persulfate and TEMED immediately before casting the gel.

- d. Pour the resolving gel mixture into the gel plates upto a level 2 cm below the top of the shorter plate.
- e. Place a layer of Isopropyl alcohol over the top of the resolving gel to prevent the formation of meniscus in the resolving gel.
- f. Allow the resolving gel to stand for 30 minutes at RT.
- g. Remove the layer of Isopropyl alcohol from top of the resolving gel properly. Rinse the gel with DDI H₂O. Drain the DDI H₂O, and wick any remaining DDI H₂O away using a Kimwipe.
- h. **Preparation for the Stacking gel:** The components for the stacking gel were mixed as described below

Stacking Gel (4 %) Composition		
	1 gel	2 gels
DDI H ₂ O	1.95 ml	3.9 ml
1.0 M Tris-HCl, pH 6.8 (Stacking Gel Buffer)	250 µl	500 µl

40% Acrylamide Stock	250 μ l	500 μ l
20 % SDS	50 μ l	100 μ l
30% Ammonium Persulfate	8 μ l	16 μ l
TEMED	4 μ l	8 μ l

Note: Always add Ammonium Persulfate and TEMED immediately before casting gel.

- i. Pour the stacking gel solution into gel plates (on top of the running gel i.e., the resolving gel), completely filling the gel plate. Insert comb on the top of the spacers.
- j. Allow the stacking gel to stand for at least 1 hour at RT, and check if the gel is solidified.

B) Protein sample preparation for SDS-PAGE

- a. Place a volume of protein solution (5 μ l of sample) into a microfuge tube, such that there is 5 - 10 μ g of protein in the solution (Check the protein concentration using BCA assay).
- b. Add an equal volume of 2x sample loading buffer.
- c. Incubate the tubes at 90°C for 5 minutes.
- d. Cool and Centrifuge at 4000 rpm for 30s.

C) Resolving extracted proteins on the Gel

- a. Remove comb and assemble cast gel into Mini-Protean II apparatus (Bio-Rad).
- b. Add freshly prepared 1x running buffer (500 ml) to both chambers of the apparatus.
- c. Load the prepared samples into the wells of the gel.
- d. Run the gel at 100 V on Power pack provided by Bio-Rad (PowerPack Basic) until the dye front migrates into the Resolving gel from stacking gel (~15 minutes), and then increase to 200 V until the dye front reaches the bottom of the resolving gel.

D) Staining & Destaining the Gel

- a. Remove the run gel from the apparatus and remove the spacers and glass plates. Place the gel into a small tray.
- b. Add ~ 50 ml staining solution and stain for 1 hour with gentle shaking or overnight if not on shaker.
- c. Discard the staining solution.

- d. Add ~50 ml of destaining solution and destain for ~ 15 minutes with gentle shaking in between.
- e. Remove the destain solution and again add ~ 50 ml of fresh destain solution.
- f. Destain with gentle shaking until the gel is visibly destained.
- g. Discard the destain solution.
- h. Add ~100 ml DDI H₂O and rinse for 5 minutes with gentle shaking.
- i. Observe the gel under Gel Document System.

4.15. REDUCTION, ALKYLATION, AND PROTEIN DIGESTION

4.15.1. Materials

- a. 1,4-Dithiothreitol (DTT) - (Sigma)
- b. Iodoacetamide (IAA) – (Sigma)
- c. Acetone – (Merck)
- d. 50 mM triethyl ammonium bicarbonate buffer – (Thermo Scientific)
- e. Trypsin/Lys-C Mix, Mass Spectrometry Grade – (Promega)

4.15.2. Protocol

- a. To reduce the cysteine bridges, samples were incubated with 10 mM of DTT for 30 minutes at 60°C.
- b. Post reduction, the samples were alkylated with 20 mM of IAA for 20 minutes under the dark at RT to stop the rearrangement of cysteine bridges.
- c. All ten samples, post-reduction, and alkylation were subjected to acetone precipitation. The samples were transferred to chilled acetone for overnight protein precipitation.
- d. The next day, the samples were centrifuged at 10,000 rpm for 30 minutes, the supernatant was removed and the pellet was dissolved in 200 µl of 50 mM TEABC buffer.
- e. Further, the samples were checked for their pH and optimized to pH 7.0 with 50 mM TEABC and water.
- f. The sequencing grade trypsin was prepared at a concentration of 20 µg/µl for protein digestion.
- g. To the tubes incubated on ice, enough amount of trypsin solution was added and incubated at 37°C overnight.

4.16. POST-DIGESTION CHECK BY SDS-PAGE

4.16.1. Protocol

- a. Next day, SDS-PAGE was performed for digested samples.
- b. 10 µl of the digested samples were taken and run using 10% of the SDS-PAGE for 2 hours at 80V and 120V.
- c. The gel was separated and stained using Colloidal coomassie blue solution for 15 minutes, followed by destaining using 50:40:10 of Methanol: Water: Acetic acid. The gel is observed under Observe the gel under Gel Document System (Bio-Rad)

4.17. DESALTING BY WATERS SEPPAK C18 1CC CARTRIDGE:

4.17.1. Materials:

- a. SepPak C18 1cc cartridge - (Waters)
- b. Acetonitrile - LC-MS grade - (Merck)
- c. Formic Acid - LC-MS grade - (Merck)
- d. C18 StageTips – (Thermo scientific)
- e. Water - LC-MS grade - (Merck)

4.17.2. Protocol

- a. The peptides formed are desalted to remove all the DTT, IAA, and SDS present in the samples by Waters SepPak C18 1cc cartridge.
- b. Samples with 200 µg equivalent peptides were diluted further 10 times using 0.1% FA in water.
- c. Desalting was initiated with activation of the C18 bed by passing through 100% acetonitrile, followed by conditioning with 0.1% FA twice.
- d. Later, peptide mixture samples were loaded and passed through the C18 cartridge slowly, hence all the peptides will be available for the C18 bind.
- e. Washing of C18 cartridges post-sample loading was performed twice using 0.1% FA to remove DTT, IAA, and SDS.
- f. Now, peptides bound to the C18 cartridge were eluted slowly by passing 50% ACN in 0.1% FA solution twice.
- g. The complete desalting step using C18 StageTips was performed with the help of MicroCL 21R Microcentrifuge at the speed of 500 g under RT.

4.18. PEPTIDE ESTIMATION AND NORMALIZATION

4.18.1. Protocol

- a. The samples were resuspended with 200 μ L of 50 mM TEABC buffer and vortexed for 2 minutes.
- b. The peptides in the samples were quantified using Peirce Quantitative Colorimetric Peptide Assay (Thermo Scientific) as per vendor protocol.
- c. Briefly, the BSA digest standard provided in the kit was used to prepare 7 standards by serial dilution for the calibration curve.
- d. Samples were diluted 20 times by adding Milli-Q water and 20 μ l of the same was taken for the estimation.
- e. Both standard, sample and blank were mixed with 180 μ L of working reagent (50:48:2 of Reagent A: Reagent B: Reagent C) in the wells of 96 well plate by brief rotation and finally, kept for incubation at 22°C for 15 minutes.
- f. Post incubation, the plate was subjected to colorimetric estimation at 480 nm wavelength using Multiskan Sky Microplate Spectrophotometer (Thermo Scientific).
- g. The absorbance from the standard was used to plot the standard curve and the sample absorbance was used to calculate their concentration against the standard curve.

4.19. ISOBARIC TAGGING FOR RELATIVE AND ABSOLUTE QUANTITATION (ITRAQ) LABELLING AND LABEL CHECK:

4.19.1. Protocol

- a. Based on peptide estimation, the sample volume was normalized to lowest concentration carrying sample. The iTRAQ reaction was performed as per vendor protocol.
- b. Briefly, samples were resuspended in equal volumes of resuspension buffer and iTRAQ labels dissolved in 100% isopropanol were added to the sample, vortexed for 30 seconds, centrifuged at 8,000 rpm for 2 minutes and kept for incubation at room temperature for 2 hours.
- c. After 2 hours of incubation, 5 μ l of sample from each were aliquoted, and pooled.
- d. Please find the table 8 for details of the iTRAQ and samples.

- e. All the samples were pooled together and kept for drying by vacuum evaporation (Savant™ SPD1010 SpeedVac Concentrator, Thermo Scientific) at RT.

Table 8: Details of iTRAQ tag used for each sample

iTRAQ tag	Sample ID	Sample
113	C	Control
114	B_0 hrs	Influenza B
115	B_8 hrs	Influenza B
116	H1_0 hrs	pH1N1
117	H1_8 hrs	pH1N1
118	H3_0 hrs	H3N2
119	H3_8 hrs	H3N2

4.20. LC-MS/MS ACQUISITION AND DATABASE SEARCH OF ITRAQ

LABEL CHECK SAMPLE:

4.20.1. Protocol

- a. The iTRAQ labeled samples were desalted using C18 StageTips as mentioned in Methods > Section 5.
- b. The desalted labeled peptide mixture samples were resuspended with 0.1% FA in water (LC-MS grade) and transferred to a 96-well plate.
- c. The plate is placed in the Thermo Scientific EASY nLC 1200 liquid chromatogram system, which is connected to the Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific).
- d. The sample was acquired in a 30 minutes DDA method, where peptides were eluted by passing Mobile Phase B (0.1% FA in 80% ACN) in gradient flow at a 300 nL/min flow rate.
- e. The acquired raw data files were searched against a Human RefSeq protein database.

- f. The search was performed using Proteome Discoverer (version 2.2, Thermo Scientific) using Sequest HT and the Mascot search engine.
- g. All the other search parameters were set as mentioned in table 8.
- h. Based on the label check data, the normalization factor was calculated for each iTRAQ channel.
- i. As per the normalization factor, the volume of the sample to be taken for iTRAQ labeling was calculated. Accordingly, all the samples were pooled.

4.21. PHOSPHOPEPTIDE ENRICHMENT USING THE IMMOBILIZED METAL ION AFFINITY CHROMATOGRAPHY (SIMAC) METHOD AND FRACTIONATION

4.21.1. Protocol

- a. Phosphopeptide enrichment was carried out using the SIMAC method [FeNTA method followed by TiO₂ method] as per vendor protocol.
- b. The enriched phosphopeptide samples were then subjected for fractionation.

4.21.2. Phospho-enrichment of peptides using Fe-NTA beads

- a. Enrichment of phosphopeptides was done using Fe-NTA Phosphopeptide Enrichment Kit (Thermo Fisher Scientific, A32992).
- b. Enrichment was done according to the manufacturer's protocol. Briefly, the vacuum-dried peptides were resuspended in a binding buffer provided in the Fe-NTA Kit and loaded to the column provided in the kit.
- c. It was further incubated for 30 minutes at room temperature with gentle taping every 5 minutes. After washing off unbound peptides, phosphopeptides were eluted with 50 µl elution buffer.
- d. The eluted phosphopeptides were vacuum dried and stored at – 20°C until further use.

4.21.3. Phospho-enrichment of peptides using TiO₂ beads

- a. TiO₂ was used to enrich phosphopeptides using the method that was previously mentioned. After being dried in a vacuum concentrator, the phospho Fe-NTA enrichment flow-through was resuspended in a solution containing 2,5-dihydroxybenzoic acid (DHB) (5% DHB, 80% ACN, and 3% TFA; HPLC grade).

- b. Dehydrated TiO₂ beads (GL Science 5020-75010) were then resuspended in a 5% DHB solution. On a rotator, the beads were incubated at room temperature for 15 minutes.
- c. Equal amounts of the produced beads were added to each sample before being incubated for an hour on a rotator at room temperature.
- d. After the incubation, the beads were spun down and successively washed with three different solutions, including wash solution 2 (80% ACN, 1% TFA, and LCMS grade), solution 3, and solution 1 (80% ACN, 0.1% TFA, and LCMS grade).
- e. The phosphopeptides were then extracted by adding elution solution (4% NH₄OH, LCMS grade water) to a microfuge tube that already contained 10 µl of 3% formic acid that had been placed on ice. The dried, vacuum-concentrated phosphopeptides were then kept at -20°C until further usage.

4.22. FRACTIONATION OF THE LABELLED SAMPLES BY C18 STAGETIPS:

4.22.1. Protocol

- a. A 200 µl addition of 0.2% TFA and a 60-second vortexing procedure were used to reconstitute the dried mixture of tagged peptides from the samples.
- b. A StageTip with 8 discs that contained C18 sorbent was also created in tandem. By passing the discs through 100 µL of 100 % LC-MS grade ACN and 0.2 % TFA, respectively, the discs were activated and equilibrated.
- c. After that, the reconstituted samples were put into the StageTip that had been prepared and run through twice.
- d. The unbound peptide-containing flow-through was gathered and kept in a deep freezer. The samples were twice washed with 100 µl of 0.2 % TFA to remove salts and surfactants.
- e. In contrast, the polarity of the peptides that were attached to the C18 sorbent determined how gradually they were eluted.
- f. For this elution procedure, 100 µl of 24 elution buffers containing various ACN concentrations were passed through 10 mM TEABC in water.
- g. Lastly, 100 µl of 80% ACN in 10 mM TEABC in water was passed to elute the residual peptides.

- h. The StageTips were centrifuged at 500 g for 15 minutes at room temperature to complete the aforementioned procedures.

Table 9: Composition of buffers used for SCX fractionation

Fraction	Acetonitrile	Volume mobile phase A	Volume mobile phase B
number	(%)	(μl)	(μl)
1	4	96	4
2	6	94	6
3	8	92	8
4	11	89	11
5	13	87	13
6	15	85	15
7	17	83	17
8	19	81	19
9	23	77	23
10	25	75	25
11	28	72	28
12	30	70	30
13	33	67	33
14	36	64	36
15	40	60	40
16	45	55	45
17	48	52	48
18	50	50	50
19	52	48	52

20	56	44	56
21	61	39	61
22	69	31	69
23	75	25	75
24	80	20	80

After the fractionation, 24 fractions were reduced down to 6 by concatenate pooling. Where, fractions 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 were pooled with 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 and 24 respectively and were again reduced to 6 fractions by pooling 1, 2, 3, 4, 5, and 6 with 7, 8, 9, 10, 11 and 12. All 6 fractions were kept for drying by vacuum evaporation (SpeedVac, Thermo Scientific), respectively.

4.23. LC-MS/MS DATA ACQUISITION:

4.23.1. Protocol

- Thermo Scientific Orbitrap Fusion Tribrid mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) connected to Easy-nLC-1200 nanoflow liquid chromatography system (Thermo Scientific) was used for data acquisition as described previously.
- The peptides were reconstituted in 0.1 % formic acid and loaded onto a 2 cm trap column (nanoViper, 3 μ m C18 Aq) (164213, Thermo Fisher Scientific, USA). Peptide separation was done using a 50 cm analytical column (EASY-Spray column PepMap RSLC, C18, 2 μ m, 100A, 75 μ m x 15 cm) at a flow rate of 300 nl/min.
- The solvent gradients were set as a linear gradient of 5-35 % solvent B (80 % acetonitrile in 0.1 % formic acid) for 90 min.
- The total run time for each fraction was 120 min. Global MS survey scan was carried out at a scan range of 400-1600 m/z mass range (120,000 mass resolution at 200 m/z) in a data-dependent mode using an Orbitrap mass analyzer.
- The maximum injection time was 20 ms and the AGC target was set to 2.0e5.
- Only peptides with charge states 2-7 were considered for analysis, and the dynamic exclusion rate was set to 30 s.

- g. For MS/MS analysis, data was acquired at top speed with the most intense precursor selection scan event 1 and subjected to higher collision energy dissociation with 35 % normalized collision energy.
- h. MS/MS scans were carried out at a range of 110-2000 m/z using an Orbitrap mass analyzer at a resolution of 50,000 at 200 m/z. The maximum injection time was 200 ms.

4.24. DATABASE SEARCH:

4.24.1. Protocol

- a. All the raw files were searched for peptides and proteins separately using Mascot and Sequest HT search engines present in Proteome Discoverer 2.2.
- b. The proteomic database search was performed against the *Homo sapiens* proteome database (RefSeq version 108). Protein sequences were theoretically digested using protease trypsin with a maximum of 2 missed cleavages and searched against the LC-MS/MS generated peptide precursor and fragment ions (b and y ions) with 10 ppm and 0.05 Da peptide and fragment mass tolerance respectively.
- c. Modifications such as carbamidomethylation at cysteine (C), iTRAQ at lysine and peptide N-termini, oxidation at (M), and acetylation (Protein N-termini) were included as fixed and variable modifications.
- d. For phosphoproteomics search, phosphorylation at serine (S), tyrosine (Y), and threonine (T) was added as dynamic modifications, and phosphoRS node for the identification of PTMs with site probability of greater than 75%.
- e. To calculate the FDR, a decoy database was generated by reversing the amino acid sequences at peptide and protein levels. The PSMs identified with FDR less than 1% (q-value <0.01) are considered true matches.

Table 10: Database search parameters used in Proteome Discoverer 2.2

Parameters	
Database	<i>Homo sapiens</i> (RefSeq Version 109)
Search engines	Mascot and Sequest HT
Enzyme	Trypsin

Max. missed cleavage	2
Precursor mass tolerance (ppm)	5
Fragment mass tolerance (Da)	0.02
iTRAQ (Fixed)	K
iTRAQ (Fixed)	Peptide N-termini
Oxidation (Variable)	M
Acetylation: +42.011 Da (Variable)	Protein N-Termini
Phosphorylation +79.9799 Da (Variable)	S, T, Y
Carbamidomethylation: +57.021 Da (Fixed)	C

4.25. Data analysis

The Proteome Discoverer results file was utilized for additional analysis. Fold change values were computed, and heat maps were generated using the Multiple Array viewer. Gene Ontology analysis was performed using the DAVID 8.6 functional annotation tool to classify and categorize the proteins. Additionally, pathway analysis was conducted using the KEGG to further understand the pathways associated with the identified proteins. The proteins were also classified based on their functions by referring to data from PANTHER database.

CHAPTER 5

RESULTS

5. RESULTS

5.1. Cell Culture Propagation

The Madin-Darby Canine Kidney (MDCK) and Adenocarcinomic human alveolar basal epithelial (A549) cells were revived, propagated, and maintained at 37°C with 5% CO₂.

5.1.1. Madin-Darby Canine Kidney (MDCK) cells

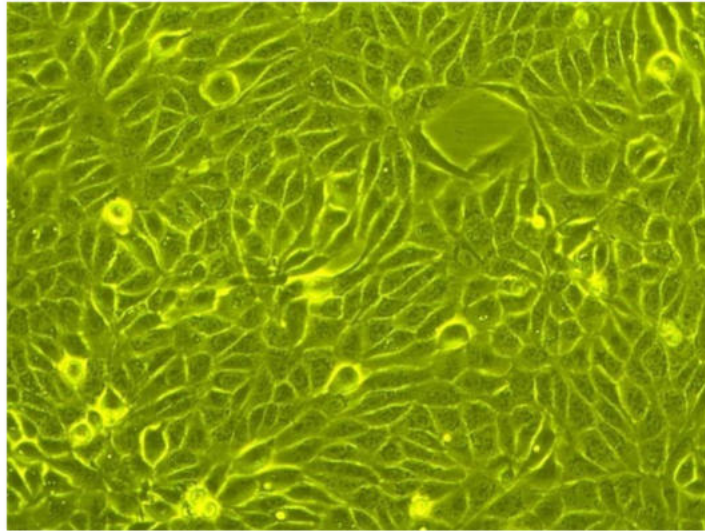


Figure 7: MDCK cells observed under Carl Zeiss Axiovert 40 CFL (200X magnification)

5.1.2. Adenocarcinomic human alveolar basal epithelial (A549) cells

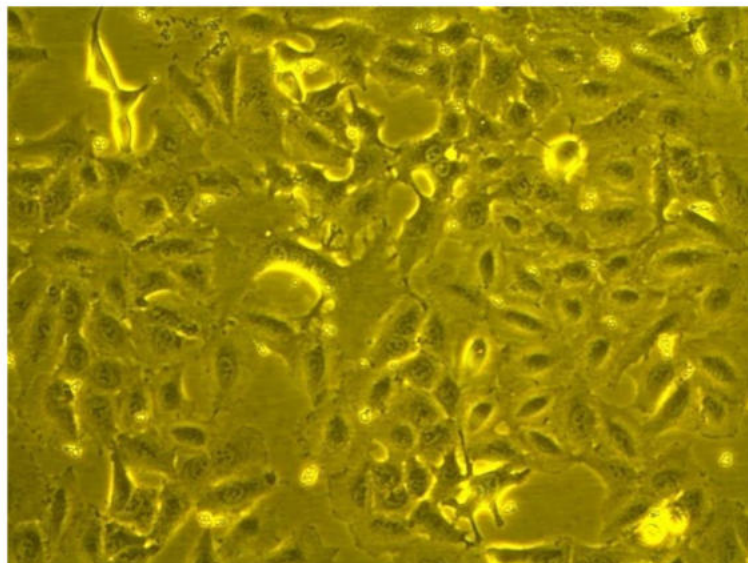


Figure 8: A549 cells observed under Carl Zeiss Axiovert 40 CFL (200X magnification)

5.2. Virus Propagation

For the experiments, different strains of influenza i.e., Influenza A (pH1N1, H3N2) and Influenza B Influenza Virus were used. Strains of influenza virus was propagated on MDCK cells and then acclimatized to A549 cells. Virus titer was measured using Haemagglutination and tissue culture infective dose (TCID₅₀) titration assay. The Spearman Karber formula was used to calculate TCID₅₀. (96)

5.2.1. Virus propagation on MDCK cells:

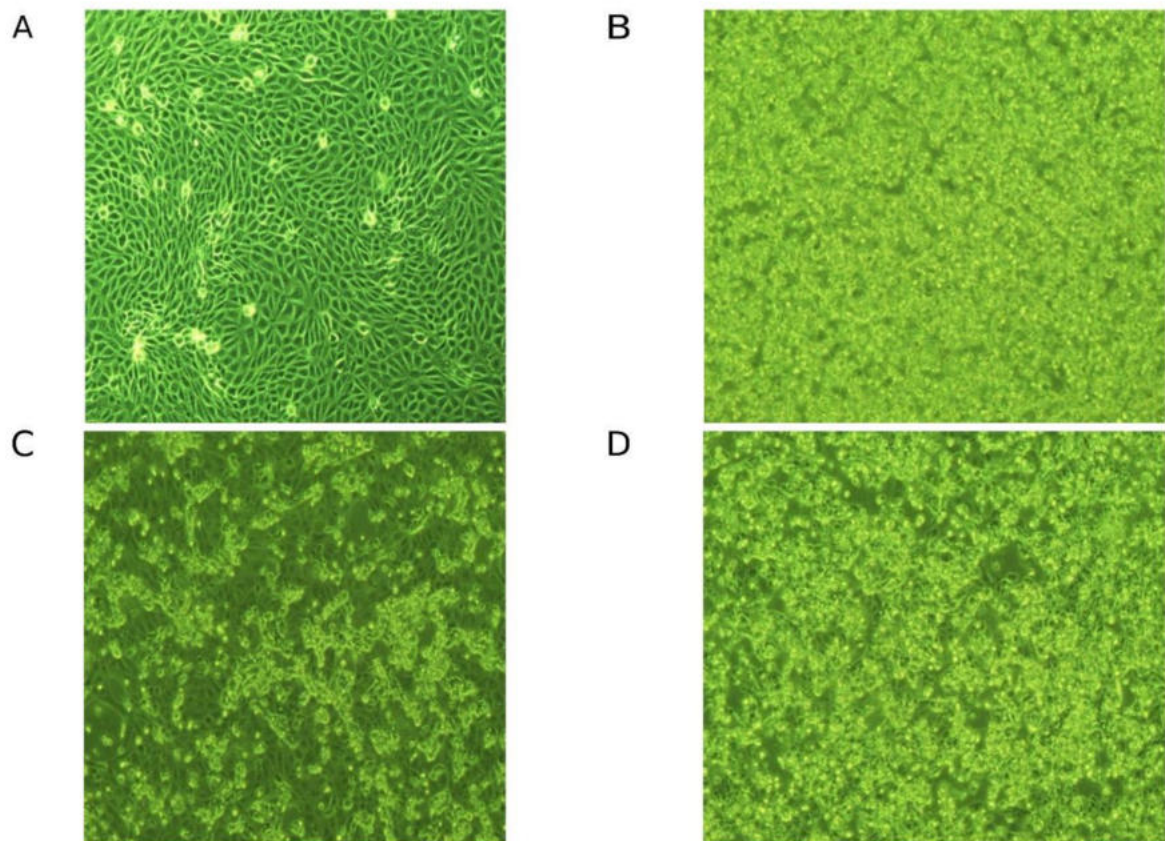


Figure 9: **A** – Mock infected A549 cells observed under Carl Zeiss Axiovert 40 CFL (100X magnification) **B** – A549 cells infected with pH1N1 virus observed under Carl Zeiss Axiovert 40 CFL (100X magnification) **C** - A549 cells infected with Influenza B virus, observed under Carl Zeiss Axiovert 40 CFL (100X magnification) **D** - A549 cells infected with H3N2 virus, observed under Carl Zeiss Axiovert 40 CFL (100X magnification)

5.2.2. Virus propagation on A549 cells:

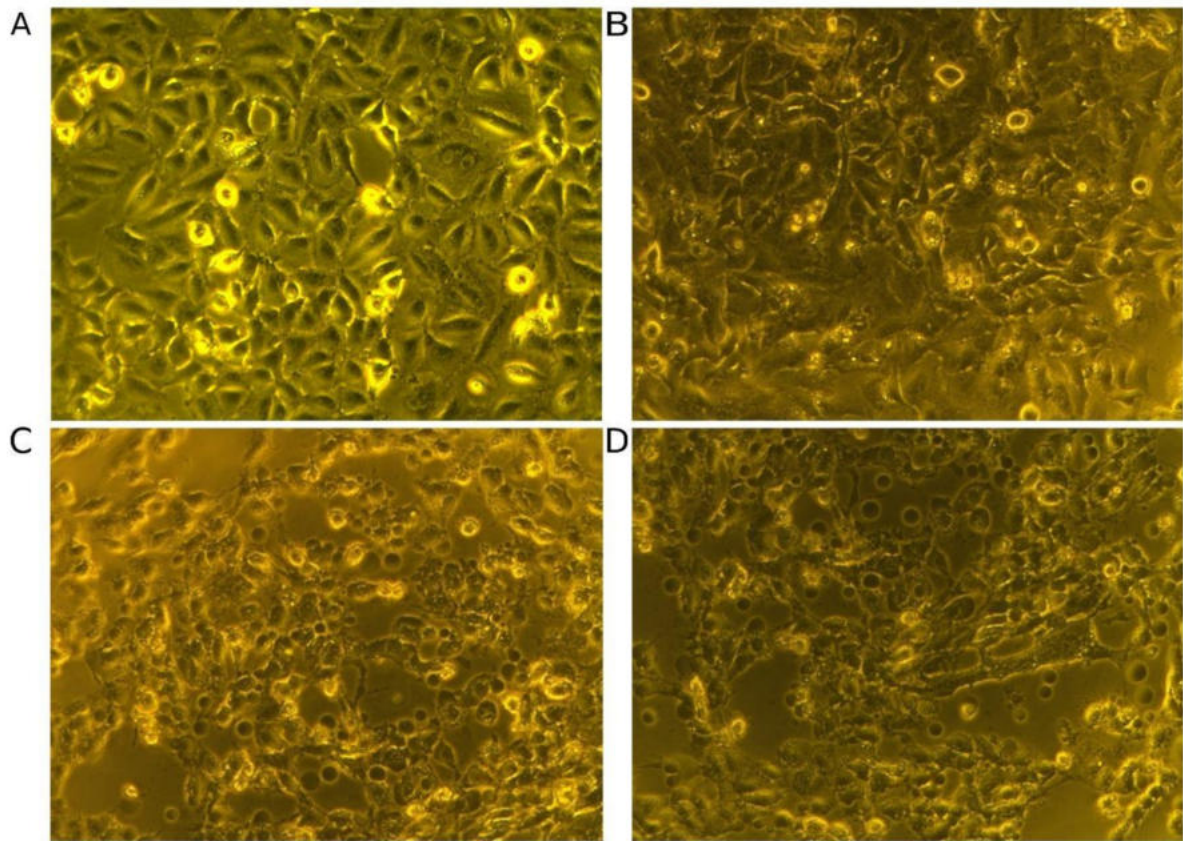
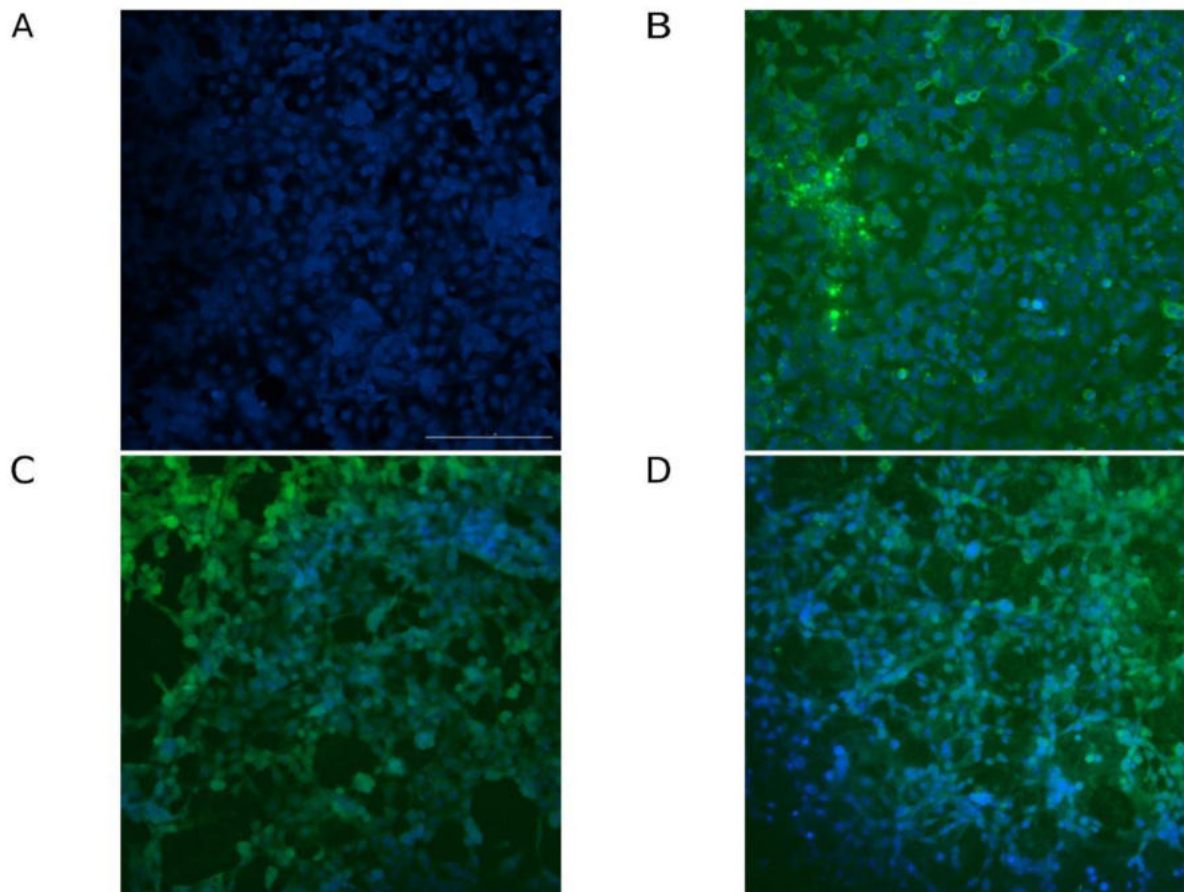


Figure 10: **A** - Mock infected A549 cells observed under Carl Zeiss Axiovert 40 CFL (200X magnification) **B** - Influenza A virus strain pH1N1 infected A549 cells observed under Carl Zeiss Axiovert 40 CFL (200X magnification) **C** - Influenza A virus strain H3N2 infected A549 cells observed under Carl Zeiss Axiovert 40 CFL (200X magnification) **D** - Influenza virus strain B infected A549 cells observed under Carl Zeiss Axiovert 40 CFL (200X magnification)

5.3. Immunofluorescence Staining



KEY: **A** – Mock infected A549 cells stained with DAPI. Observed under Cytation 7 (200X magnification) **B** – A549 cells infected with pH1N1 virus, stained with FITC conjugated antibody against the nucleoprotein of Influenza A and DAPI. Observed under Cytation 7 (200X magnification). **C** - A549 cells infected with Influenza B virus, stained with FITC conjugated antibody against the nucleoprotein of Influenza B and DAPI. Observed under Cytation 7 (200X magnification). **D** - A549 cells infected with H3N2 virus, stained with FITC conjugated antibody against the nucleoprotein of Influenza A and DAPI. Observed under Cytation 7 (200X magnification)

5.4. Hemagglutination Assay

Hemagglutination is a characteristic of all influenza viruses, which is predicated on the fact that they will agglutinate RBCs. This is influenced by the binding of haemagglutinin viral protein receptors to the sialic acid residue on the membrane of RBCs, which causes visible agglutination and serves as the basis for viral particle detection. It is utilised to determine the virus titer for viral stock harvesting. The endpoint of a HA titration is the maximum dilution of viruses that induce total hemagglutination. The HA titer for all the three viruses was found to be 1:256 HA units.

5.4.1. Influenza A (pH1N1)

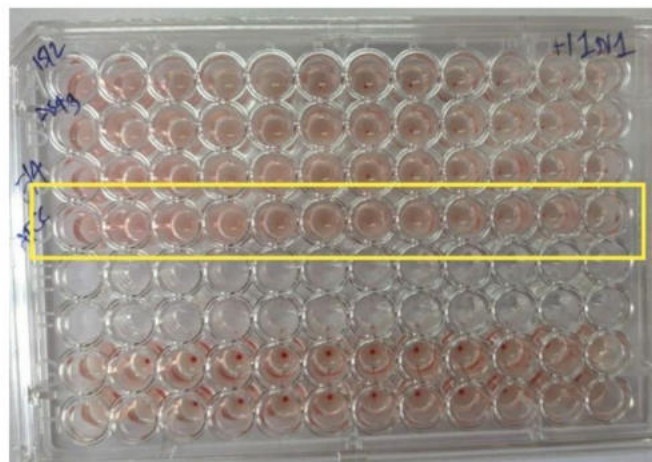


Figure 11: Result for HA performed using influenza pH1N1 virus - 1:256

5.4.2. Influenza A (H3N2)

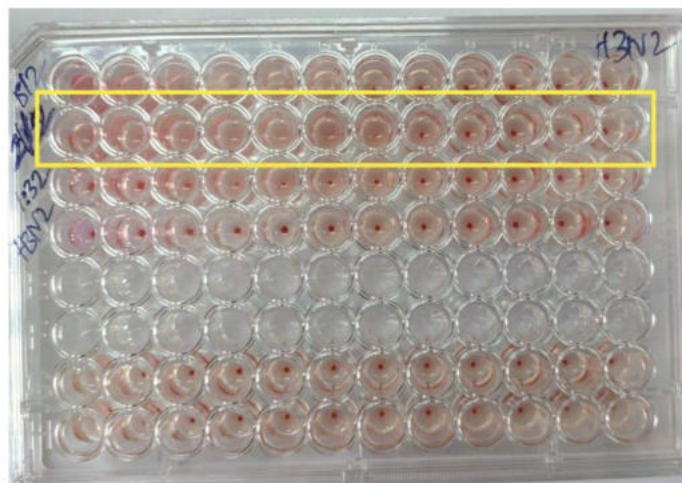


Figure 12: Result for HA performed using influenza H3N2 virus - 1:256

5.4.3. Influenza B

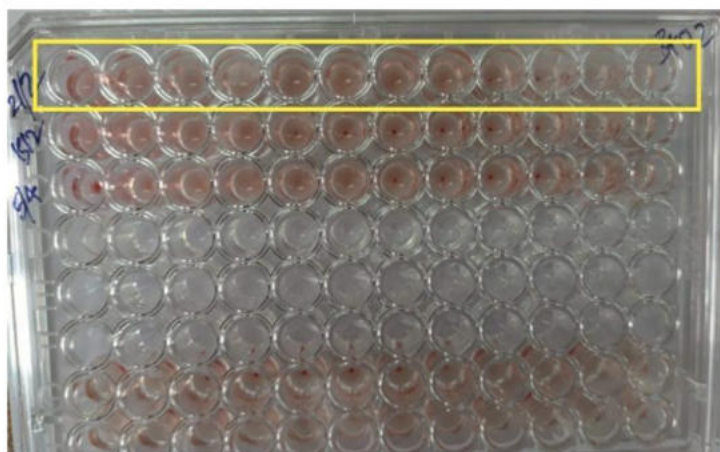


Figure 13: Result for HA performed using influenza B virus - 1:64

5.5. GENE EXPRESSION ANALYSIS

The present study demonstrates that the host gene expression responses to H1N1 and H3N2 were qualitatively similar but quantitatively different. The different time points of virus infection played an important role in the host gene expression responses. At 24 hrs increase in expression of all the genes was observed as compared to than 4 hours. With an exception of IL10, IFITM 2 and IFITM 3. For IL10 the fold change was 1.67 times greater in 4 hrs with respect to 24 hrs. On the contrary, CCL5 expressed was upregulated at 4 hours in Influenza A whereas, it was downregulated during Influenza B infection. There was increase in expression of IFITM 2 and IFITM 3 at 24 hrs in comparison to 4 hrs. TNF- α , CCL5, IL6 was found to be upregulated post 24hrs of infection with H1N1 (atleast 35 fold) and H3N2 (atleast 20 fold).

5.5.1. TNF- α

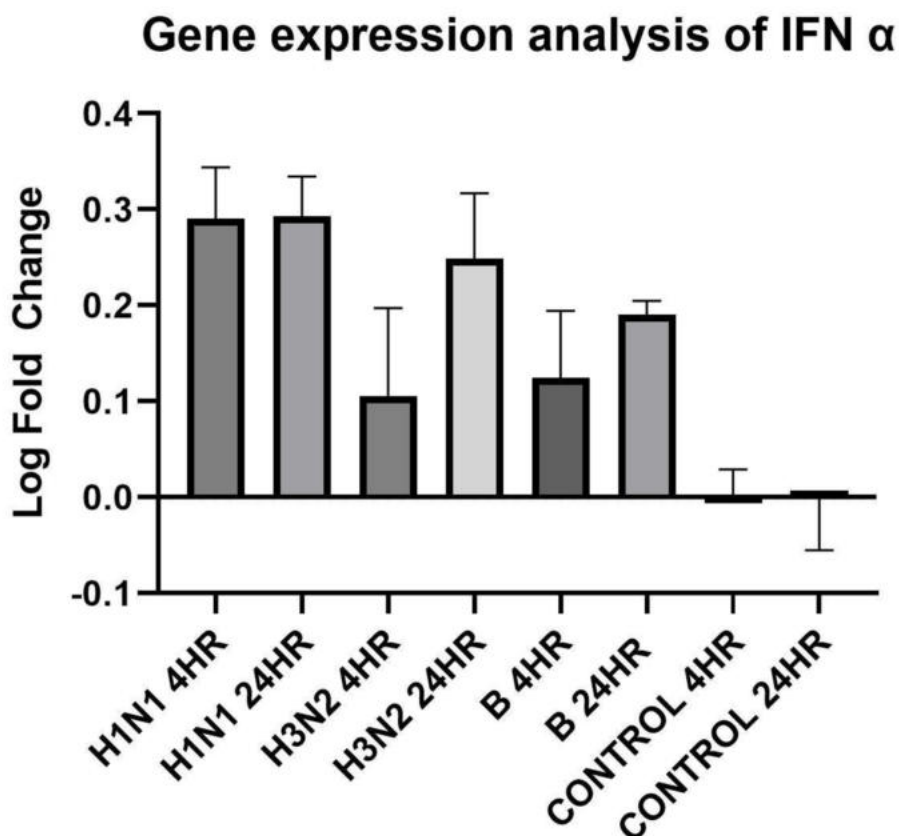


Figure 14. Log of fold change in TNF- α at 4hpi and 24hpi

Sample group	Fold change	
	4 hpi	24 hpi
H1N1	+4.30	+65.4
H3N2	+4.0	+45.97
Influenza B	+1.30	-6.25

Table 11. Gene expression of TNF- α at 4hrs and 24hrs with respect to their respective controls.

5.5.2. IL8

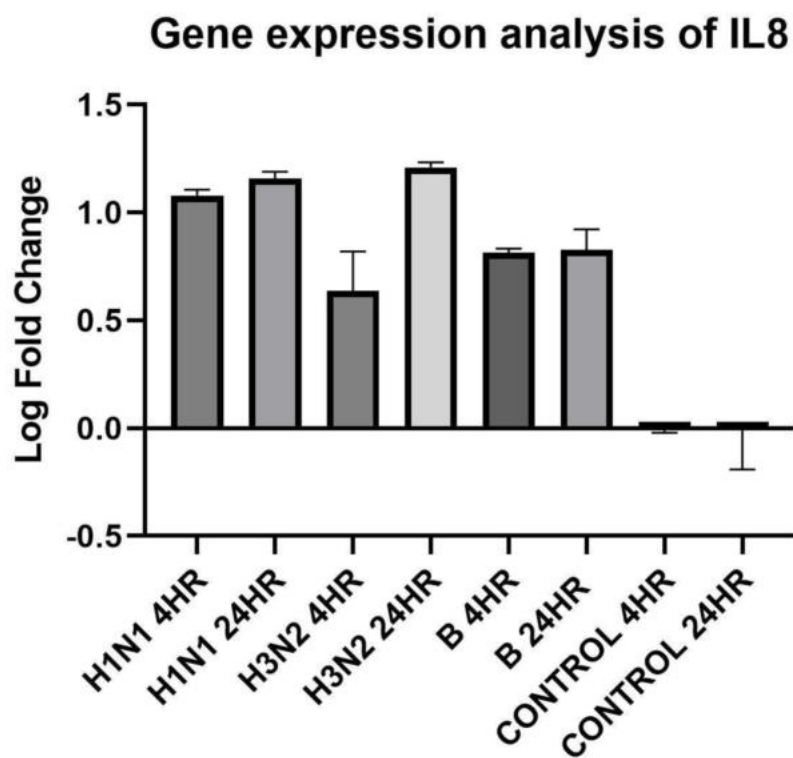


Figure 15. Log of fold change in IL8 at 4 hpi and 24 hpi

Sample group	Fold change	
	4 hpi	24 hpi
H1N1	+11.9	+14.4
H3N2	+4.5	+16.2
Influenza B	+6.49	+6.8

Table 12. Gene expression of IL8 at 4 hr and 24 hr with respect to their respective controls.

5.5.3. IL10

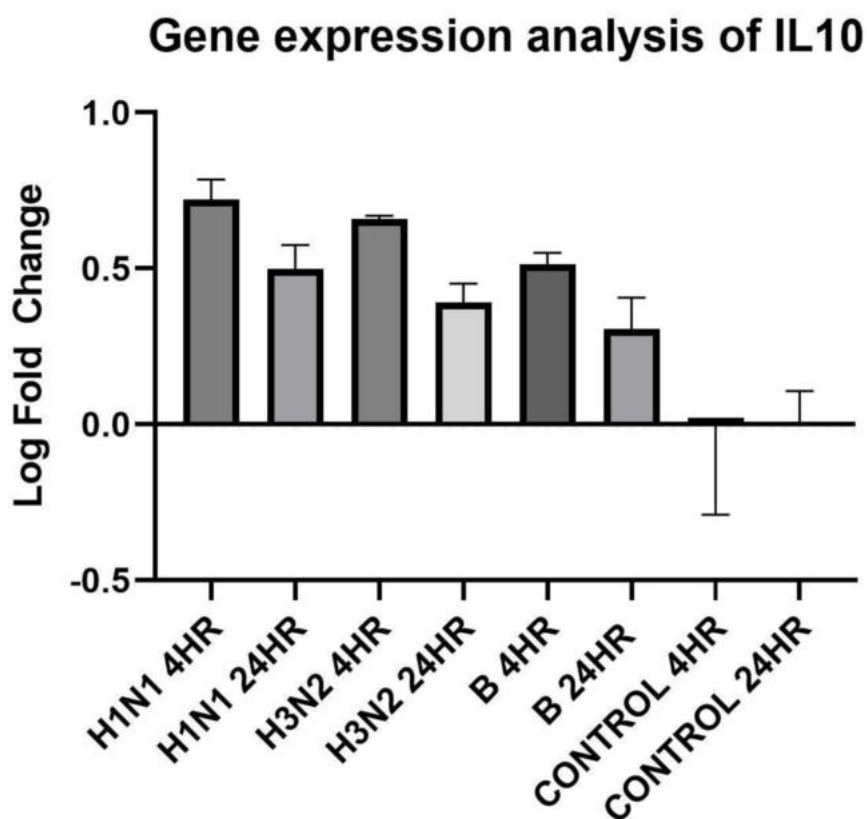


Figure 16. Log of fold change in IL10 at 4 hpi and 24 hpi

Sample group	Fold change	
	4 hpi	24 hpi
H1N1	+5.3	+3.18
H3N2	+4.5	+2.4
Influenza B	+3.27	+2.0

Table 13. Gene expression of IL10 at 4 hrs and 24 hrs with respect to their respective controls.

5.5.4. CCL5

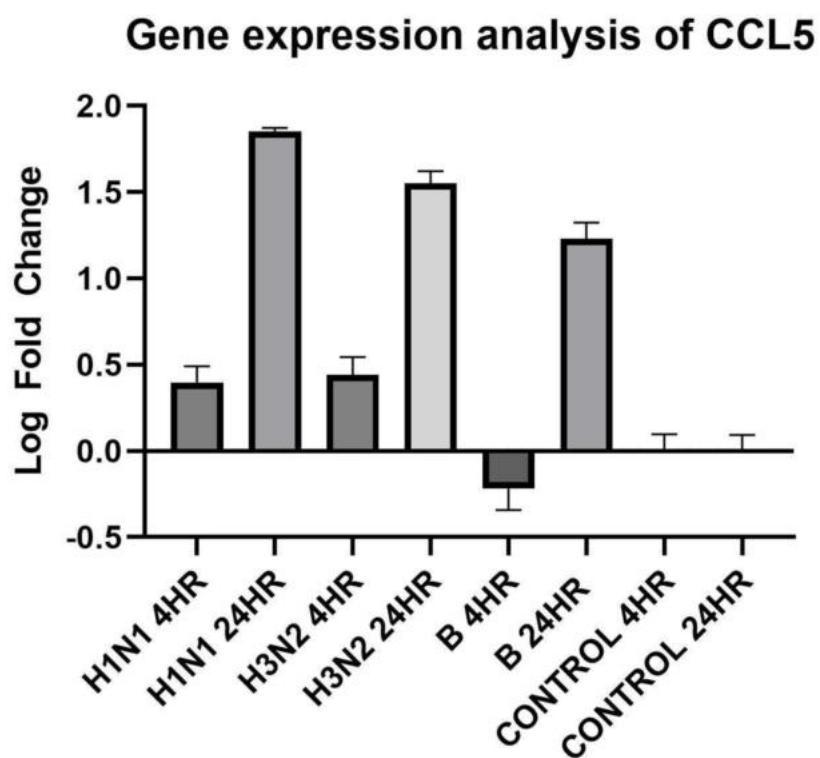


Figure 17. Log of fold change in CCL5 at 4 hpi and 24 hpi

Sample group	Fold change	
	4 hpi	24 hpi
H1N1	+2.53	+71
H3N2	+2.81	+35.87
Influenza B	-1.61	+17.2

Table 14. Gene expression of CCL5 at 4 hrs and 24 hrs with respect to their respective controls.

5.5.5. IFN- γ

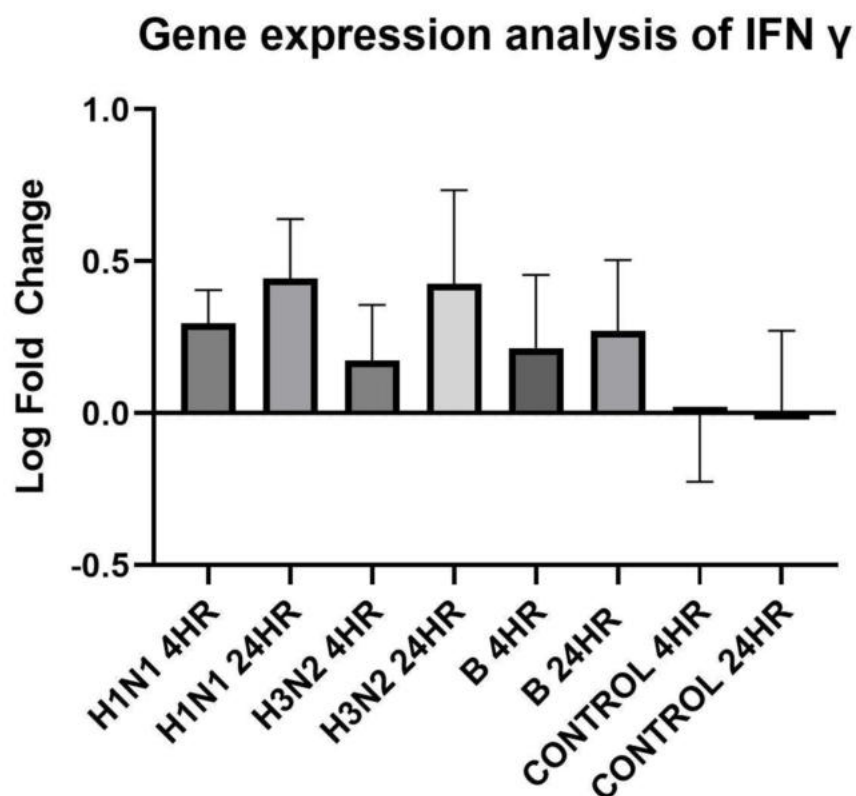


Figure 18. Log of fold change in IFN- γ at 4 hpi and 24 hpi

Sample group	Fold change	
	4 hpi	24 hpi
H1N1	+2.02	+2.93
H3N2	+1.58	+3.09
Influenza B	+1.82	+2.05

Table 15. Gene expression of IFN- γ at 4 hrs and 24 hrs with respect to their respective controls.

5.5.6. IFN- α

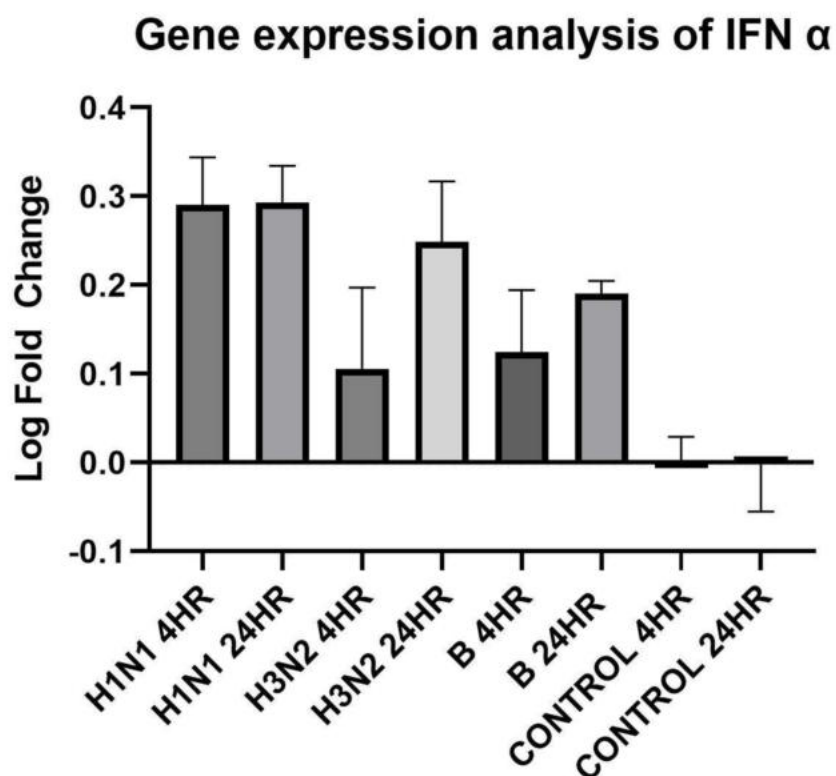


Figure 19. Log of fold change in IFN- α at 4 hpi and 24 hpi

Sample group	Fold change	
	4 hpi	24 hpi
H1N1	+1.963	+1.97
H3N2	+1.29	+1.78
Influenza B	+1.34	+1.55

Table 16. Gene expression of IFN- α at 4 hrs and 24 hrs with respect to their respective controls.

5.5.7. IL6

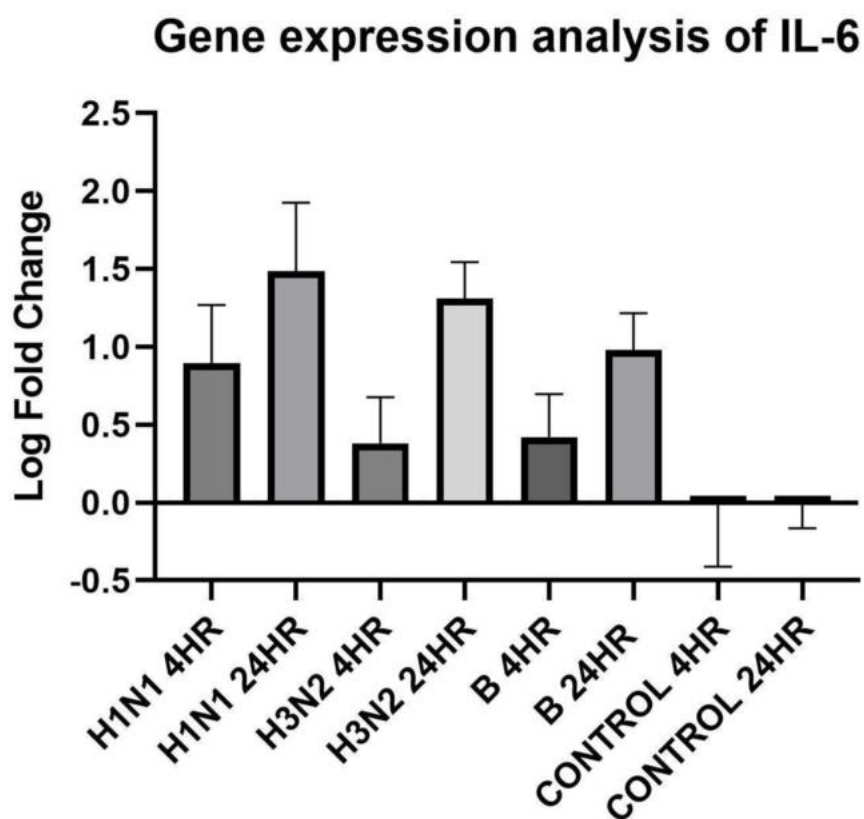


Figure 20. Log of fold change in IL6 at 4 hpi and 24 hpi

Sample group	Fold change	
	4 hpi	24 hpi
H1N1	+9.57	+39.93
H3N2	+2.75	+22.27
Influenza B	+2.96	+10.44

Table 17. Gene expression of IL6 at 4 hrs and 24 hrs with respect to their respective controls

5.5.8. IFITM2

Gene expression analysis of IFITM2

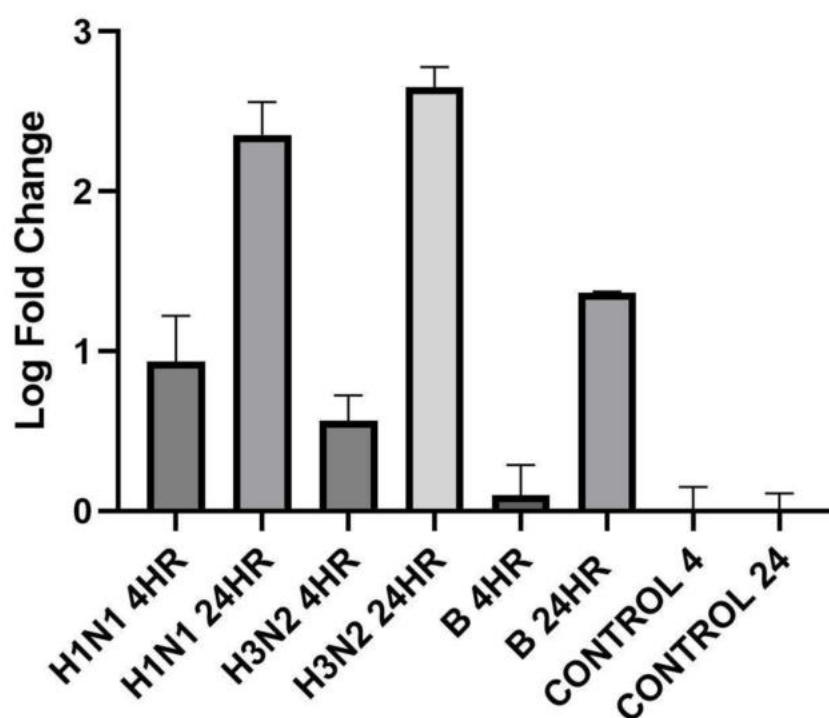


Figure 21: Log of fold change in IFITM2 at 4 hpi and 24 hpi

Sample group	Fold change	
	4 hpi	24 hpi
H1N1	+9.81	+242.68
H3N2	+3.82	+462.08
Influenza B	+1.34	+23.42

Table 18: Gene expression of IFITM2 at 4 hrs and 24 hrs with respect to their respective controls

5.5.9. IFITM3

Gene expression analysis of IFITM3

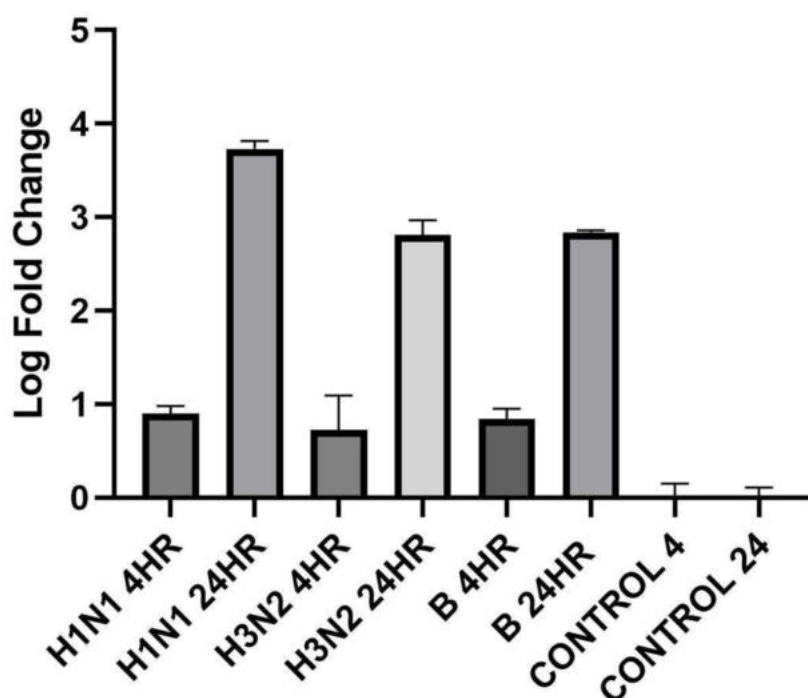


Figure 22: Log of fold change in IFITM3 at 4 hpi and 24 hpi

Sample group	Fold change	
	4 hpi	24 hpi
H1N1	+8.05	+5455.21
H3N2	+6.68	+673.87
Influenza B	+7.11	+688.89

Table 19: Gene expression of IFITM3 at 4 hrs and 24 hrs with respect to their respective controls

5.6. Total Protein Estimation using bicinchoninic acid (BCA) assay

The A549 cells after infection were lysed using 2% SDS lysis buffer. The extracted proteins were subjected to biochemical estimation of total protein using BCA assay, and the results are presented below. Bovine serum albumin (BSA) was used as standard.

Table 20: OD at 560 nms for the standard (BSA) using BCA method

Concentrations of BSA (µg/ml)	OD at 560 nms
0	0.111
125	0.109
250	0.124
500	0.151
750	0.207
1000	0.285
1500	0.403
2000	0.666

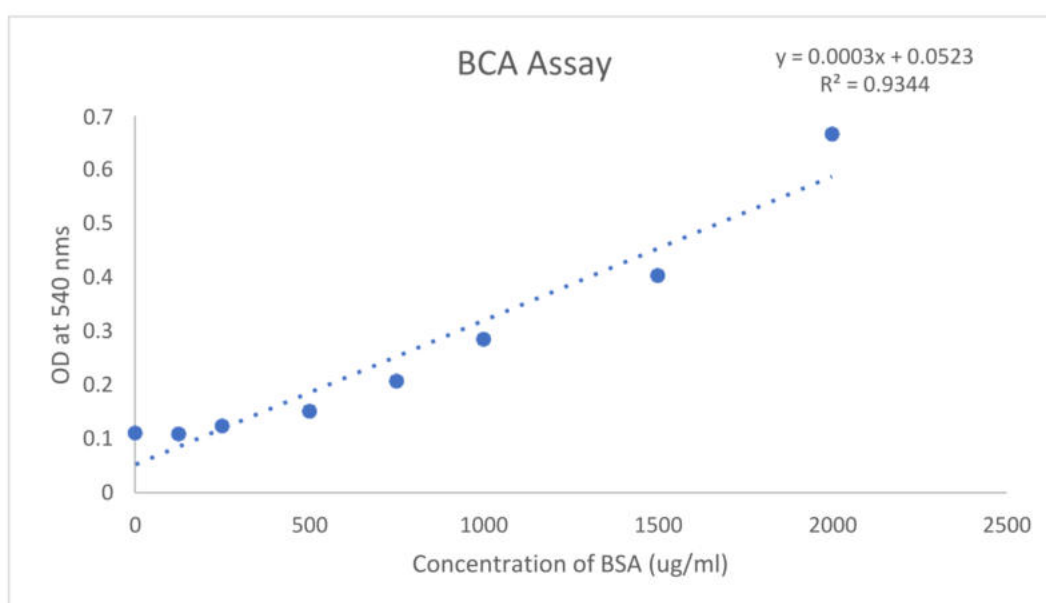


Figure 23: Graphical representation of the standard curve (BSA)

Table 21: BCA Assay – calculations for proteins extracted from A549 cells infected with different strains of Influenza virus at different time-points

Strain	OD	OD - Blank	Conc.	Dilution 1:2	µg/ul
H1_0 hrs	0.296	0.2437	812.3333	1624.667	1.624667
H3_0 hrs	0.288	0.2357	785.6667	1571.333	1.571333
B_0 hrs	0.279	0.2267	755.6667	1511.333	1.511333
H1_8hrs	0.377	0.3247	1082.333	2164.667	2.164667
H3_8hrs	0.345	0.2927	975.6667	1951.333	1.951333
B_8hrs	0.228	0.1757	585.6667	1171.333	1.171333
Control	0.243	0.1907	635.6667	1271.333	1.271333

KEY:

H1_0hrs – Concentration of proteins extracted from A549 cells after infection with pH1N1 at 0 hpi.,

H3_0hrs – Concentration of proteins extracted from A549 cells after infection with H3N2 at 0 hpi.,

B_0hrs – Concentration of proteins extracted from A549 cells after infection with Influenza B at 0 hpi.,

H1_8hrs – Concentration of proteins extracted from A549 cells after infection with pH1N1 at 8 hpi.,

H3_8hrs – Concentration of proteins extracted from A549 cells after infection with H3N2 at 8 hpi.,

B_8hrs – Concentration of proteins extracted from A549 cells after infection with Influenza B at 8 hpi.,

Control – Concentration of proteins extracted from A549 cells i.e. mock infected well.

5.7. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

Total protein estimation was done by BCA method. Total protein content was normalized using the lowest concentration of the protein. After normalization the protein extracts were resolved using 10% SDS - PAGE.

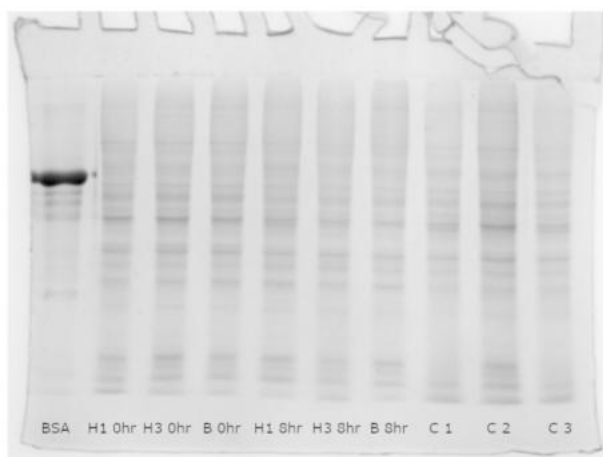


Figure 24: Protein estimation by SDS-PAGE (Gel before trypsin digestion)

(Left to right): BSA, H1 0hr – proteins extracted after infection with pH1N1 at 0 hr, H3 0hr– proteins extracted after infection with H3N2 at 0 hr, B 0hr – proteins extracted after infection with Influenza B at 0 hr, H1 8hr – proteins extracted after infection with pH1N1 at 8 hrs, H3 8hr– proteins extracted after infection with H3N2 at 8 hrs, B 8hrs – proteins extracted after infection with Influenza B at 8 hrs, C1, C2, C3 – Controls.

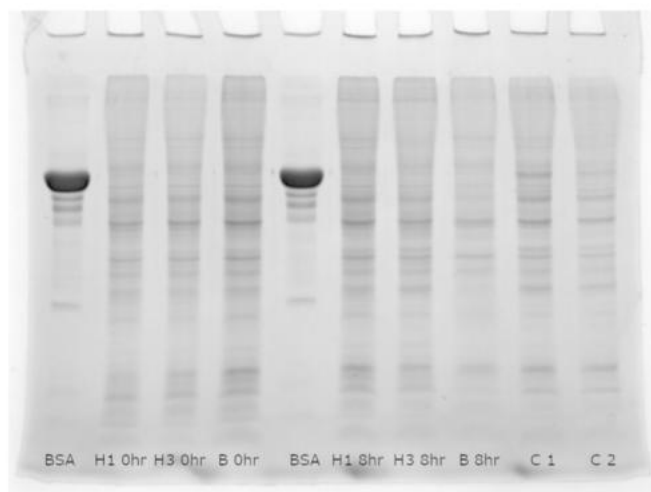


Figure 25: Protein estimation by SDS-PAGE (Gel before trypsin digestion)

(Left to right): BSA, H1 0hr – proteins extracted after infection with pH1N1 at 0 hr, H3 0hr– proteins extracted after infection with H3N2 at 0 hr, B 0hr – proteins extracted after infection with Influenza B at 0 hr, H1 8 hrs – proteins extracted after infection with pH1N1 at 8 hrs, H3 8 hrs – proteins extracted after infection with H3N2 at 8 hrs, B 8 hrs – proteins extracted after infection with Influenza B at 8 hrs, C1, C2 – Controls.

***10µg BSA was loaded as control**

After normalization the protein samples were reduced, alkylated and trypsin digested. Complete digestion of the protein samples was confirmed using 10% SDS - PAGE.

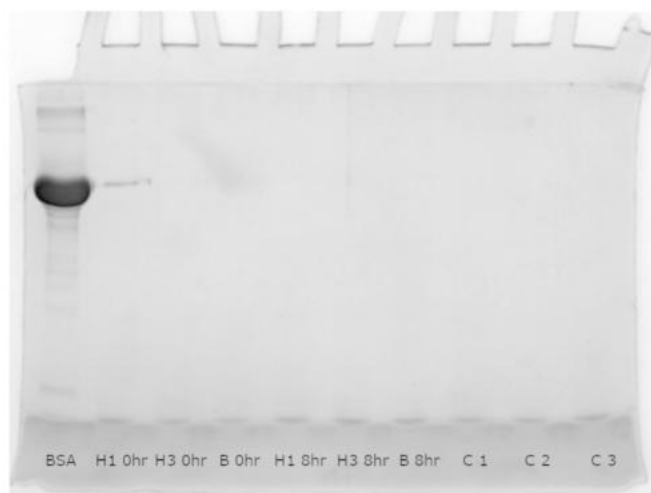


Figure 26: Protein estimation by SDS-PAGE (Gel after trypsin digestion)

(Left to right): BSA, H1 0 hr – proteins digested after infection with pH1N1 at 0 hr, H3 0hr– proteins digested after infection with H3N2 at 0 hr, B 0 hr – proteins digested after infection with Influenza B at 0 hr, H1 8 hrs – proteins digested after infection with pH1N1 at 8 hrs, H3 8 hrs – proteins digested after infection with H3N2 at 8 hrs, B 8 hrs – proteins digested after infection with Influenza B at 8 hrs, C1, C2, C3 – Controls.

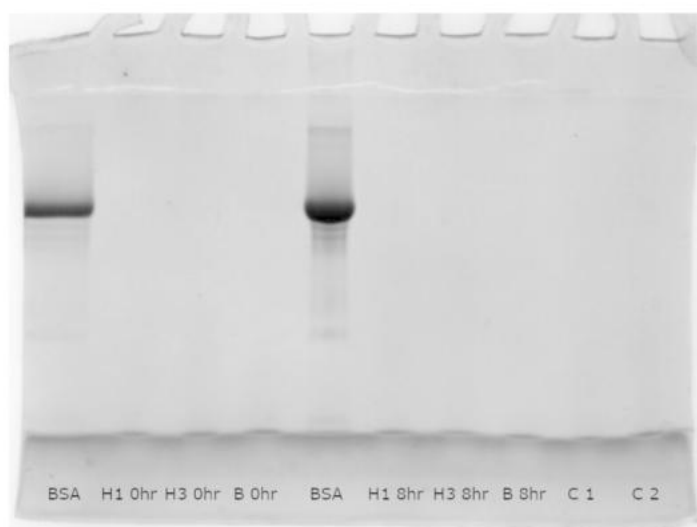


Figure 27: Protein estimation by SDS-PAGE (Gel after trypsin digestion)

(Left to right): BSA, H1 0 hr – proteins digested after infection with pH1N1 at 0 hr, H3 0hr– proteins digested after infection with H3N2 at 0 hr, B 0 hr – proteins digested after infection with Influenza B at 0 hr, H1 8 hrs – proteins digested after infection with pH1N1 at 8 hrs, H3 8 hrs– proteins digested after infection with H3N2 at 8 hrs, B 8 hrs – proteins digested after infection with Influenza B at 8 hrs, C1, C2 – Controls digested.

***10µg BSA was loaded as control**

5.8. Proteome search result:

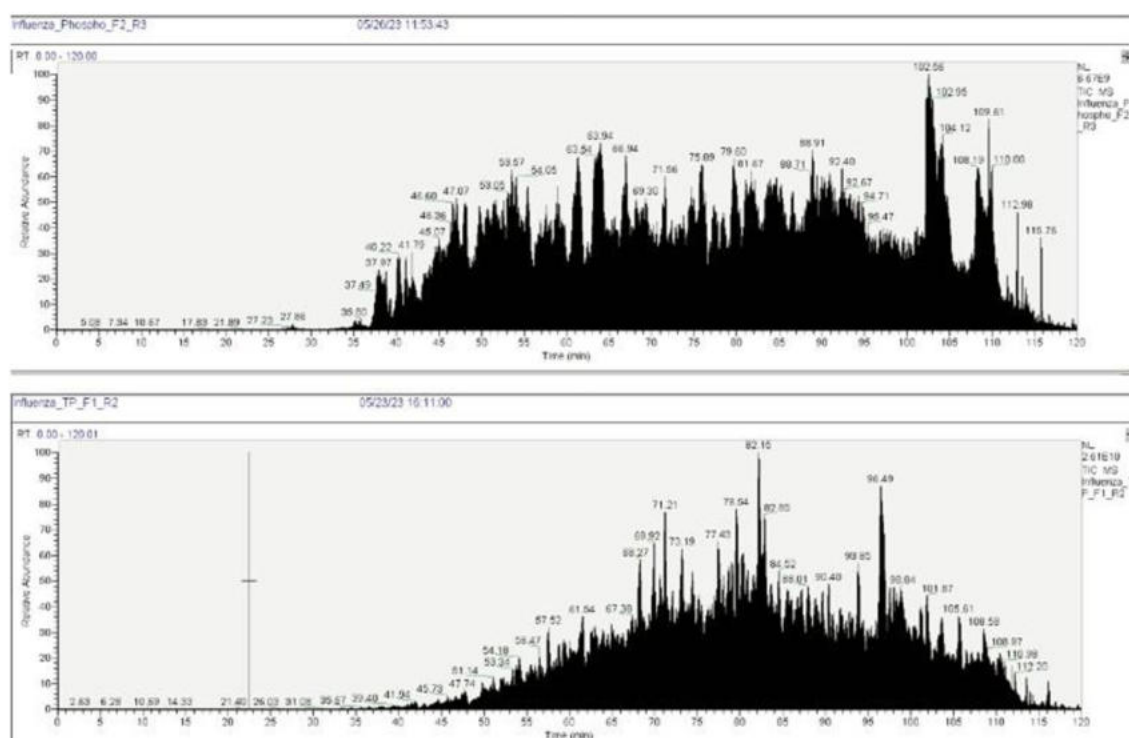


Figure 28: Representative 120 minutes chromatogram of total proteomics and phosphoproteomics

5.9. iTRAQ label check search output, iTRAQ normalization factor and labelling of complete sample:

Raw Data	Proteome Database	PSMs	Peptides	Proteins	PSM with iTRAQ tag	iTRAQ efficiency (%)
iTRAQ Label Check	RefSeq Homo sapiens v109	4443	1960	476	3531	79.46

Table 22: Summary of results from database searches of iTRAQ label check files

Sample ID	Normalization factor based on median abundance of iTRAQ channels	Sample volume (µl)
C	0.91317	128
B0	0.75952	106.33
B8	1	140
H1-0	0.73053	102.27
H1-3	0.76683	107.35
H3-0	0.71312	99.83
H3-8	0.77427	108.39

Table 23: Peptide mixture samples taken for pooling based on iTRAQ label check

KEY: C – Control, B0 – proteins labelled after infection with Influenza B at 0 hr, B8 – proteins labelled after infection with Influenza B at 8 hrs, H1-0 – proteins labelled from infection with pH1N1 at 0 hr, H1-8 – proteins labelled after infection with pH1N1 at 8 hrs, H3-0– proteins labelled after infection with H3N2 at 0 hr, H3-8– proteins labelled after infection with H3N2 at 8 hrs,

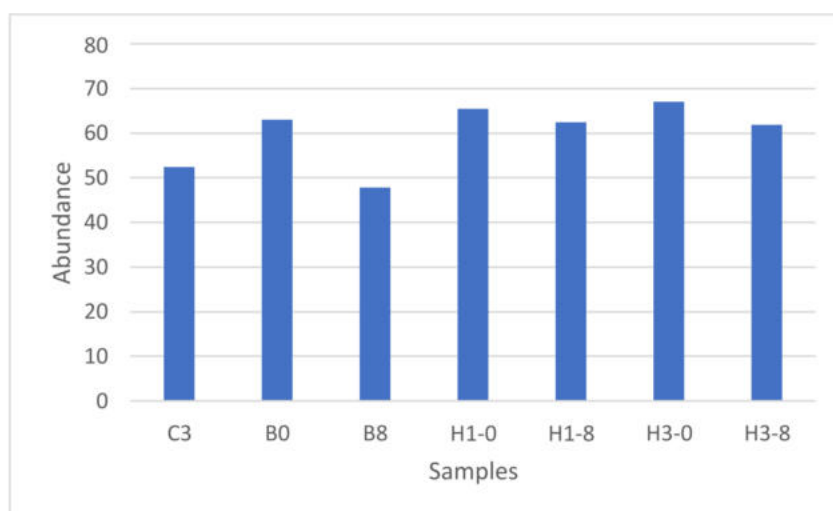


Figure 29: Sum abundance values of iTRAQ channels used for protein digest samples

5.10. Proteins and peptides from database search:

The below tables consist of proteomic and phosphoproteomics search results

Raw Data	MS/MS scans	Proteome Database	PSMs	Peptides	Proteins
Influenza Proteomics	5,96,800	RefSeq Homo sapiens v109	1,63,548	15,412	3,227

Figure 30: Summary of results from database searches of proteomics samples

5.11. Phosphoproteomics

Raw Data	MS/MS scans	Proteome Database	PSMs	Peptides	Proteins	Phosphopeptides
Influenza Phosphoproteomics	5,34,452	RefSeq Homo sapiens v109	1,36,063	13,179	3,056	843

Figure 31: Summary of results from database searches of phosphoproteomics samples

5.12. Identification of the Total Proteins and Phosphoproteins using LC-MS/MS

The expression patterns of the entire proteins and phosphoproteins profile of A549 cells infected with different strains of influenza viruses at different time-points were obtained by preprocessing the raw tandem mass spectrometry data using Mascot and Sequest HT search engines present in Proteome Discoverer 2.2. The differentially expressed proteins of A549 cells infected with different strains of influenza viruses were grouped individually. The number of upregulated and downregulated proteins and phosphoproteins were distinctly identified based on the log-fold change criteria between the two groups i.e.,

infected and control. The log-fold change values were set between (0.8 and 1.25), the proteins and phosphoproteins expressed with values less than 0.8 were downregulated and the proteins expressed with values greater than 1.25 were upregulated.

5.12.1. Total Proteins

Regulation status	B_0hrs	B_8hrs	H1_0hrs	H1_8hrs	H3_0hrs	H3_8hrs
Upregulated proteins	113	272	178	291	104	289
Downregulated proteins	158	429	177	210	87	302
Non-differentially expressed proteins	2239	1809	2155	2009	2319	1919

Table 24: Summarization of total and differentially expressed proteins identified in A549 cells infected with different strains of Influenza virus at different time - points

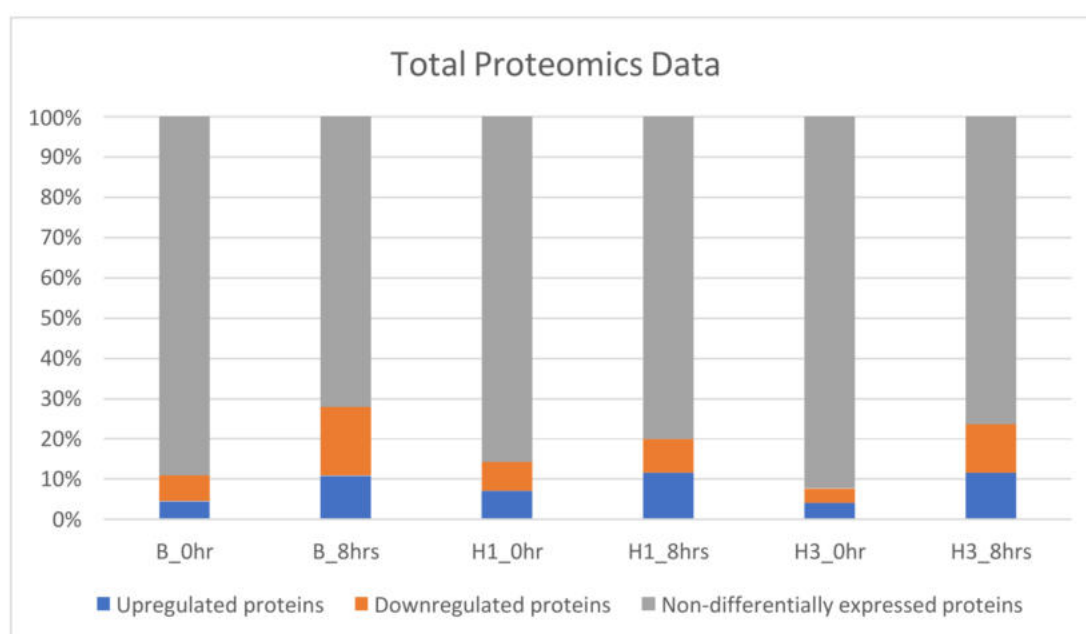


Figure 32: Percentage distribution of differentially expressed proteins identified in A549 cells infected with different strains of Influenza virus at 0 hpi and 8 hpi

5.12.2. Phosphoproteins

Regulation status	B_0hrs	B_8hrs	H1_0hrs	H1_8hrs	H3_0hrs	H3_8hrs
Upregulated proteins	85	143	98	138	81	147
Downregulated proteins	89	122	95	82	86	105
Non-differentially expressed proteins	291	200	272	245	298	213

Table 25: Summarization of total and differentially expressed phosphoproteins identified in A549 cells infected with different strains of Influenza virus at different time - points

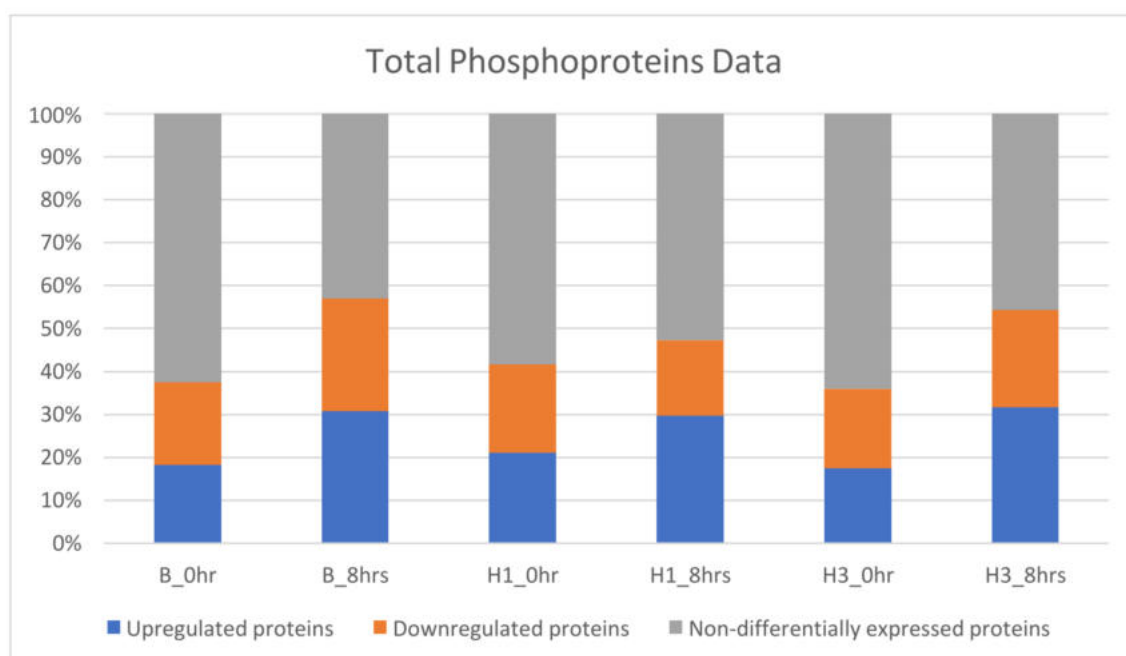


Figure 33: Percentage distribution of differentially expressed phosphoproteins identified in A549 cells infected with different strains of Influenza virus at 0 hpi and 8 hpi

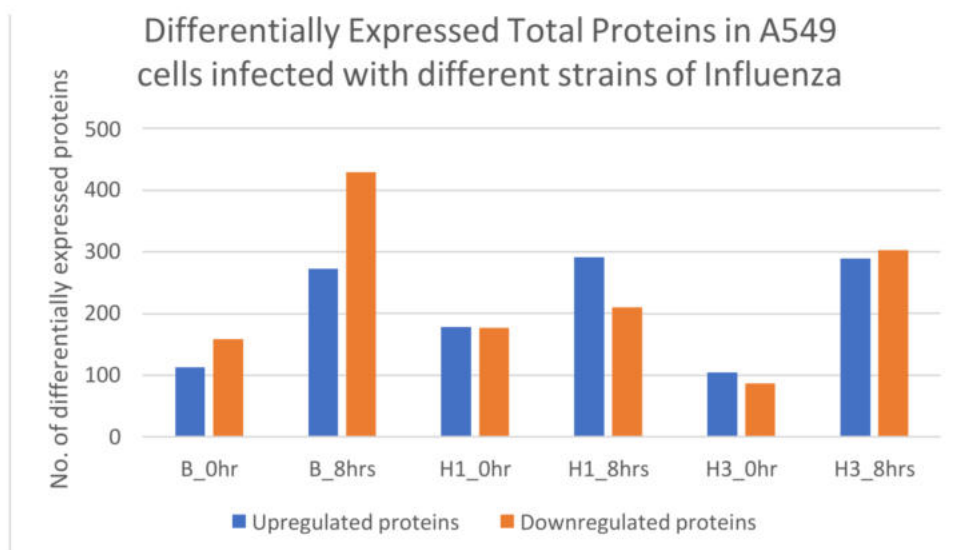


Figure 34: Summarization of differentially expressed total proteins identified in A549 cells infected with different strains of Influenza virus at different time - points

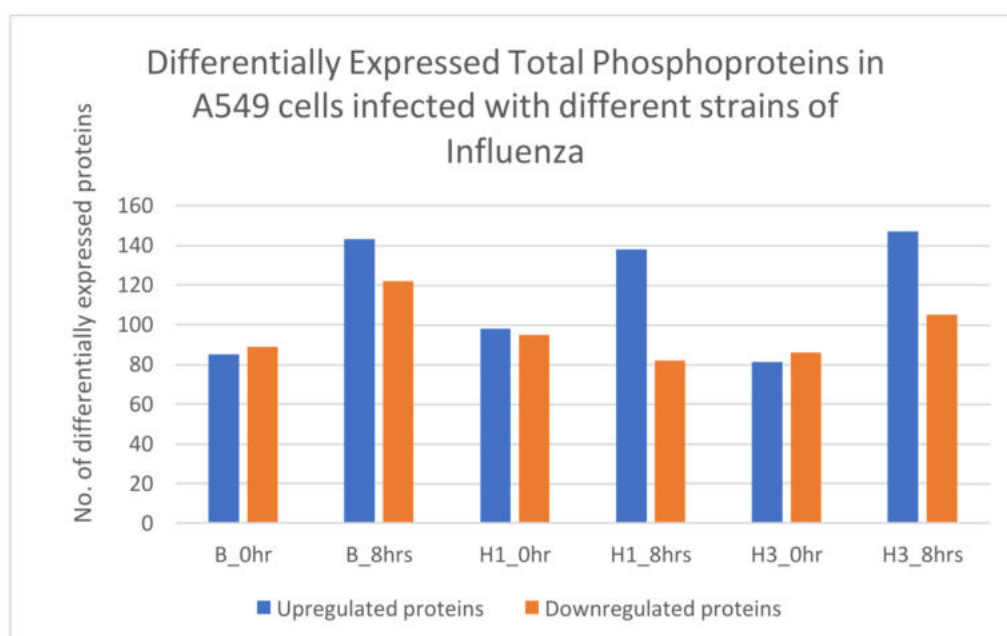


Figure 35: Summarization of differentially expressed phosphoproteins identified in A549 cells infected with different strains of Influenza virus at different time - points

Table 26: List of differentially expressed – upregulated phosphoproteins in A549 cells infected with Influenza B at 0 hrs

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q96GM8	TOE1	Target of EGR1 protein 1	mRNA polyadenylation factor (PC00146)
Q07955	SRSF1	Serine/arginine-rich splicing factor 1	RNA splicing factor (PC00148)
Q9H6F5	CCDC86	Coiled-coil domain-containing protein 86	
Q9H1E3	NUCKS1	Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1	
Q99733	NAP1L4	Nucleosome assembly protein 1-like 4	chromatin/chromatin-binding, or -regulatory protein (PC00077)
P07900	HSP90AA1	Heat shock protein HSP 90-alpha	Hsp90 family chaperone (PC00028)
Q6PKG0	LARP1	La-related protein 1	RNA metabolism protein (PC00031)
P49768	PSEN1	Presenilin-1	aspartic protease (PC00053)
Q8TAQ2	SMARCC2	SWI/SNF complex subunit SMARCC2	chromatin/chromatin-binding, or -regulatory protein (PC00077)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q13439	GOLGA4	Golgin subfamily A member 4	membrane trafficking regulatory protein (PC00151)
Q9UQ35	SRRM2	Serine/arginine repetitive matrix protein 2	
P41236	PPP1R2	Protein phosphatase inhibitor 2	phosphatase inhibitor (PC00183)
P62269	RPS18	40S ribosomal protein S18	ribosomal protein (PC00202)
P84022	SMAD3	Mothers against decapentaplegic homolog 3	DNA-binding transcription factor (PC00218)
Q15185	PTGES3	Prostaglandin E synthase 3	chaperone (PC00072)
P05388	RPLP0	60S acidic ribosomal protein P0	ribosomal protein (PC00202)
P17812	CTPS1	CTP synthase 1	ligase (PC00142)
Q96JA1	LIG1	Leucine-rich repeats and immunoglobulin-like domains protein 1	transmembrane signal receptor (PC00197)
P98175	RBM10	RNA-binding protein 10	
P52756	RBM5	RNA-binding protein 5	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q14103	HNRNPD	Heterogeneous nuclear ribonucleoprotein D0	RNA metabolism protein (PC00031)
Q9NTJ3	SMC4	Structural maintenance of chromosomes protein 4	
Q8TEM1	NUP210	Nuclear pore membrane glycoprotein 210	transporter (PC00227)
P25788	PSMA3	Proteasome subunit alpha type-3	protease (PC00190)
Q96I25	RBM17	Splicing factor 45	RNA splicing factor (PC00148)
Q13442	PDAP1	28 kDa heat- and acid-stable phosphoprotein	
P17096	HMGA1	High mobility group protein HMG-I/HMG-Y	endodeoxyribonuclease (PC00093)
Q9P035	HACD3	Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase 3	dehydratase (PC00091)
Q92911	SLC5A5	Sodium/iodide cotransporter	secondary carrier transporter (PC00258)
Q15121	PEA15	Astrocytic phosphoprotein PEA-15	
O95674	CDS2	Phosphatidate cytidylyltransferase 2	transferase (PC00220)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q9UK76	JPT1	Jupiter microtubule associated homolog 1	
P49756	RBM25	RNA-binding protein 25	
P06748	NPM1	Nucleophosmin	chaperone (PC00072)
P14923	JUP	Junction plakoglobin	
P49006	MARCKSL1	MARCKS-related protein	
P09651	HNRNPA1	Heterogeneous nuclear ribonucleoprotein A1	RNA processing factor (PC00147)
Q5H9R7	PPP6R3	Serine/threonine-protein phosphatase 6 regulatory subunit 3	phosphatase modulator (PC00184)
Q0ZGT2	NEXN	Nexilin	scaffold/adaptor protein (PC00226)
O00193	C11orf58	Small acidic protein	
P06454	PTMA	Prothymosin alpha	
Q8IZL8	PELP1	Proline-, glutamic acid- and leucine-rich protein 1	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
O95218	ZRANB2	Zinc finger Ran-binding domain-containing protein 2	RNA splicing factor (PC00148)
A2RRP1	NBAS	Neuroblastoma-amplified sequence	
P26358	DNMT1	DNA (cytosine-5)-methyltransferase 1	DNA methyltransferase (PC00013)
P27816	MAP4	Microtubule-associated protein 4	
P78527	PRKDC	DNA-dependent protein kinase catalytic subunit	non-receptor serine/threonine protein kinase (PC00167)
Q6KC79	NIPBL	Nipped-B-like protein	chromatin/chromatin-binding, or -regulatory protein (PC00077)
P54727	RAD23B	UV excision repair protein RAD23 homolog B	damaged DNA-binding protein (PC00086)
Q8IYB3	SRRM1	Serine/arginine repetitive matrix protein 1	RNA processing factor (PC00147)
Q9NZN4	EHD2	EH domain-containing protein 2	membrane traffic protein (PC00150)
Q9UJK0	TSR3	18S rRNA aminocarboxypropyltransferase	
Q96L91	EP400	E1A-binding protein p400	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
O00505	KPNA3	Importin subunit alpha-4	transporter (PC00227)
P18858	LIG1	DNA ligase 1	DNA ligase (PC00012)
Q86VM9	ZC3H18	Zinc finger CCCH domain-containing protein 18	
Q9Y2W1	THRAP3	Thyroid hormone receptor-associated protein 3	DNA metabolism protein (PC00009)
Q8IWA0	WDR75	WD repeat-containing protein 75	
Q9UHB6	LIMA1	LIM domain and actin-binding protein 1	actin or actin-binding cytoskeletal protein (PC00041)
Q9Y281	CFL2	Cofilin-2	non-motor actin binding protein (PC00165)
O00264	PGRMC1	Membrane-associated progesterone receptor component 1	transmembrane signal receptor (PC00197)
Q8TEW0	PARD3	Partitioning defective 3 homolog	
O75367	MACROH2A1	Core histone macro-H2A.1	chromatin/chromatin-binding, or -regulatory protein (PC00077)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P35221	CTNNA1	Catenin alpha-1	non-motor actin binding protein (PC00165)
P08238	HSP90AB1	Heat shock protein HSP 90-beta	Hsp90 family chaperone (PC00028)
Q8TDD1	DDX54	ATP-dependent RNA helicase DDX54	RNA helicase (PC00032)
P51858	HDGF	Hepatoma-derived growth factor	transcription cofactor (PC00217)
Q9HCN4	GPN1	GPN-loop GTPase 1	small GTPase (PC00208)
Q66PJ3	ARL6IP4	ADP-ribosylation factor-like protein 6-interacting protein 4	
E9PAV3	NACA	Nascent polypeptide-associated complex subunit alpha, muscle-specific form	basic helix-loop-helix transcription factor (PC00055)
P35611	ADD1	Alpha-adducin	non-motor actin binding protein (PC00165)
Q08945	SSRP1	FACT complex subunit SSRP1	
Q14684	RRP1B	Ribosomal RNA processing protein 1 homolog B	
Q9BX95	SGPP1	Sphingosine-1-phosphate phosphatase 1	phosphatase (PC00181)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
O75420	CDS2	GRB10-interacting GYF protein 1	

Table 27: List of differentially expressed – downregulated phosphoproteins in A549 cells infected with Influenza B at 0 hrs

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P67809	YBX1	Y-box-binding protein 1	
Q9H6F5	CCDC86	Coiled-coil domain-containing protein 86	
P11388	TOP2A	DNA topoisomerase 2-alpha	DNA metabolism protein (PC00009)
Q6ZRP7	QSOX2	Sulfhydryl oxidase 2	oxidase (PC00175)
Q6PD62	CTR9	RNA polymerase-associated protein CTR9 homolog	general transcription factor (PC00259)
Q5JTV8	TOR1AIP1	Torsin-1A-interacting protein 1	
Q92844	TRAF2	TRAF family member-associated NF-kappa-B activator	
Q8IVP5	FUNDC1	FUN14 domain-containing protein 1	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q9UQ35	SRRM2	Serine/arginine repetitive matrix protein 2	
O00559	EBAG9	Receptor-binding cancer antigen expressed on SiSo cells	
Q12766	HMGXB3	HMG domain-containing protein 3	
P61978	HNRNPK	Heterogeneous nuclear ribonucleoprotein K	RNA metabolism protein (PC00031)
P98175	RBM10	RNA-binding protein 10	
Q5T200	ZC3H13	Zinc finger CCCH domain-containing protein 13	
P17676	CEBPB	CCAAT/enhancer-binding protein beta	basic leucine zipper transcription factor (PC00056)
Q8N163	CCAR2	Cell cycle and apoptosis regulator protein 2	primary active transporter (PC00068)
Q9BU76	C1orf35	Multiple myeloma tumor-associated protein 2	
Q13501	SQSTM1	Sequestosome-1	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P46100	ATRX	Transcriptional regulator ATRX	DNA-binding transcription factor (PC00218)
O15061	SYNM	Synemin	
P38432	COIL	Coilin	DNA metabolism protein (PC00009)
Q8WVC0	LEO1	RNA polymerase-associated protein LEO1	DNA-directed RNA polymerase (PC00019)
Q8N9T8	KRI1	Protein KRI1 homolog	
Q2TB10	ZNF800	Zinc finger protein 800	
Q15796	SMAD2	Mothers against decapentaplegic homolog 2	DNA-binding transcription factor (PC00218)
P47755	CAPZA2	F-actin-capping protein subunit alpha-2	non-motor actin binding protein (PC00165)
Q16637	SMN1	Survival motor neuron protein	RNA splicing factor (PC00148)
Q13435	SF3B2	Splicing factor 3B subunit 2	RNA splicing factor (PC00148)
Q12933	TRAF2	TNF receptor-associated factor 2	scaffold/adaptor protein (PC00226)
Q09666	AHNAK	Neuroblast differentiation-associated protein AHNAK	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q5T8D3	ACBD5	Acyl-CoA-binding domain-containing protein 5	transfer/carrier protein (PC00219)
Q6ZRV2	FAM83H	Protein FAM83H	
P42166	TMPO	Lamina-associated polypeptide 2, isoform alpha	peptide hormone (PC00179)
P08195	SLC3A2	4F2 cell-surface antigen heavy chain	
P05787	KRT8	Keratin, type II cytoskeletal 8	
Q96JM3	CHAMP1	Chromosome alignment-maintaining phosphoprotein 1	
P16989	YBX3	Y-box-binding protein 3	
Q8TEU7	RAPGEF6	Rap guanine nucleotide exchange factor 6	guanyl-nucleotide exchange factor (PC00113)
Q12888	TP53BP1	TP53-binding protein 1	DNA metabolism protein (PC00009)
P08670	VIM	Vimentin	intermediate filament (PC00129)
P10451	SPP1	Osteopontin	cytokine (PC00083)
P13611	VCAN	Versican core protein	extracellular matrix glycoprotein (PC00100)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q15154	PCM1	Pericentriolar material 1 protein	
Q8IVT2	MISP	Mitotic interactor and substrate of PLK1	
Q92522	H1-10	Histone H1.10	chromatin/chromatin-binding, or -regulatory protein (PC00077)
P30038	ALDH4A1	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial	dehydrogenase (PC00092)
Q7Z417	NUFIP2	Nuclear fragile X mental retardation-interacting protein 2	
Q659A1	ICE2	Little elongation complex subunit 2	
Q9NQC3	RTN4	Reticulon-4	
O43760	SYNGR2	Synaptogyrin-2	membrane trafficking regulatory protein (PC00151)
Q96I59	SYNM	Probable asparagine--tRNA ligase, mitochondrial	aminoacyl-tRNA synthetase (PC00047)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P51636	CAV2	Caveolin-2	scaffold/adaptor protein (PC00226)
P16403	H1-2	Histone H1.2	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q9H3Z4	DNAJC5	DnaJ homolog subfamily C member 5	chaperone (PC00072)
Q9NS69	TOMM22	Mitochondrial import receptor subunit TOM22 homolog	primary active transporter (PC00068)
Q13263	TRIM28	Transcription intermediary factor 1-beta	ubiquitin-protein ligase (PC00234)
P25205	MCM3	DNA replication licensing factor MCM3	DNA metabolism protein (PC00009)
O00567	NOP56	Nucleolar protein 56	RNA metabolism protein (PC00031)
O43493	TGOLN2	Trans-Golgi network integral membrane protein 2	membrane traffic protein (PC00150)
Q9NU22	MDN1	Midasin	
O60832	DKC1	H/ACA ribonucleoprotein complex subunit DKC1	centromere DNA-binding protein (PC00071)
P20042	EIF2S2	Eukaryotic translation initiation factor 2 subunit 2	translation initiation factor (PC00224)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P02545	LMNA	Prelamin-A/C	
Q9Y4B5	MTCL1	Microtubule cross-linking factor 1	
Q9H3N1	TMX1	Thioredoxin-related transmembrane protein 1	
P35659	DEK	Protein DEK	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q8NDC0	MAPK1IP1L	MAPK-interacting and spindle-stabilizing protein-like	
O76021	RSL1D1	Ribosomal L1 domain-containing protein 1	ribosomal protein (PC00202)
Q96N64	PWWP2A	PWWP domain-containing protein 2A	DNA methyltransferase (PC00013)
Q9UBH6	XPR1	Xenotropic and polytropic retrovirus receptor 1	secondary carrier transporter (PC00258)
P46821	MAP1B	Microtubule-associated protein 1B	non-motor microtubule binding protein (PC00166)
Q9NTI5	PDS5B	Sister chromatid cohesion protein PDS5 homolog B	chromatin/chromatin-binding, or -regulatory protein (PC00077)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P10412	H1-4	Histone H1.4	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q9BX95	SPP1	Sphingosine-1-phosphate phosphatase 1	phosphatase (PC00181)

Table 28: List of differentially expressed – upregulated phosphoproteins in A549 cells infected with Influenza B at 8 hrs

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q14137	BOP1	Ribosome biogenesis protein BOP1	ribosomal protein (PC00202)
Q07955	SRSF1	Serine/arginine-rich splicing factor 1	RNA splicing factor (PC00148)
Q9H6F5	CCDC86	Coiled-coil domain-containing protein 86	
Q9H1E3	NUCKS1	Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1	
P11388	TOP2A	DNA topoisomerase 2-alpha	DNA metabolism protein (PC00009)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q15149	PLEC	Plectin	intermediate filament binding protein (PC00130)
Q9H4G0	EPB41L1	Band 4.1-like protein 1	non-motor actin binding protein (PC00165)
Q6PKG0	LARP1	La-related protein 1	RNA metabolism protein (PC00031)
Q8WX93	PALLD	Palladin	scaffold/adaptor protein (PC00226)
Q7Z6Z7	HUWE1	E3 ubiquitin-protein ligase HUWE1	ubiquitin-protein ligase (PC00234)
P49585	PCYT1A	Choline-phosphate cytidylyltransferase A	transferase (PC00220)
Q7L014	DDX46	Probable ATP-dependent RNA helicase DDX46	RNA helicase (PC00032)
P05386	RPLP1	60S acidic ribosomal protein P1	ribosomal protein (PC00202)
Q9UQ35	SRRM2	Serine/arginine repetitive matrix protein 2	
P41236	PPP1R2	Protein phosphatase inhibitor 2	phosphatase inhibitor (PC00183)
Q86UP2	KTN1	Kinectin	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q9BQA1	WDR77	Methylosome protein 50	
Q7L4I2	RSRC2	Arginine/serine-rich coiled-coil protein 2	
P05388	RPLP0	60S acidic ribosomal protein P0	ribosomal protein (PC00202)
O75821	EIF3G	Eukaryotic translation initiation factor 3 subunit G	translation initiation factor (PC00224)
P61978	HNRNPK	Heterogeneous nuclear ribonucleoprotein K	RNA metabolism protein (PC00031)
P61247	RPS3A	40S ribosomal protein S3a	ribosomal protein (PC00202)
Q5T200	ZC3H13	Zinc finger CCCH domain-containing protein 13	
Q8N163	CCAR2	Cell cycle and apoptosis regulator protein 2	primary active transporter (PC00068)
Q9BU76	C1orf35	Multiple myeloma tumor-associated protein 2	
P31943	HNRNPH1	Heterogeneous nuclear ribonucleoprotein H	RNA splicing factor (PC00148)
Q9H0D6	XRN2	5'-3' exoribonuclease 2	exoribonuclease (PC00099)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q13501	SQSTM1	Sequestosome-1	
Q9NTJ3	SMC4	Structural maintenance of chromosomes protein 4	
Q02880	TOP2B	DNA topoisomerase 2-beta	DNA metabolism protein (PC00009)
Q8IVF2	AHNAK2	Protein AHNAK2	
Q7RTP6	MICAL3	[F-actin]-monooxygenase MICAL3	scaffold/adaptor protein (PC00226)
Q8TEM1	NUP210	Nuclear pore membrane glycoprotein 210	transporter (PC00227)
P25788	PSMA3	Proteasome subunit alpha type-3	protease (PC00190)
Q96I25	RBM17	Splicing factor 45	RNA splicing factor (PC00148)
Q13442	PDAP1	28 kDa heat- and acid-stable phosphoprotein	
P17096	HMGA1	High mobility group protein HMG-I/HMG-Y	endodeoxyribonuclease (PC00093)
Q9P035	HACD3	Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase 3	dehydratase (PC00091)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q09666	AHNAK	Neuroblast differentiation-associated protein AHNAK	
Q92911	SLC5A5	Sodium/iodide cotransporter	secondary carrier transporter (PC00258)
P21333	FLNA	Filamin-A	
Q5T8D3	ACBD5	Acyl-CoA-binding domain-containing protein 5	transfer/carrier protein (PC00219)
Q01082	SPTBN1	Spectrin beta chain, non-erythrocytic 1	actin or actin-binding cytoskeletal protein (PC00041)
P05787	KRT8	Keratin, type II cytoskeletal 8	
P23396	RPS3	40S ribosomal protein S3	ribosomal protein (PC00202)
Q9Y3T9	NOC2L	Nucleolar complex protein 2 homolog	
Q9BQG0	MYBBP1A	Myb-binding protein 1A	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q9NWH9	SLTM	SAFB-like transcription modulator	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
O95400	CD2BP2	CD2 antigen cytoplasmic tail-binding protein 2	RNA splicing factor (PC00148)
P14923	JUP	Junction plakoglobin	
Q12888	TP53BP1	TP53-binding protein 1	DNA metabolism protein (PC00009)
P07910	HNRNPC	Heterogeneous nuclear ribonucleoproteins C1/C2	RNA metabolism protein (PC00031)
B5ME19	EIF3CL	Eukaryotic translation initiation factor 3 subunit C-like protein	translation initiation factor (PC00224)
Q9BUJ2	HNRNPUL1	Heterogeneous nuclear ribonucleoprotein U-like protein 1	
P19338	NCL	Nucleolin	RNA metabolism protein (PC00031)
Q13185	CBX3	Chromobox protein homolog 3	
Q5H9R7	PPP6R3	Serine/threonine-protein phosphatase 6 regulatory subunit 3	phosphatase modulator (PC00184)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
O00193	C11orf58	Small acidic protein	
Q9Y388	RBMX2	RNA-binding motif protein, X-linked 2	
Q9NQC3	RTN4	Reticulon-4	
O95218	ZRANB2	Zinc finger Ran-binding domain-containing protein 2	RNA splicing factor (PC00148)
Q8IY81	FTSJ3	pre-rRNA 2'-O-ribose RNA methyltransferase FTSJ3	RNA methyltransferase (PC00033)
A2RRP1	NBAS	Neuroblastoma-amplified sequence	
P27816	MAP4	Microtubule-associated protein 4	
O60716	CTNND1	Catenin delta-1	intermediate filament binding protein (PC00130)
P40222	TXLNA	Alpha-taxilin	membrane traffic protein (PC00150)
Q6KC79	NIPBL	Nipped-B-like protein	chromatin/chromatin-binding, or -regulatory protein (PC00077)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q9Y2X7	GIT1	ARF GTPase-activating protein GIT1	
P26368	U2AF2	Splicing factor U2AF 65 kDa subunit	
P54727	RAD23B	UV excision repair protein RAD23 homolog B	damaged DNA-binding protein (PC00086)
Q8IYB3	SRRM1	Serine/arginine repetitive matrix protein 1	RNA processing factor (PC00147)
Q9UIG0	BAZ1B	Tyrosine-protein kinase BAZ1B	non-receptor tyrosine protein kinase (PC00168)
Q13112	CHAF1B	Chromatin assembly factor 1 subunit B	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q8TCJ2	STT3B	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3B	glycosyltransferase (PC00111)
P23588	EIF4B	Eukaryotic translation initiation factor 4B	translation initiation factor (PC00224)
O00505	KPNA3	Importin subunit alpha-4	transporter (PC00227)
P29692	EEF1D	Elongation factor 1-delta	translation elongation factor (PC00222)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
O60832	DKC1	H/ACA ribonucleoprotein complex subunit DKC1	centromere DNA-binding protein (PC00071)
Q9NYF8	BCLAF1	Bcl-2-associated transcription factor 1	DNA metabolism protein (PC00009)
P18583	SON	Protein SON	RNA splicing factor (PC00148)
Q14498	RBM39	RNA-binding protein 39	RNA splicing factor (PC00148)
Q9Y2W1	THRAP3	Thyroid hormone receptor-associated protein 3	DNA metabolism protein (PC00009)
P02545	LMNA	Prelamin-A/C	
P08651	NFIC	Nuclear factor 1 C-type	DNA-binding transcription factor (PC00218)
Q8IWA0	WDR75	WD repeat-containing protein 75	
Q9H3N1	TMX1	Thioredoxin-related transmembrane protein 1	
P00533	EGFR	Epidermal growth factor receptor	transmembrane signal receptor (PC00197)
Q9UHB6	LIMA1	LIM domain and actin-binding protein 1	actin or actin-binding cytoskeletal protein (PC00041)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
O15173	PGRMC2	Membrane-associated progesterone receptor component 2	transmembrane signal receptor (PC00197)
Q8TEW0	PARD3	Partitioning defective 3 homolog	
Q1KMD3	HNRNPUL2	Heterogeneous nuclear ribonucleoprotein U-like protein 2	
P35221	CTNNA1	Catenin alpha-1	non-motor actin binding protein (PC00165)
Q8TDD1	DDX54	ATP-dependent RNA helicase DDX54	RNA helicase (PC00032)
Q53GS9	USP39	U4/U6.U5 tri-snRNP-associated protein 2	cysteine protease (PC00081)
Q96S55	WRNIP1	ATPase WRNIP1	DNA helicase (PC00011)
P27824	CANX	Calnexin	chaperone (PC00072)
Q9UBH6	XPR1	Xenotropic and polytropic retrovirus receptor 1	secondary carrier transporter (PC00258)
P46821	MAP1B	Microtubule-associated protein 1B	non-motor microtubule binding protein (PC00166)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q66PJ3	ARL6IP4	ADP-ribosylation factor-like protein 6-interacting protein 4	
Q92769	HDAC2	Histone deacetylase 2	histone modifying enzyme (PC00261)
E9PAV3	NACA	Nascent polypeptide-associated complex subunit alpha, muscle-specific form	basic helix-loop-helix transcription factor (PC00055)
P35611	ADD1	Alpha-adducin	non-motor actin binding protein (PC00165)
Q08945	SSRP1	FACT complex subunit SSRP1	

Table 29: List of differentially expressed – downregulated phosphoproteins in A549 cells infected with Influenza B at 8 hrs

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q16513	PKN2	Serine/threonine-protein kinase N2	non-receptor serine/threonine protein kinase (PC00167)
O76075	CAD	DNA fragmentation factor subunit beta	DNA metabolism protein (PC00009)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P11388	TOP2A	DNA topoisomerase 2-alpha	DNA metabolism protein (PC00009)
Q99733	NAP1L4	Nucleosome assembly protein 1-like 4	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q6ZRP7	QSOX2	Sulfhydryl oxidase 2	oxidase (PC00175)
Q6PD62	CTR9	RNA polymerase-associated protein CTR9 homolog	general transcription factor (PC00259)
Q5JTV8	TOR1AIP1	Torsin-1A-interacting protein 1	
P07900	HSP90AA1	Heat shock protein HSP 90-alpha	Hsp90 family chaperone (PC00028)
P49768	PSEN1	Presenilin-1	aspartic protease (PC00053)
Q92844	TRAF2	TRAF family member-associated NF-kappa-B activator	
Q8TAQ2	SMARCC2	SWI/SNF complex subunit SMARCC2	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q03701	CEBPZ	CCAAT/enhancer-binding protein zeta	DNA-binding transcription factor (PC00218)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q8IVP5	FUNDC1	FUN14 domain-containing protein 1	
Q13439	GOLGA4	Golgin subfamily A member 4	membrane trafficking regulatory protein (PC00151)
P16401	H1-5	Histone H1.5	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q5VTR2	RNF20	E3 ubiquitin-protein ligase BRE1A	
Q9UQ35	SRRM2	Serine/arginine repetitive matrix protein 2	
P62269	RPS18	40S ribosomal protein S18	ribosomal protein (PC00202)
P35579	MYH9	Myosin-9	actin or actin-binding cytoskeletal protein (PC00041)
P84022	SMAD3	Mothers against decapentaplegic homolog 3	DNA-binding transcription factor (PC00218)
Q9NWD8	TMEM248	Transmembrane protein 248	
Q15185	PTGES3	Prostaglandin E synthase 3	chaperone (PC00072)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q9H6S0	YTHDC2	3'-5' RNA helicase YTHDC2	RNA helicase (PC00032)
Q12766	HMGXB3	HMG domain-containing protein 3	
P61978	HNRNPK	Heterogeneous nuclear ribonucleoprotein K	RNA metabolism protein (PC00031)
P17812	CTPS1	CTP synthase 1	ligase (PC00142)
Q96JA1	LIG1	Leucine-rich repeats and immunoglobulin-like domains protein 1	transmembrane signal receptor (PC00197)
Q14103	HNRNPD	Heterogeneous nuclear ribonucleoprotein D0	RNA metabolism protein (PC00031)
Q8N163	CCAR2	Cell cycle and apoptosis regulator protein 2	primary active transporter (PC00068)
Q01650	SLC7A5	Large neutral amino acids transporter small subunit 1	transporter (PC00227)
O15061	SYNM	Synemin	
A6NK06	CAD	Cis-aconitate decarboxylase	dehydratase (PC00091)
P38432	COIL	Coilin	DNA metabolism protein (PC00009)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q7Z2W4	ZC3HAV1	Zinc finger CCCH-type antiviral protein 1	
Q05209	PTPN12	Tyrosine-protein phosphatase non-receptor type 12	
Q2TB10	ZNF800	Zinc finger protein 800	
P00352	ALDH1A1	Retinal dehydrogenase 1	dehydrogenase (PC00092)
P16402	H1-3	Histone H1.3	chromatin/chromatin-binding, or -regulatory protein (PC00077)
P17096	HMGA1	High mobility group protein HMG-I/HMG-Y	endodeoxyribonuclease (PC00093)
Q9H0S4	DDX47	Probable ATP-dependent RNA helicase DDX47	RNA helicase (PC00032)
Q13435	SF3B2	Splicing factor 3B subunit 2	RNA splicing factor (PC00148)
Q12933	TRAF2	TNF receptor-associated factor 2	scaffold/adaptor protein (PC00226)
Q05682	CAD	Caldesmon	non-motor actin binding protein (PC00165)
Q15121	PEA15	Astrocytic phosphoprotein PEA-15	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P42166	TMPO	Lamina-associated polypeptide 2, isoform alpha	peptide hormone (PC00179)
Q9UQ80	PA2G4	Proliferation-associated protein 2G4	protease (PC00190)
P54105	CLNS1A	Methylosome subunit pICln	transporter (PC00227)
P29966	MARCKS	Myristoylated alanine-rich C-kinase substrate	
Q8NE71	ABCF1	ATP-binding cassette sub-family F member 1	translation elongation factor (PC00222)
P16989	YBX3	Y-box-binding protein 3	
P49736	MCM2	DNA replication licensing factor MCM2	DNA metabolism protein (PC00009)
Q8TEU7	RAPGEF6	Rap guanine nucleotide exchange factor 6	guanyl-nucleotide exchange factor (PC00113)
P06748	NPM1	Nucleophosmin	chaperone (PC00072)
Q07157	TJP1	Tight junction protein ZO-1	tight junction (PC00214)
P08670	VIM	Vimentin	intermediate filament (PC00129)
P07910	HNRNPC	Heterogeneous nuclear ribonucleoproteins C1/C2	RNA metabolism protein (PC00031)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
O60231	DHX16	Pre-mRNA-splicing factor ATP-dependent RNA helicase DHX16	RNA helicase (PC00032)
P13611	VCAN	Versican core protein	extracellular matrix glycoprotein (PC00100)
P27708	CAD	CAD protein	ligase (PC00142)
Q9Y6M7	SLC4A7	Sodium bicarbonate cotransporter 3	secondary carrier transporter (PC00258)
Q92522	H1-10	Histone H1.10	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q15459	SF3A1	Splicing factor 3A subunit 1	RNA splicing factor (PC00148)
P30038	ALDH4A1	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial	dehydrogenase (PC00092)
Q7Z417	NUFIP2	Nuclear fragile X mental retardation-interacting protein 2	
Q0ZGT2	NEXN	Nexilin	scaffold/adaptor protein (PC00226)
P06454	PTMA	Prothymosin alpha	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q659A1	ICE2	Little elongation complex subunit 2	
Q5JTD0	TJAP1	Tight junction-associated protein 1	tight junction (PC00214)
Q9NQC3	RTN4	Reticulon-4	
P33993	MCM2	DNA replication licensing factor MCM7	DNA metabolism protein (PC00009)
Q8IY81	FTSJ3	pre-rRNA 2'-O-ribose RNA methyltransferase FTSJ3	RNA methyltransferase (PC00033)
P63165	SUMO1	Small ubiquitin-related modifier 1	
Q96I59	SYNM	Probable asparagine--tRNA ligase, mitochondrial	aminoacyl-tRNA synthetase (PC00047)
Q13895	BYSL	Bystin	
P26358	DNMT1	DNA (cytosine-5)-methyltransferase 1	DNA methyltransferase (PC00013)
P27816	MAP4	Microtubule-associated protein 4	
Q13425	SNTB2	Beta-2-syntrophin	scaffold/adaptor protein (PC00226)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P51636	CAV2	Caveolin-2	scaffold/adaptor protein (PC00226)
P16403	H1-2	Histone H1.2	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q9H3Z4	DNAJC5	DnaJ homolog subfamily C member 5	chaperone (PC00072)
Q13263	TRIM28	Transcription intermediary factor 1-beta	ubiquitin-protein ligase (PC00234)
P25205	MCM3	DNA replication licensing factor MCM3	DNA metabolism protein (PC00009)
Q9BVG4	PBDC1	Protein PBDC1	
O00567	NOP56	Nucleolar protein 56	RNA metabolism protein (PC00031)
O43493	TGOLN2	Trans-Golgi network integral membrane protein 2	membrane traffic protein (PC00150)
Q9NU22	MDN1	Midasin	
Q9NZN4	EHD2	EH domain-containing protein 2	membrane traffic protein (PC00150)
Q5VT52	RPRD2	Regulation of nuclear pre-mRNA domain-containing protein 2	kinase inhibitor (PC00139)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
O60832	DKC1	H/ACA ribonucleoprotein complex subunit DKC1	centromere DNA-binding protein (PC00071)
P18858	LIG1	DNA ligase 1	DNA ligase (PC00012)
P16949	STMN1	Stathmin	non-motor microtubule binding protein (PC00166)
P20042	EIF2S2	Eukaryotic translation initiation factor 2 subunit 2	translation initiation factor (PC00224)
Q96K21	ZFYVE19	Abscission/NoCut checkpoint regulator	
P02545	LMNA	Prelamin-A/C	
Q9H3N1	TMX1	Thioredoxin-related transmembrane protein 1	
O75822	EIF3J	Eukaryotic translation initiation factor 3 subunit J	translation initiation factor (PC00224)
Q96T23	RSF1	Remodeling and spacing factor 1	
Q9Y6G9	DYNC1LI1	Cytoplasmic dynein 1 light intermediate chain 1	microtubule or microtubule-binding cytoskeletal protein (PC00157)
Q9Y281	CFL2	Cofilin-2	non-motor actin binding protein (PC00165)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P08238	HSP90AB1	Heat shock protein HSP 90-beta	Hsp90 family chaperone (PC00028)
Q96N64	PWWP2A	PWWP domain-containing protein 2A	DNA methyltransferase (PC00013)
O43169	CYB5B	Cytochrome b5 type B	oxidoreductase (PC00176)
Q9C0C2	TNKS1BP1	182 kDa tankyrase-1-binding protein	
P51858	HDGF	Hepatoma-derived growth factor	transcription cofactor (PC00217)
P27824	CANX	Calnexin	chaperone (PC00072)
Q9HCN4	GPN1	GPN-loop GTPase 1	small GTPase (PC00208)
P46821	MAP1B	Microtubule-associated protein 1B	non-motor microtubule binding protein (PC00166)
Q14247	CTTN	Src substrate cortactin	scaffold/adaptor protein (PC00226)
P10412	H1-4	Histone H1.4	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q14684	RRP1B	Ribosomal RNA processing protein 1 homolog B	

Table 30: List of differentially expressed – upregulated phosphoproteins in A549 cells infected with Influenza A – pH1N1 at 0 hr

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q96GM8	TOE1	Target of EGR1 protein 1	mRNA polyadenylation factor (PC00146)
Q07955	SRSF1	Serine/arginine-rich splicing factor 1	RNA splicing factor (PC00148)
Q9H6F5	CCDC86	Coiled-coil domain-containing protein 86	
Q9H1E3	NUCKS1	Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1	
P11388	TOP2A	DNA topoisomerase 2-alpha	DNA metabolism protein (PC00009)
Q99733	NAP1L4	Nucleosome assembly protein 1-like 4	chromatin/chromatin-binding, or -regulatory protein (PC00077)
P23193	TCEA1	Transcription elongation factor A protein 1	general transcription factor (PC00259)
Q9H4G0	EPB41L1	Band 4.1-like protein 1	non-motor actin binding protein (PC00165)
Q6PKG0	LARP1	La-related protein 1	RNA metabolism protein (PC00031)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q8WX93	PALLD	Palladin	scaffold/adaptor protein (PC00226)
Q03701	CEBPZ	CCAAT/enhancer-binding protein zeta	DNA-binding transcription factor (PC00218)
P05386	RPLP1	60S acidic ribosomal protein P1	ribosomal protein (PC00202)
Q12906	ILF3	Interleukin enhancer-binding factor 3	RNA metabolism protein (PC00031)
P41236	PPP1R2	Protein phosphatase inhibitor 2	phosphatase inhibitor (PC00183)
Q03135	CAV1	Caveolin-1	scaffold/adaptor protein (PC00226)
P62269	RPS18	40S ribosomal protein S18	ribosomal protein (PC00202)
Q15185	PTGES3	Prostaglandin E synthase 3	chaperone (PC00072)
Q7L4I2	RSRC2	Arginine/serine-rich coiled-coil protein 2	
Q16666	IFI16	Gamma-interferon-inducible protein 16	DNA-binding transcription factor (PC00218)
Q9H6S0	YTHDC2	3'-5' RNA helicase YTHDC2	RNA helicase (PC00032)
P05388	RPLP0	60S acidic ribosomal protein P0	ribosomal protein (PC00202)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P61978	HNRNPK	Heterogeneous nuclear ribonucleoprotein K	RNA metabolism protein (PC00031)
P17812	CTPS1	CTP synthase 1	ligase (PC00142)
Q96JA1	LIG1	Leucine-rich repeats and immunoglobulin-like domains protein 1	transmembrane signal receptor (PC00197)
P98175	RBM10	RNA-binding protein 10	
P52756	RBM5	RNA-binding protein 5	
O95365	ZBTB7A	Zinc finger and BTB domain-containing protein 7A	C2H2 zinc finger transcription factor (PC00248)
Q14103	HNRNPD	Heterogeneous nuclear ribonucleoprotein D0	RNA metabolism protein (PC00031)
P61247	RPS3A	40S ribosomal protein S3a	ribosomal protein (PC00202)
P31943	HNRNPH1	Heterogeneous nuclear ribonucleoprotein H	RNA splicing factor (PC00148)
O75569	PRKRA	Interferon-inducible double-stranded RNA-dependent protein kinase activator A	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q9NTJ3	SMC4	Structural maintenance of chromosomes protein 4	
P25788	PSMA3	Proteasome subunit alpha type-3	protease (PC00190)
Q86XP3	DDX42	ATP-dependent RNA helicase DDX42	RNA helicase (PC00032)
Q13442	PDAP1	28 kDa heat- and acid-stable phosphoprotein	
P16402	H1-3	Histone H1.3	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q9BZZ5	API5	Apoptosis inhibitor 5	
P17096	HMGA1	High mobility group protein HMG-I/HMG-Y	endodeoxyribonuclease (PC00093)
Q92911	SLC5A5	Sodium/iodide cotransporter	secondary carrier transporter (PC00258)
Q15121	PEA15	Astrocytic phosphoprotein PEA-15	
P21333	FLNA	Filamin-A	
Q9UK76	JPT1	Jupiter microtubule associated homolog 1	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q9UQ80	PA2G4	Proliferation-associated protein 2G4	protease (PC00190)
P49756	RBM25	RNA-binding protein 25	
P29966	MARCKS	Myristoylated alanine-rich C-kinase substrate	
Q8NE71	ABCF1	ATP-binding cassette sub-family F member 1	translation elongation factor (PC00222)
Q9Y3T9	NOC2L	Nucleolar complex protein 2 homolog	
O95400	CD2BP2	CD2 antigen cytoplasmic tail-binding protein 2	RNA splicing factor (PC00148)
P06748	NPM1	Nucleophosmin	chaperone (PC00072)
P14923	JUP	Junction plakoglobin	
P09651	HNRNPA1	Heterogeneous nuclear ribonucleoprotein A1	RNA processing factor (PC00147)
P19338	NCL	Nucleolin	RNA metabolism protein (PC00031)
Q13185	CBX3	Chromobox protein homolog 3	
Q0ZGT2	NEXN	Nexilin	scaffold/adaptor protein (PC00226)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P06454	PTMA	Prothymosin alpha	
O95218	ZRANB2	Zinc finger Ran-binding domain-containing protein 2	RNA splicing factor (PC00148)
Q8IY81	FTSJ3	pre-rRNA 2'-O-ribose RNA methyltransferase FTSJ3	RNA methyltransferase (PC00033)
Q13895	BYSL	Bystin	
P26358	DNMT1	DNA (cytosine-5)-methyltransferase 1	DNA methyltransferase (PC00013)
P27816	MAP4	Microtubule-associated protein 4	
Q9Y2X7	GIT1	ARF GTPase-activating protein GIT1	
P26368	U2AF2	Splicing factor U2AF 65 kDa subunit	
P54727	RAD23B	UV excision repair protein RAD23 homolog B	damaged DNA-binding protein (PC00086)
Q8IYB3	SRRM1	Serine/arginine repetitive matrix protein 1	RNA processing factor (PC00147)
O00567	NOP56	Nucleolar protein 56	RNA metabolism protein (PC00031)
Q9NZN4	EHD2	EH domain-containing protein 2	membrane traffic protein (PC00150)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q13112	CHAF1B	Chromatin assembly factor 1 subunit B	chromatin/chromatin-binding, or -regulatory protein (PC00077)
P21127	CDK11B	Cyclin-dependent kinase 11B	non-receptor serine/threonine protein kinase (PC00167)
Q8TCJ2	STT3B	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3B	glycosyltransferase (PC00111)
P23588	EIF4B	Eukaryotic translation initiation factor 4B	translation initiation factor (PC00224)
O00505	KPNA3	Importin subunit alpha-4	transporter (PC00227)
Q9NYF8	BCLAF1	Bcl-2-associated transcription factor 1	DNA metabolism protein (PC00009)
P18858	LIG1	DNA ligase 1	DNA ligase (PC00012)
Q7Z4V5	HDGFL2	Hepatoma-derived growth factor-related protein 2	transcription cofactor (PC00217)
P08651	NFIC	Nuclear factor 1 C-type	DNA-binding transcription factor (PC00218)
Q8IWA0	WDR75	WD repeat-containing protein 75	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
O75475	PSIP1	PC4 and SFRS1-interacting protein	transcription cofactor (PC00217)
P35659	DEK	Protein DEK	chromatin/chromatin-binding, or -regulatory protein (PC00077)
P51608	MECP2	Methyl-CpG-binding protein 2	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q1KMD3	HNRNPUL2	Heterogeneous nuclear ribonucleoprotein U-like protein 2	
O75367	MACROH2A1	Core histone macro-H2A.1	chromatin/chromatin-binding, or -regulatory protein (PC00077)
P08238	HSP90AB1	Heat shock protein HSP 90-beta	Hsp90 family chaperone (PC00028)
O43169	CYB5B	Cytochrome b5 type B	oxidoreductase (PC00176)
Q8TDD1	DDX54	ATP-dependent RNA helicase DDX54	RNA helicase (PC00032)
P51858	HDGF	Hepatoma-derived growth factor	transcription cofactor (PC00217)
P27824	CANX	Calnexin	chaperone (PC00072)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P46821	MAP1B	Microtubule-associated protein 1B	non-motor microtubule binding protein (PC00166)
Q66PJ3	ARL6IP4	ADP-ribosylation factor-like protein 6-interacting protein 4	
E9PAV3	NACA	Nascent polypeptide-associated complex subunit alpha, muscle-specific form	basic helix-loop-helix transcription factor (PC00055)
Q08945	SSRP1	FACT complex subunit SSRP1	

Table 31: List of differentially expressed – downregulated phosphoproteins in A549 cells infected with Influenza A – pH1N1 at 0 hr

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P24557	TBXAS1	Thromboxane-A synthase	isomerase (PC00135)
Q16513	PKN2	Serine/threonine-protein kinase N2	non-receptor serine/threonine protein kinase (PC00167)
O76075	CAD	DNA fragmentation factor subunit beta	DNA metabolism protein (PC00009)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P16615	ATP2A2	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	primary active transporter (PC00068)
P11388	TOP2A	DNA topoisomerase 2-alpha	DNA metabolism protein (PC00009)
Q15149	PLEC	Plectin	intermediate filament binding protein (PC00130)
Q6ZRP7	QSOX2	Sulfhydryl oxidase 2	oxidase (PC00175)
Q6PD62	CTR9	RNA polymerase-associated protein CTR9 homolog	general transcription factor (PC00259)
Q5JTV8	TOR1AIP1	Torsin-1A-interacting protein 1	
Q04721	NOTCH2	Neurogenic locus notch homolog protein 2	
Q92844	TRAF2	TRAF family member-associated NF-kappa-B activator	
Q8IVP5	FUNDC1	FUN14 domain-containing protein 1	
P49585	PCYT1A	Choline-phosphate cytidylyltransferase A	transferase (PC00220)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P16401	H1-5	Histone H1.5	chromatin/chromatin-binding, or -regulatory protein (PC00077)
P05386	RPLP1	60S acidic ribosomal protein P1	ribosomal protein (PC00202)
Q8NC44	RETREG2	Reticulophagy regulator 2	
Q5VTR2	RNF20	E3 ubiquitin-protein ligase BRE1A	
Q9UQ35	SRRM2	Serine/arginine repetitive matrix protein 2	
Q86UP2	KTN1	Kinectin	
P84022	SMAD3	Mothers against decapentaplegic homolog 3	DNA-binding transcription factor (PC00218)
P05388	RPLP0	60S acidic ribosomal protein P0	ribosomal protein (PC00202)
Q12766	HMGXB3	HMG domain-containing protein 3	
P17676	CEBPB	CCAAT/enhancer-binding protein beta	basic leucine zipper transcription factor (PC00056)
Q8N163	CCAR2	Cell cycle and apoptosis regulator protein 2	primary active transporter (PC00068)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q01650	SLC7A5	Large neutral amino acids transporter small subunit 1	transporter (PC00227)
Q13501	SQSTM1	Sequestosome-1	
P46100	ATRX	Transcriptional regulator ATRX	DNA-binding transcription factor (PC00218)
O15061	SYNM	Synemin	
A6NK06	CAD	Cis-aconitate decarboxylase	dehydratase (PC00091)
Q8WVC0	LEO1	RNA polymerase-associated protein LEO1	DNA-directed RNA polymerase (PC00019)
Q05209	PTPN12	Tyrosine-protein phosphatase non-receptor type 12	
Q2TB10	ZNF800	Zinc finger protein 800	
Q15796	SMAD2	Mothers against decapentaplegic homolog 2	DNA-binding transcription factor (PC00218)
P47755	CAPZA2	F-actin-capping protein subunit alpha-2	non-motor actin binding protein (PC00165)
Q16637	SMN1	Survival motor neuron protein	RNA splicing factor (PC00148)
Q12933	TRAF2	TNF receptor-associated factor 2	scaffold/adaptor protein (PC00226)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q09666	AHNAK	Neuroblast differentiation-associated protein AHNAK	
Q05682	CAD	Caldesmon	non-motor actin binding protein (PC00165)
Q6ZRV2	FAM83H	Protein FAM83H	
P42166	TMPO	Lamina-associated polypeptide 2, isoform alpha	peptide hormone (PC00179)
Q9P1Y6	PHRF1	PHD and RING finger domain-containing protein 1	
P29317	EPHA2	Ephrin type-A receptor 2	transmembrane signal receptor (PC00197)
Q5JTH9	RRP12	RRP12-like protein	
P05787	KRT8	Keratin, type II cytoskeletal 8	
Q96JM3	CHAMP1	Chromosome alignment-maintaining phosphoprotein 1	
Q12888	TP53BP1	TP53-binding protein 1	DNA metabolism protein (PC00009)
Q07157	TJP1	Tight junction protein ZO-1	tight junction (PC00214)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P08670	VIM	Vimentin	intermediate filament (PC00129)
P10451	SPP1	Osteopontin	cytokine (PC00083)
O60231	DHX16	Pre-mRNA-splicing factor ATP-dependent RNA helicase DHX16	RNA helicase (PC00032)
P13611	VCAN	Versican core protein	extracellular matrix glycoprotein (PC00100)
B5ME19	EIF3CL	Eukaryotic translation initiation factor 3 subunit C-like protein	translation initiation factor (PC00224)
Q9BUJ2	HNRNPUL1	Heterogeneous nuclear ribonucleoprotein U-like protein 1	
P27708	CAD	CAD protein	ligase (PC00142)
O94826	TOMM70	Mitochondrial import receptor subunit TOM70	primary active transporter (PC00068)
Q92522	H1-10	Histone H1.10	chromatin/chromatin-binding, or -regulatory protein (PC00077)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P30038	ALDH4A1	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial	dehydrogenase (PC00092)
Q8IZL8	PELP1	Proline-, glutamic acid- and leucine-rich protein 1	
Q5JTD0	TJAP1	Tight junction-associated protein 1	tight junction (PC00214)
Q9NQC3	RTN4	Reticulon-4	
O43760	SYNGR2	Synaptogyrin-2	membrane trafficking regulatory protein (PC00151)
P63165	SUMO1	Small ubiquitin-related modifier 1	
A2RRP1	NBAS	Neuroblastoma-amplified sequence	
Q96I59	SYNM	Probable asparagine--tRNA ligase, mitochondrial	aminoacyl-tRNA synthetase (PC00047)
P16403	H1-2	Histone H1.2	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q9H3Z4	DNAJC5	DnaJ homolog subfamily C member 5	chaperone (PC00072)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q13263	TRIM28	Transcription intermediary factor 1-beta	ubiquitin-protein ligase (PC00234)
Q9P2G1	ANKIB1	Ankyrin repeat and IBR domain-containing protein 1	ubiquitin-protein ligase (PC00234)
P25205	MCM3	DNA replication licensing factor MCM3	DNA metabolism protein (PC00009)
O43493	TGOLN2	Trans-Golgi network integral membrane protein 2	membrane traffic protein (PC00150)
Q9H6X2	ANTXR1	Anthrax toxin receptor 1	cell adhesion molecule (PC00069)
Q9H7N4	SCAF1	Splicing factor, arginine/serine-rich 19	RNA splicing factor (PC00148)
Q96K21	ZFYVE19	Abscission/NoCut checkpoint regulator	
P02545	LMNA	Prelamin-A/C	
Q9H3N1	TMX1	Thioredoxin-related transmembrane protein 1	
Q96T23	RSF1	Remodeling and spacing factor 1	
Q9Y281	CFL2	Cofilin-2	non-motor actin binding protein (PC00165)
O76021	RSL1D1	Ribosomal L1 domain-containing protein 1	ribosomal protein (PC00202)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q96N64	PWWP2A	PWWP domain-containing protein 2A	DNA methyltransferase (PC00013)
Q9UBH6	XPR1	Xenotropic and polytropic retrovirus receptor 1	secondary carrier transporter (PC00258)
Q9HCN4	GPN1	GPN-loop GTPase 1	small GTPase (PC00208)
P46821	MAP1B	Microtubule-associated protein 1B	non-motor microtubule binding protein (PC00166)
P23528	CFL1	Cofilin-1	non-motor actin binding protein (PC00165)
Q14684	RRP1B	Ribosomal RNA processing protein 1 homolog B	
Q9BX95	SPP1	Sphingosine-1-phosphate phosphatase 1	phosphatase (PC00181)

Table 32: List of differentially expressed – upregulated phosphoproteins in A549 cells infected with Influenza A – pH1N1 at 8 hr

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q96GM8	TOE1	Target of EGR1 protein 1	mRNA polyadenylation factor (PC00146)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q96ST3	SIN3A	Paired amphipathic helix protein Sin3a	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q07955	SRSF1	Serine/arginine-rich splicing factor 1	RNA splicing factor (PC00148)
Q9H6F5	CCDC86	Coiled-coil domain-containing protein 86	
Q9H1E3	NUCKS1	Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1	
Q9UKV3	ACIN1	Apoptotic chromatin condensation inducer in the nucleus	
P11388	TOP2A	DNA topoisomerase 2-alpha	DNA metabolism protein (PC00009)
Q99733	NAP1L4	Nucleosome assembly protein 1-like 4	chromatin/chromatin-binding, or -regulatory protein (PC00077)
P23193	TCEA1	Transcription elongation factor A protein 1	general transcription factor (PC00259)
Q9H4G0	EPB41L1	Band 4.1-like protein 1	non-motor actin binding protein (PC00165)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P07900	HSP90AA1	Heat shock protein HSP 90-alpha	Hsp90 family chaperone (PC00028)
Q6PKG0	LARP1	La-related protein 1	RNA metabolism protein (PC00031)
Q8WX93	PALLD	Palladin	scaffold/adaptor protein (PC00226)
Q8TAQ2	SMARCC2	SWI/SNF complex subunit SMARCC2	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q7Z6Z7	HUWE1	E3 ubiquitin-protein ligase HUWE1	ubiquitin-protein ligase (PC00234)
Q03701	CEBPZ	CCAAT/enhancer-binding protein zeta	DNA-binding transcription factor (PC00218)
Q13439	GOLGA4	Golgin subfamily A member 4	membrane trafficking regulatory protein (PC00151)
Q7L014	DDX46	Probable ATP-dependent RNA helicase DDX46	RNA helicase (PC00032)
P05386	RPLP1	60S acidic ribosomal protein P1	ribosomal protein (PC00202)
Q12906	ILF3	Interleukin enhancer-binding factor 3	RNA metabolism protein (PC00031)
Q8ND56	LSM14A	Protein LSM14 homolog A	RNA metabolism protein (PC00031)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P62269	RPS18	40S ribosomal protein S18	ribosomal protein (PC00202)
P35579	MYH9	Myosin-9	actin or actin-binding cytoskeletal protein (PC00041)
P84022	SMAD3	Mothers against decapentaplegic homolog 3	DNA-binding transcription factor (PC00218)
Q9NWD8	TMEM248	Transmembrane protein 248	
Q7L4I2	RSRC2	Arginine/serine-rich coiled-coil protein 2	
Q16666	IFI16	Gamma-interferon-inducible protein 16	DNA-binding transcription factor (PC00218)
P05388	RPLP0	60S acidic ribosomal protein P0	ribosomal protein (PC00202)
O75821	EIF3G	Eukaryotic translation initiation factor 3 subunit G	translation initiation factor (PC00224)
P61978	HNRNPK	Heterogeneous nuclear ribonucleoprotein K	RNA metabolism protein (PC00031)
P17812	CTPS1	CTP synthase 1	ligase (PC00142)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q96JA1	LIG1	Leucine-rich repeats and immunoglobulin-like domains protein 1	transmembrane signal receptor (PC00197)
P98175	RBM10	RNA-binding protein 10	
P52756	RBM5	RNA-binding protein 5	
O95365	ZBTB7A	Zinc finger and BTB domain-containing protein 7A	C2H2 zinc finger transcription factor (PC00248)
Q14103	HNRNPD	Heterogeneous nuclear ribonucleoprotein D0	RNA metabolism protein (PC00031)
P61247	RPS3A	40S ribosomal protein S3a	ribosomal protein (PC00202)
Q9BU76	C1orf35	Multiple myeloma tumor-associated protein 2	
Q9H0D6	XRN2	5'-3' exoribonuclease 2	exoribonuclease (PC00099)
O75569	PRKRA	Interferon-inducible double-stranded RNA-dependent protein kinase activator A	
Q02880	TOP2B	DNA topoisomerase 2-beta	DNA metabolism protein (PC00009)
P46100	ATRX	Transcriptional regulator ATRX	DNA-binding transcription factor (PC00218)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q7RTP6	MICAL3	[F-actin]-monooxygenase MICAL3	scaffold/adaptor protein (PC00226)
P25788	PSMA3	Proteasome subunit alpha type-3	protease (PC00190)
Q15796	SMAD2	Mothers against decapentaplegic homolog 2	DNA-binding transcription factor (PC00218)
Q86XP3	DDX42	ATP-dependent RNA helicase DDX42	RNA helicase (PC00032)
Q13442	PDAP1	28 kDa heat- and acid-stable phosphoprotein	
P16402	H1-3	Histone H1.3	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q9BZZ5	API5	Apoptosis inhibitor 5	
P17096	HMGA1	High mobility group protein HMG-I/HMG-Y	endodeoxyribonuclease (PC00093)
Q16637	SMN1	Survival motor neuron protein	RNA splicing factor (PC00148)
Q9H0S4	DDX47	Probable ATP-dependent RNA helicase DDX47	RNA helicase (PC00032)
Q15121	PEA15	Astrocytic phosphoprotein PEA-15	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q9UK76	JPT1	Jupiter microtubule associated homolog 1	
Q9UQ80	PA2G4	Proliferation-associated protein 2G4	protease (PC00190)
P49756	RBM25	RNA-binding protein 25	
P29966	MARCKS	Myristoylated alanine-rich C-kinase substrate	
Q8NE71	ABCF1	ATP-binding cassette sub-family F member 1	translation elongation factor (PC00222)
P23396	RPS3	40S ribosomal protein S3	ribosomal protein (PC00202)
O95400	CD2BP2	CD2 antigen cytoplasmic tail-binding protein 2	RNA splicing factor (PC00148)
P06748	NPM1	Nucleophosmin	chaperone (PC00072)
P14923	JUP	Junction plakoglobin	
Q12888	TP53BP1	TP53-binding protein 1	DNA metabolism protein (PC00009)
P07910	HNRNPC	Heterogeneous nuclear ribonucleoproteins C1/C2	RNA metabolism protein (PC00031)
P09651	HNRNPA1	Heterogeneous nuclear ribonucleoprotein A1	RNA processing factor (PC00147)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P19338	NCL	Nucleolin	RNA metabolism protein (PC00031)
Q13185	CBX3	Chromobox protein homolog 3	
Q15459	SF3A1	Splicing factor 3A subunit 1	RNA splicing factor (PC00148)
Q7Z417	NUFIP2	Nuclear fragile X mental retardation-interacting protein 2	
Q0ZGT2	NEXN	Nexilin	scaffold/adaptor protein (PC00226)
Q00839	HNRNPU	Heterogeneous nuclear ribonucleoprotein U	
P06454	PTMA	Prothymosin alpha	
O95218	ZRANB2	Zinc finger Ran-binding domain-containing protein 2	RNA splicing factor (PC00148)
Q8IY81	FTSJ3	pre-rRNA 2'-O-ribose RNA methyltransferase FTSJ3	RNA methyltransferase (PC00033)
Q13895	BYSL	Bystin	
P26358	DNMT1	DNA (cytosine-5)-methyltransferase 1	DNA methyltransferase (PC00013)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P27816	MAP4	Microtubule-associated protein 4	
Q16643	DBN1	Drebrin	
P26368	U2AF2	Splicing factor U2AF 65 kDa subunit	
P54727	RAD23B	UV excision repair protein RAD23 homolog B	damaged DNA-binding protein (PC00086)
Q13263	TRIM28	Transcription intermediary factor 1-beta	ubiquitin-protein ligase (PC00234)
Q8IYB3	SRRM1	Serine/arginine repetitive matrix protein 1	RNA processing factor (PC00147)
Q9BVG4	PBDC1	Protein PBDC1	
O00567	NOP56	Nucleolar protein 56	RNA metabolism protein (PC00031)
Q9NZN4	EHD2	EH domain-containing protein 2	membrane traffic protein (PC00150)
Q14978	NOLC1	Nucleolar and coiled-body phosphoprotein 1	
Q13112	CHAF1B	Chromatin assembly factor 1 subunit B	chromatin/chromatin-binding, or -regulatory protein (PC00077)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P21127	CDK11B	Cyclin-dependent kinase 11B	non-receptor serine/threonine protein kinase (PC00167)
Q96L91	EP400	E1A-binding protein p400	
P23588	EIF4B	Eukaryotic translation initiation factor 4B	translation initiation factor (PC00224)
O00505	KPNA3	Importin subunit alpha-4	transporter (PC00227)
P29692	EEF1D	Elongation factor 1-delta	translation elongation factor (PC00222)
P18858	LIG1	DNA ligase 1	DNA ligase (PC00012)
Q7Z4V5	HDGFL2	Hepatoma-derived growth factor-related protein 2	transcription cofactor (PC00217)
Q14498	RBM39	RNA-binding protein 39	RNA splicing factor (PC00148)
Q9Y2W1	THRAP3	Thyroid hormone receptor-associated protein 3	DNA metabolism protein (PC00009)
P20042	EIF2S2	Eukaryotic translation initiation factor 2 subunit 2	translation initiation factor (PC00224)
P08651	NFIC	Nuclear factor 1 C-type	DNA-binding transcription factor (PC00218)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q8IWA0	WDR75	WD repeat-containing protein 75	
Q9UHD8	SEPTIN9	Septin-9	cytoskeletal protein (PC00085)
O75822	EIF3J	Eukaryotic translation initiation factor 3 subunit J	translation initiation factor (PC00224)
Q15019	SEPTIN2	Septin-2	cytoskeletal protein (PC00085)
O75475	PSIP1	PC4 and SFRS1-interacting protein	transcription cofactor (PC00217)
P35659	DEK	Protein DEK	chromatin/chromatin-binding, or -regulatory protein (PC00077)
P51608	MECP2	Methyl-CpG-binding protein 2	chromatin/chromatin-binding, or -regulatory protein (PC00077)
O00264	PGRMC1	Membrane-associated progesterone receptor component 1	transmembrane signal receptor (PC00197)
Q92466	DDB2	DNA damage-binding protein 2	damaged DNA-binding protein (PC00086)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q1KMD3	HNRNPUL2	Heterogeneous nuclear ribonucleoprotein U-like protein 2	
O75367	MACROH2A1	Core histone macro-H2A.1	chromatin/chromatin-binding, or -regulatory protein (PC00077)
O43169	CYB5B	Cytochrome b5 type B	oxidoreductase (PC00176)
P51858	HDGF	Hepatoma-derived growth factor	transcription cofactor (PC00217)
P46821	MAP1B	Microtubule-associated protein 1B	non-motor microtubule binding protein (PC00166)
Q66PJ3	ARL6IP4	ADP-ribosylation factor-like protein 6-interacting protein 4	
Q9NTI5	PDS5B	Sister chromatid cohesion protein PDS5 homolog B	chromatin/chromatin-binding, or -regulatory protein (PC00077)
E9PAV3	NACA	Nascent polypeptide-associated complex subunit alpha, muscle-specific form	basic helix-loop-helix transcription factor (PC00055)

Table 33: List of differentially expressed – downregulated phosphoproteins in A549 cells infected with Influenza A – pH1N1 at 8 hr

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q16513	PKN2	Serine/threonine-protein kinase N2	non-receptor serine/threonine protein kinase (PC00167)
P16615	ATP2A2	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	primary active transporter (PC00068)
Q15149	PLEC	Plectin	intermediate filament binding protein (PC00130)
Q6ZRP7	QSOX2	Sulfhydryl oxidase 2	oxidase (PC00175)
Q6PD62	CTR9	RNA polymerase-associated protein CTR9 homolog	general transcription factor (PC00259)
Q5JTV8	TOR1AIP1	Torsin-1A-interacting protein 1	
Q04721	NOTCH2	Neurogenic locus notch homolog protein 2	
Q92844	TRAF2	TRAF family member-associated NF-kappa-B activator	
Q8IVP5	FUNDC1	FUN14 domain-containing protein 1	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P49585	PCYT1A	Choline-phosphate cytidylyltransferase A	transferase (PC00220)
Q8IXM2	C17orf49	Chromatin complexes subunit BAP18	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q8NC44	RETREG2	Reticulophagy regulator 2	
Q9UQ35	SRRM2	Serine/arginine repetitive matrix protein 2	
Q03135	CAV1	Caveolin-1	scaffold/adaptor protein (PC00226)
Q15185	PTGES3	Prostaglandin E synthase 3	chaperone (PC00072)
O75369	FLNB	Filamin-B	
P98175	RBM10	RNA-binding protein 10	
Q01650	SLC7A5	Large neutral amino acids transporter small subunit 1	transporter (PC00227)
Q13501	SQSTM1	Sequestosome-1	
O15061	SYNM	Synemin	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q8WVC0	LEO1	RNA polymerase-associated protein LEO1	DNA-directed RNA polymerase (PC00019)
Q05209	PTPN12	Tyrosine-protein phosphatase non-receptor type 12	
Q8IVF2	AHNAK2	Protein AHNAK2	
Q2TB10	ZNF800	Zinc finger protein 800	
P47755	CAPZA2	F-actin-capping protein subunit alpha-2	non-motor actin binding protein (PC00165)
Q12933	TRAF2	TNF receptor-associated factor 2	scaffold/adaptor protein (PC00226)
Q09666	AHNAK	Neuroblast differentiation-associated protein AHNAK	
O95674	CDS2	Phosphatidate cytidylyltransferase 2	transferase (PC00220)
P42166	TMPO	Lamina-associated polypeptide 2, isoform alpha	peptide hormone (PC00179)
P29966	MARCKS	Myristoylated alanine-rich C-kinase substrate	
Q9P1Y6	PHRF1	PHD and RING finger domain-containing protein 1	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P29317	EPHA2	Ephrin type-A receptor 2	transmembrane signal receptor (PC00197)
Q5JTH9	RRP12	RRP12-like protein	
Q96JM3	CHAMP1	Chromosome alignment-maintaining phosphoprotein 1	
Q9Y3T9	NOC2L	Nucleolar complex protein 2 homolog	
P13861	PRKAR2A	cAMP-dependent protein kinase type II-alpha regulatory subunit	kinase modulator (PC00140)
Q9NWH9	SLTM	SAFB-like transcription modulator	
Q07157	TJP1	Tight junction protein ZO-1	tight junction (PC00214)
P08670	VIM	Vimentin	intermediate filament (PC00129)
P10451	SPP1	Osteopontin	cytokine (PC00083)
O60231	DHX16	Pre-mRNA-splicing factor ATP-dependent RNA helicase DHX16	RNA helicase (PC00032)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P13611	VCAN	Versican core protein	extracellular matrix glycoprotein (PC00100)
Q15154	PCM1	Pericentriolar material 1 protein	
P04792	HSPB1	Heat shock protein beta-1	chaperone (PC00072)
Q9Y6M7	SLC4A7	Sodium bicarbonate cotransporter 3	secondary carrier transporter (PC00258)
Q92522	H1-10	Histone H1.10	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q8ND76	CCNY	Cyclin-Y	
Q7Z417	NUFIP2	Nuclear fragile X mental retardation-interacting protein 2	
Q8IZL8	PELP1	Proline-, glutamic acid- and leucine-rich protein 1	
Q9NQC3	RTN4	Reticulon-4	
O43760	SYNGR2	Synaptogyrin-2	membrane trafficking regulatory protein (PC00151)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q96I59	SYNM	Probable asparagine--tRNA ligase, mitochondrial	aminoacyl-tRNA synthetase (PC00047)
Q96TA1	NIBAN2	Protein Niban 2	
Q6UN15	FIP1L1	Pre-mRNA 3'-end-processing factor FIP1	
O60716	CTNND1	Catenin delta-1	intermediate filament binding protein (PC00130)
Q9H3Z4	DNAJC5	DnaJ homolog subfamily C member 5	chaperone (PC00072)
Q9Y2X7	GIT1	ARF GTPase-activating protein GIT1	
Q9NS69	TOMM22	Mitochondrial import receptor subunit TOM22 homolog	primary active transporter (PC00068)
Q9H6X2	ANTXR1	Anthrax toxin receptor 1	cell adhesion molecule (PC00069)
Q9H7N4	SCAF1	Splicing factor, arginine/serine-rich 19	RNA splicing factor (PC00148)
P02545	LMNA	Prelamin-A/C	
Q9H3N1	TMX1	Thioredoxin-related transmembrane protein 1	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
O00264	PGRMC1	Membrane-associated progesterone receptor component 1	transmembrane signal receptor (PC00197)
O76021	RSL1D1	Ribosomal L1 domain-containing protein 1	ribosomal protein (PC00202)
Q9C0C2	TNKS1BP1	182 kDa tankyrase-1-binding protein	
Q96S55	WRNIP1	ATPase WRNIP1	DNA helicase (PC00011)
P27824	CANX	Calnexin	chaperone (PC00072)
Q9UBH6	XPR1	Xenotropic and polytropic retrovirus receptor 1	secondary carrier transporter (PC00258)
P46821	MAP1B	Microtubule-associated protein 1B	non-motor microtubule binding protein (PC00166)
Q14684	RRP1B	Ribosomal RNA processing protein 1 homolog B	
Q9BX95	SPP1	Sphingosine-1-phosphate phosphatase 1	phosphatase (PC00181)
O75420	CDS2	GRB10-interacting GYF protein 1	

Table 34: List of differentially expressed – upregulated phosphoproteins in A549 cells infected with Influenza A – H3N2 at 0 hr

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q96GM8	TOE1	Target of EGR1 protein 1	mRNA polyadenylation factor (PC00146)
Q96ST3	SIN3A	Paired amphipathic helix protein Sin3a	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q14137	BOP1	Ribosome biogenesis protein BOP1	ribosomal protein (PC00202)
Q07955	SRSF1	Serine/arginine-rich splicing factor 1	RNA splicing factor (PC00148)
Q9H1E3	NUCKS1	Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1	
P11388	TOP2A	DNA topoisomerase 2-alpha	DNA metabolism protein (PC00009)
Q99733	NAP1L4	Nucleosome assembly protein 1-like 4	chromatin/chromatin-binding, or -regulatory protein (PC00077)
P23193	TCEA1	Transcription elongation factor A protein 1	general transcription factor (PC00259)
P49768	PSEN1	Presenilin-1	aspartic protease (PC00053)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P63218	GNG5	Guanine nucleotide-binding protein G (I)/G (S)/G (O) subunit gamma-5	heterotrimeric G-protein (PC00117)
Q03701	CEBPZ	CCAAT/enhancer-binding protein zeta	DNA-binding transcription factor (PC00218)
Q13439	GOLGA4	Golgin subfamily A member 4	membrane trafficking regulatory protein (PC00151)
P05386	RPLP1	60S acidic ribosomal protein P1	ribosomal protein (PC00202)
Q12906	ILF3	Interleukin enhancer-binding factor 3	RNA metabolism protein (PC00031)
P62269	RPS18	40S ribosomal protein S18	ribosomal protein (PC00202)
Q7L4I2	RSRC2	Arginine/serine-rich coiled-coil protein 2	
P05388	RPLP0	60S acidic ribosomal protein P0	ribosomal protein (PC00202)
Q12766	HMGXB3	HMG domain-containing protein 3	
P61978	HNRNPK	Heterogeneous nuclear ribonucleoprotein K	RNA metabolism protein (PC00031)
P17812	CTPS1	CTP synthase 1	ligase (PC00142)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q96JA1	LIG1	Leucine-rich repeats and immunoglobulin-like domains protein 1	transmembrane signal receptor (PC00197)
O95365	ZBTB7A	Zinc finger and BTB domain-containing protein 7A	C2H2 zinc finger transcription factor (PC00248)
Q14103	HNRNPD	Heterogeneous nuclear ribonucleoprotein D0	RNA metabolism protein (PC00031)
P61247	RPS3A	40S ribosomal protein S3a	ribosomal protein (PC00202)
Q9H0D6	XRN2	5'-3' exoribonuclease 2	exoribonuclease (PC00099)
P00352	ALDH1A1	Retinal dehydrogenase 1	dehydrogenase (PC00092)
P25788	PSMA3	Proteasome subunit alpha type-3	protease (PC00190)
Q13442	PDAP1	28 kDa heat- and acid-stable phosphoprotein	
P16402	H1-3	Histone H1.3	chromatin/chromatin-binding, or -regulatory protein (PC00077)
P17096	HMGA1	High mobility group protein HMG-I/HMG-Y	endodeoxyribonuclease (PC00093)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q9P035	HACD3	Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase 3	dehydratase (PC00091)
Q9H0S4	DDX47	Probable ATP-dependent RNA helicase DDX47	RNA helicase (PC00032)
Q15121	PEA15	Astrocytic phosphoprotein PEA-15	
Q9UK76	JPT1	Jupiter microtubule associated homolog 1	
Q9UQ80	PA2G4	Proliferation-associated protein 2G4	protease (PC00190)
P29966	MARCKS	Myristoylated alanine-rich C-kinase substrate	
P23396	RPS3	40S ribosomal protein S3	ribosomal protein (PC00202)
P06748	NPM1	Nucleophosmin	chaperone (PC00072)
P14923	JUP	Junction plakoglobin	
P09651	HNRNPA1	Heterogeneous nuclear ribonucleoprotein A1	RNA processing factor (PC00147)
O00193	C11orf58	Small acidic protein	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P06454	PTMA	Prothymosin alpha	
Q9NQC3	RTN4	Reticulon-4	
O95218	ZRANB2	Zinc finger Ran-binding domain-containing protein 2	RNA splicing factor (PC00148)
Q15942	ZYX	Zyxin	actin or actin-binding cytoskeletal protein (PC00041)
Q8IY81	FTSJ3	pre-rRNA 2'-O-ribose RNA methyltransferase FTSJ3	RNA methyltransferase (PC00033)
P27816	MAP4	Microtubule-associated protein 4	
Q9Y2X7	GIT1	ARF GTPase-activating protein GIT1	
Q9NS69	TOMM22	Mitochondrial import receptor subunit TOM22 homolog	primary active transporter (PC00068)
Q8IYB3	SRRM1	Serine/arginine repetitive matrix protein 1	RNA processing factor (PC00147)
Q9BVG4	PBDC1	Protein PBDC1	
Q9NU22	MDN1	Midasin	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q9NZN4	EHD2	EH domain-containing protein 2	membrane traffic protein (PC00150)
Q9UJK0	TSR3	18S rRNA aminocarboxypropyltransferase	
Q96L91	EP400	E1A-binding protein p400	
P23588	EIF4B	Eukaryotic translation initiation factor 4B	translation initiation factor (PC00224)
O00505	KPNA3	Importin subunit alpha-4	transporter (PC00227)
P29692	EEF1D	Elongation factor 1-delta	translation elongation factor (PC00222)
P18858	LIG1	DNA ligase 1	DNA ligase (PC00012)
Q7Z4V5	HDGFL2	Hepatoma-derived growth factor-related protein 2	transcription cofactor (PC00217)
P08651	NFIC	Nuclear factor 1 C-type	DNA-binding transcription factor (PC00218)
Q8IWA0	WDR75	WD repeat-containing protein 75	
O75475	PSIP1	PC4 and SFRS1-interacting protein	transcription cofactor (PC00217)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P35659	DEK	Protein DEK	chromatin/chromatin-binding, or -regulatory protein (PC00077)
O00264	PGRMC1	Membrane-associated progesterone receptor component 1	transmembrane signal receptor (PC00197)
Q8TEW0	PARD3	Partitioning defective 3 homolog	
Q1KMD3	HNRNPUL2	Heterogeneous nuclear ribonucleoprotein U-like protein 2	
P35221	CTNNA1	Catenin alpha-1	non-motor actin binding protein (PC00165)
P08238	HSP90AB1	Heat shock protein HSP 90-beta	Hsp90 family chaperone (PC00028)
O43169	CYB5B	Cytochrome b5 type B	oxidoreductase (PC00176)
P51858	HDGF	Hepatoma-derived growth factor	transcription cofactor (PC00217)
P46821	MAP1B	Microtubule-associated protein 1B	non-motor microtubule binding protein (PC00166)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q66PJ3	ARL6IP4	ADP-ribosylation factor-like protein 6-interacting protein 4	
Q9NTI5	PDS5B	Sister chromatid cohesion protein PDS5 homolog B	chromatin/chromatin-binding, or -regulatory protein (PC00077)
E9PAV3	NACA	Nascent polypeptide-associated complex subunit alpha, muscle-specific form	basic helix-loop-helix transcription factor (PC00055)
Q08945	SSRP1	FACT complex subunit SSRP1	

Table 35: List of differentially expressed – downregulated phosphoproteins in A549 cells infected with Influenza A – H3N2 at 0 hr

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q16513	PKN2	Serine/threonine-protein kinase N2	non-receptor serine/threonine protein kinase (PC00167)
O76075	CAD	DNA fragmentation factor subunit beta	DNA metabolism protein (PC00009)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P16615	ATP2A2	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	primary active transporter (PC00068)
P11388	TOP2A	DNA topoisomerase 2-alpha	DNA metabolism protein (PC00009)
Q15149	PLEC	Plectin	intermediate filament binding protein (PC00130)
Q6P158	DHX57	Putative ATP-dependent RNA helicase DHX57	RNA helicase (PC00032)
Q6ZRP7	QSOX2	Sulfhydryl oxidase 2	oxidase (PC00175)
Q5JTV8	TOR1AIP1	Torsin-1A-interacting protein 1	
Q04721	NOTCH2	Neurogenic locus notch homolog protein 2	
Q92844	TRAF2	TRAF family member-associated NF-kappa-B activator	
Q8IVP5	FUNDC1	FUN14 domain-containing protein 1	
P49585	PCYT1A	Choline-phosphate cytidylyltransferase A	transferase (PC00220)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P16401	H1-5	Histone H1.5	chromatin/chromatin-binding, or -regulatory protein (PC00077)
P05386	RPLP1	60S acidic ribosomal protein P1	ribosomal protein (PC00202)
Q8NC44	RETREG2	Reticulophagy regulator 2	
Q9UQ35	SRRM2	Serine/arginine repetitive matrix protein 2	
O75369	FLNB	Filamin-B	
Q16666	IFI16	Gamma-interferon-inducible protein 16	DNA-binding transcription factor (PC00218)
Q5T200	ZC3H13	Zinc finger CCCH domain-containing protein 13	
P17676	CEBPB	CCAAT/enhancer-binding protein beta	basic leucine zipper transcription factor (PC00056)
Q8N163	CCAR2	Cell cycle and apoptosis regulator protein 2	primary active transporter (PC00068)
Q9BU76	C1orf35	Multiple myeloma tumor-associated protein 2	
Q6Y7W6	GIGYF2	GRB10-interacting GYF protein 2	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q13501	SQSTM1	Sequestosome-1	
Q02880	TOP2B	DNA topoisomerase 2-beta	DNA metabolism protein (PC00009)
P46100	ATRX	Transcriptional regulator ATRX	DNA-binding transcription factor (PC00218)
O15061	SYNM	Synemin	
A6NK06	CAD	Cis-aconitate decarboxylase	dehydratase (PC00091)
Q8WVC0	LEO1	RNA polymerase-associated protein LEO1	DNA-directed RNA polymerase (PC00019)
Q9Y5J1	UTP18	U3 small nucleolar RNA-associated protein 18 homolog	
Q05209	PTPN12	Tyrosine-protein phosphatase non-receptor type 12	
Q2TB10	ZNF800	Zinc finger protein 800	
P47755	CAPZA2	F-actin-capping protein subunit alpha-2	non-motor actin binding protein (PC00165)
Q16637	SMN1	Survival motor neuron protein	RNA splicing factor (PC00148)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q13435	SF3B2	Splicing factor 3B subunit 2	RNA splicing factor (PC00148)
Q12933	TRAF2	TNF receptor-associated factor 2	scaffold/adaptor protein (PC00226)
Q09666	AHNAK	Neuroblast differentiation-associated protein AHNAK	
Q05682	CAD	Caldesmon	non-motor actin binding protein (PC00165)
Q8NEY8	PPHLN1	Periphrin-1	
Q5T8D3	ACBD5	Acyl-CoA-binding domain-containing protein 5	transfer/carrier protein (PC00219)
Q6ZRV2	FAM83H	Protein FAM83H	
P42166	TMPO	Lamina-associated polypeptide 2, isoform alpha	peptide hormone (PC00179)
P07814	EPRS1	Bifunctional glutamate/proline--tRNA ligase	aminoacyl-tRNA synthetase (PC00047)
Q8NE71	ABCF1	ATP-binding cassette sub-family F member 1	translation elongation factor (PC00222)
Q5JTH9	RRP12	RRP12-like protein	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q96JM3	CHAMP1	Chromosome alignment-maintaining phosphoprotein 1	
Q07157	TJP1	Tight junction protein ZO-1	tight junction (PC00214)
P08670	VIM	Vimentin	intermediate filament (PC00129)
P10451	SPP1	Osteopontin	cytokine (PC00083)
P13611	VCAN	Versican core protein	extracellular matrix glycoprotein (PC00100)
Q15154	PCM1	Pericentriolar material 1 protein	
B5ME19	EIF3CL	Eukaryotic translation initiation factor 3 subunit C-like protein	translation initiation factor (PC00224)
P27708	CAD	CAD protein	ligase (PC00142)
Q92522	H1-10	Histone H1.10	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q7Z417	NUFIP2	Nuclear fragile X mental retardation-interacting protein 2	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q8IY81	FTSJ3	pre-rRNA 2'-O-ribose RNA methyltransferase FTSJ3	RNA methyltransferase (PC00033)
A2RRP1	NBAS	Neuroblastoma-amplified sequence	
Q96I59	SYNM	Probable asparagine--tRNA ligase, mitochondrial	aminoacyl-tRNA synthetase (PC00047)
P26358	DNMT1	DNA (cytosine-5)-methyltransferase 1	DNA methyltransferase (PC00013)
P27816	MAP4	Microtubule-associated protein 4	
Q6UN15	FIP1L1	Pre-mRNA 3'-end-processing factor FIP1	
P16403	H1-2	Histone H1.2	chromatin/chromatin-binding, or -regulatory protein (PC00077)
P78527	PRKDC	DNA-dependent protein kinase catalytic subunit	non-receptor serine/threonine protein kinase (PC00167)
Q13263	TRIM28	Transcription intermediary factor 1-beta	ubiquitin-protein ligase (PC00234)
P25205	MCM3	DNA replication licensing factor MCM3	DNA metabolism protein (PC00009)
O43493	TGOLN2	Trans-Golgi network integral membrane protein 2	membrane traffic protein (PC00150)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q5VT52	RPRD2	Regulation of nuclear pre-mRNA domain-containing protein 2	kinase inhibitor (PC00139)
Q9H6X2	ANTXR1	Anthrax toxin receptor 1	cell adhesion molecule (PC00069)
Q9Y520	PRRC2C	Protein PRRC2C	RNA metabolism protein (PC00031)
P02545	LMNA	Prelamin-A/C	
Q9Y4B5	MTCL1	Microtubule cross-linking factor 1	
Q9H3N1	TMX1	Thioredoxin-related transmembrane protein 1	
Q8NDC0	MAPK1IP1L	MAPK-interacting and spindle-stabilizing protein-like	
Q9UBH6	XPR1	Xenotropic and polytropic retrovirus receptor 1	secondary carrier transporter (PC00258)
Q9HCN4	GPN1	GPN-loop GTPase 1	small GTPase (PC00208)
P46821	MAP1B	Microtubule-associated protein 1B	non-motor microtubule binding protein (PC00166)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P23528	CFL1	Cofilin-1	non-motor actin binding protein (PC00165)
Q9BX95	SPP1	Sphingosine-1-phosphate phosphatase 1	phosphatase (PC00181)

Table 36: List of differentially expressed – upregulated phosphoproteins in A549 cells infected with Influenza A – H3N2 at 8 hrs

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q96GM8	TOE1	Target of EGR1 protein 1	mRNA polyadenylation factor (PC00146)
Q96ST3	SIN3A	Paired amphipathic helix protein Sin3a	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q14137	BOP1	Ribosome biogenesis protein BOP1	ribosomal protein (PC00202)
Q07955	SRSF1	Serine/arginine-rich splicing factor 1	RNA splicing factor (PC00148)
Q9H6F5	CCDC86	Coiled-coil domain-containing protein 86	
Q9H1E3	NUCKS1	Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P24557	TBXAS1	Thromboxane-A synthase	isomerase (PC00135)
P11388	TOP2A	DNA topoisomerase 2-alpha	DNA metabolism protein (PC00009)
Q99733	NAP1L4	Nucleosome assembly protein 1-like 4	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q6P158	DHX57	Putative ATP-dependent RNA helicase DHX57	RNA helicase (PC00032)
P23193	TCEA1	Transcription elongation factor A protein 1	general transcription factor (PC00259)
Q9H4G0	EPB41L1	Band 4.1-like protein 1	non-motor actin binding protein (PC00165)
Q6PKG0	LARP1	La-related protein 1	RNA metabolism protein (PC00031)
Q8WX93	PALLD	Palladin	scaffold/adaptor protein (PC00226)
Q13283	G3BP1	Ras GTPase-activating protein-binding protein 1	RNA metabolism protein (PC00031)
P49768	PSEN1	Presenilin-1	aspartic protease (PC00053)
Q8TAQ2	SMARCC2	SWI/SNF complex subunit SMARCC2	chromatin/chromatin-binding, or -regulatory protein (PC00077)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q03701	CEBPZ	CCAAT/enhancer-binding protein zeta	DNA-binding transcription factor (PC00218)
P49585	PCYT1A	Choline-phosphate cytidylyltransferase A	transferase (PC00220)
P05386	RPLP1	60S acidic ribosomal protein P1	ribosomal protein (PC00202)
Q12906	ILF3	Interleukin enhancer-binding factor 3	RNA metabolism protein (PC00031)
Q8ND56	LSM14A	Protein LSM14 homolog A	RNA metabolism protein (PC00031)
Q9UQ35	SRRM2	Serine/arginine repetitive matrix protein 2	
P35579	MYH9	Myosin-9	actin or actin-binding cytoskeletal protein (PC00041)
P84022	SMAD3	Mothers against decapentaplegic homolog 3	DNA-binding transcription factor (PC00218)
Q9NWD8	TMEM248	Transmembrane protein 248	
Q7L4I2	RSRC2	Arginine/serine-rich coiled-coil protein 2	
P05388	RPLP0	60S acidic ribosomal protein P0	ribosomal protein (PC00202)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
O75821	EIF3G	Eukaryotic translation initiation factor 3 subunit G	translation initiation factor (PC00224)
P61978	HNRNPK	Heterogeneous nuclear ribonucleoprotein K	RNA metabolism protein (PC00031)
P17812	CTPS1	CTP synthase 1	ligase (PC00142)
Q96JA1	LIG1	Leucine-rich repeats and immunoglobulin-like domains protein 1	transmembrane signal receptor (PC00197)
P98175	RBM10	RNA-binding protein 10	
O95365	ZBTB7A	Zinc finger and BTB domain-containing protein 7A	C2H2 zinc finger transcription factor (PC00248)
Q14103	HNRNPD	Heterogeneous nuclear ribonucleoprotein D0	RNA metabolism protein (PC00031)
P61247	RPS3A	40S ribosomal protein S3a	ribosomal protein (PC00202)
Q8N163	CCAR2	Cell cycle and apoptosis regulator protein 2	primary active transporter (PC00068)
Q9BU76	C1orf35	Multiple myeloma tumor-associated protein 2	
Q9H0D6	XRN2	5'-3' exoribonuclease 2	exoribonuclease (PC00099)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q13428	TCOF1	Treacle protein	RNA metabolism protein (PC00031)
O75643	SNRNP200	U5 small nuclear ribonucleoprotein 200 kDa helicase	scaffold/adaptor protein (PC00226)
Q8TEM1	NUP210	Nuclear pore membrane glycoprotein 210	transporter (PC00227)
Q15796	SMAD2	Mothers against decapentaplegic homolog 2	DNA-binding transcription factor (PC00218)
Q96I25	RBM17	Splicing factor 45	RNA splicing factor (PC00148)
Q13442	PDAP1	28 kDa heat- and acid-stable phosphoprotein	
Q9BZZ5	API5	Apoptosis inhibitor 5	
P17096	HMGA1	High mobility group protein HMG-I/HMG-Y	endodeoxyribonuclease (PC00093)
Q9P035	HACD3	Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase 3	dehydratase (PC00091)
Q9H0S4	DDX47	Probable ATP-dependent RNA helicase DDX47	RNA helicase (PC00032)
Q92911	SLC5A5	Sodium/iodide cotransporter	secondary carrier transporter (PC00258)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P21333	FLNA	Filamin-A	
Q5T8D3	ACBD5	Acyl-CoA-binding domain-containing protein 5	transfer/carrier protein (PC00219)
Q9UQ80	PA2G4	Proliferation-associated protein 2G4	protease (PC00190)
P49756	RBM25	RNA-binding protein 25	
O15439	ABCC4	ATP-binding cassette sub-family C member 4	ATP-binding cassette (ABC) transporter (PC00003)
Q8NE71	ABCF1	ATP-binding cassette sub-family F member 1	translation elongation factor (PC00222)
Q01082	SPTBN1	Spectrin beta chain, non-erythrocytic 1	actin or actin-binding cytoskeletal protein (PC00041)
P23396	RPS3	40S ribosomal protein S3	ribosomal protein (PC00202)
Q9Y3T9	NOC2L	Nucleolar complex protein 2 homolog	
Q9BQG0	MYBBP1A	Myb-binding protein 1A	chromatin/chromatin-binding, or -regulatory protein (PC00077)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
O95400	CD2BP2	CD2 antigen cytoplasmic tail-binding protein 2	RNA splicing factor (PC00148)
Q8TEU7	RAPGEF6	Rap guanine nucleotide exchange factor 6	guanyl-nucleotide exchange factor (PC00113)
P06748	NPM1	Nucleophosmin	chaperone (PC00072)
P14923	JUP	Junction plakoglobin	
P07910	HNRNPC	Heterogeneous nuclear ribonucleoproteins C1/C2	RNA metabolism protein (PC00031)
P13611	VCAN	Versican core protein	extracellular matrix glycoprotein (PC00100)
Q9BUJ2	HNRNPUL1	Heterogeneous nuclear ribonucleoprotein U-like protein 1	
P09651	HNRNPA1	Heterogeneous nuclear ribonucleoprotein A1	RNA processing factor (PC00147)
P19338	NCL	Nucleolin	RNA metabolism protein (PC00031)
Q15459	SF3A1	Splicing factor 3A subunit 1	RNA splicing factor (PC00148)
Q96T37	RBM15	RNA-binding protein 15	RNA metabolism protein (PC00031)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
O00193	C11orf58	Small acidic protein	
Q9Y388	RBMX2	RNA-binding motif protein, X-linked 2	
O95218	ZRANB2	Zinc finger Ran-binding domain-containing protein 2	RNA splicing factor (PC00148)
A2RRP1	NBAS	Neuroblastoma-amplified sequence	
Q13895	BYSL	Bystin	
P26358	DNMT1	DNA (cytosine-5)-methyltransferase 1	DNA methyltransferase (PC00013)
P27816	MAP4	Microtubule-associated protein 4	
O60716	CTNND1	Catenin delta-1	intermediate filament binding protein (PC00130)
P40222	TXLNA	Alpha-taxilin	membrane traffic protein (PC00150)
Q6KC79	NIPBL	Nipped-B-like protein	chromatin/chromatin-binding, or -regulatory protein (PC00077)
P26368	U2AF2	Splicing factor U2AF 65 kDa subunit	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q8IYB3	SRRM1	Serine/arginine repetitive matrix protein 1	RNA processing factor (PC00147)
P25205	MCM3	DNA replication licensing factor MCM3	DNA metabolism protein (PC00009)
O00567	NOP56	Nucleolar protein 56	RNA metabolism protein (PC00031)
Q9NU22	MDN1	Midasin	
Q9NZN4	EHD2	EH domain-containing protein 2	membrane traffic protein (PC00150)
Q9UJK0	TSR3	18S rRNA aminocarboxypropyltransferase	
Q13112	CHAF1B	Chromatin assembly factor 1 subunit B	chromatin/chromatin-binding, or -regulatory protein (PC00077)
P21127	CDK11B	Cyclin-dependent kinase 11B	non-receptor serine/threonine protein kinase (PC00167)
Q96L91	EP400	E1A-binding protein p400	
O00505	KPNA3	Importin subunit alpha-4	transporter (PC00227)
P29692	EEF1D	Elongation factor 1-delta	translation elongation factor (PC00222)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
O60832	DKC1	H/ACA ribonucleoprotein complex subunit DKC1	centromere DNA-binding protein (PC00071)
Q9NYF8	BCLAF1	Bcl-2-associated transcription factor 1	DNA metabolism protein (PC00009)
P18858	LIG1	DNA ligase 1	DNA ligase (PC00012)
Q14498	RBM39	RNA-binding protein 39	RNA splicing factor (PC00148)
Q9Y2W1	THRAP3	Thyroid hormone receptor-associated protein 3	DNA metabolism protein (PC00009)
P20042	EIF2S2	Eukaryotic translation initiation factor 2 subunit 2	translation initiation factor (PC00224)
P08651	NFIC	Nuclear factor 1 C-type	DNA-binding transcription factor (PC00218)
Q8IWA0	WDR75	WD repeat-containing protein 75	
Q9UHD8	SEPTIN9	Septin-9	cytoskeletal protein (PC00085)
O75822	EIF3J	Eukaryotic translation initiation factor 3 subunit J	translation initiation factor (PC00224)
Q15019	SEPTIN2	Septin-2	cytoskeletal protein (PC00085)
O75475	PSIP1	PC4 and SFRS1-interacting protein	transcription cofactor (PC00217)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P35659	DEK	Protein DEK	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q9P2K5	MYEF2	Myelin expression factor 2	
P51608	MECP2	Methyl-CpG-binding protein 2	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q9Y281	CFL2	Cofilin-2	non-motor actin binding protein (PC00165)
Q8NDC0	MAPK1IP1L	MAPK-interacting and spindle-stabilizing protein-like	
Q92466	DDB2	DNA damage-binding protein 2	damaged DNA-binding protein (PC00086)
Q1KMD3	HNRNPUL2	Heterogeneous nuclear ribonucleoprotein U-like protein 2	
O75367	MACROH2A1	Core histone macro-H2A.1	chromatin/chromatin-binding, or -regulatory protein (PC00077)
P35221	CTNNA1	Catenin alpha-1	non-motor actin binding protein (PC00165)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q8TDD1	DDX54	ATP-dependent RNA helicase DDX54	RNA helicase (PC00032)
Q53GS9	USP39	U4/U6.U5 tri-snRNP-associated protein 2	cysteine protease (PC00081)
P51858	HDGF	Hepatoma-derived growth factor	transcription cofactor (PC00217)
P27824	CANX	Calnexin	chaperone (PC00072)
P46821	MAP1B	Microtubule-associated protein 1B	non-motor microtubule binding protein (PC00166)
Q66PJ3	ARL6IP4	ADP-ribosylation factor-like protein 6-interacting protein 4	
E9PAV3	NACA	Nascent polypeptide-associated complex subunit alpha, muscle-specific form	basic helix-loop-helix transcription factor (PC00055)
Q08945	SSRP1	FACT complex subunit SSRP1	
P35611	ADD1	Alpha-adducin	non-motor actin binding protein (PC00165)

Table 37: List of differentially expressed – downregulated phosphoproteins in A549 cells infected with Influenza A – H3N2 at 8 hr

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q9H6F5	CCDC86	Coiled-coil domain-containing protein 86	
Q9H1E3	NUCKS1	Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1	
O76075	CAD	DNA fragmentation factor subunit beta	DNA metabolism protein (PC00009)
P16615	ATP2A2	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	primary active transporter (PC00068)
Q99733	NAP1L4	Nucleosome assembly protein 1-like 4	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q6ZRP7	QSOX2	Sulfhydryl oxidase 2	oxidase (PC00175)
Q02952	AKAP12	A-kinase anchor protein 12	scaffold/adaptor protein (PC00226)
Q5JTV8	TOR1AIP1	Torsin-1A-interacting protein 1	
Q04721	NOTCH2	Neurogenic locus notch homolog protein 2	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q8WX93	PALLD	Palladin	scaffold/adaptor protein (PC00226)
Q92844	TRAF2	TRAF family member-associated NF-kappa-B activator	
Q8IVP5	FUNDC1	FUN14 domain-containing protein 1	
P05386	RPLP1	60S acidic ribosomal protein P1	ribosomal protein (PC00202)
Q8NC44	RETREG2	Reticulophagy regulator 2	
Q9UQ35	SRRM2	Serine/arginine repetitive matrix protein 2	
P62269	RPS18	40S ribosomal protein S18	ribosomal protein (PC00202)
Q15185	PTGES3	Prostaglandin E synthase 3	chaperone (PC00072)
O75369	FLNB	Filamin-B	
O00559	EBAG9	Receptor-binding cancer antigen expressed on SiSo cells	
Q12766	HMGXB3	HMG domain-containing protein 3	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q96JA1	LIG1	Leucine-rich repeats and immunoglobulin-like domains protein 1	transmembrane signal receptor (PC00197)
P17676	CEBPB	CCAAT/enhancer-binding protein beta	basic leucine zipper transcription factor (PC00056)
Q8N163	CCAR2	Cell cycle and apoptosis regulator protein 2	primary active transporter (PC00068)
O75569	PRKRA	Interferon-inducible double-stranded RNA-dependent protein kinase activator A	
Q13501	SQSTM1	Sequestosome-1	
P46100	ATRX	Transcriptional regulator ATRX	DNA-binding transcription factor (PC00218)
O15061	SYNM	Synemin	
A6NK06	CAD	Cis-aconitate decarboxylase	dehydratase (PC00091)
P38432	COIL	Coilin	DNA metabolism protein (PC00009)
Q9Y5J1	UTP18	U3 small nucleolar RNA-associated protein 18 homolog	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q8N9T8	KRI1	Protein KRI1 homolog	
Q05209	PTPN12	Tyrosine-protein phosphatase non-receptor type 12	
Q2TB10	ZNF800	Zinc finger protein 800	
P00352	ALDH1A1	Retinal dehydrogenase 1	dehydrogenase (PC00092)
P47755	CAPZA2	F-actin-capping protein subunit alpha-2	non-motor actin binding protein (PC00165)
P17096	HMGA1	High mobility group protein HMG-I/HMG-Y	endodeoxyribonuclease (PC00093)
Q12933	TRAF2	TNF receptor-associated factor 2	scaffold/adaptor protein (PC00226)
Q09666	AHNAK	Neuroblast differentiation-associated protein AHNAK	
Q05682	CAD	Caldesmon	non-motor actin binding protein (PC00165)
Q15121	PEA15	Astrocytic phosphoprotein PEA-15	
P21333	FLNA	Filamin-A	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P42166	TMPO	Lamina-associated polypeptide 2, isoform alpha	peptide hormone (PC00179)
P29966	MARCKS	Myristoylated alanine-rich C-kinase substrate	
P29317	EPHA2	Ephrin type-A receptor 2	transmembrane signal receptor (PC00197)
Q96JM3	CHAMP1	Chromosome alignment-maintaining phosphoprotein 1	
P16989	YBX3	Y-box-binding protein 3	
P13861	PRKAR2A	cAMP-dependent protein kinase type II-alpha regulatory subunit	kinase modulator (PC00140)
Q9NWH9	SLTM	SAFB-like transcription modulator	
Q07157	TJP1	Tight junction protein ZO-1	tight junction (PC00214)
P10451	SPP1	Osteopontin	cytokine (PC00083)
Q8IVT2	MISP	Mitotic interactor and substrate of PLK1	
P04792	HSPB1	Heat shock protein beta-1	chaperone (PC00072)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P27708	CAD	CAD protein	ligase (PC00142)
Q7Z417	NUFIP2	Nuclear fragile X mental retardation-interacting protein 2	
P06454	PTMA	Prothymosin alpha	
Q659A1	ICE2	Little elongation complex subunit 2	
Q9NQC3	RTN4	Reticulon-4	
O43760	SYNGR2	Synaptogyrin-2	membrane trafficking regulatory protein (PC00151)
Q8IY81	FTSJ3	pre-rRNA 2'-O-ribose RNA methyltransferase FTSJ3	RNA methyltransferase (PC00033)
P63165	SUMO1	Small ubiquitin-related modifier 1	
Q96I59	SYNM	Probable asparagine--tRNA ligase, mitochondrial	aminoacyl-tRNA synthetase (PC00047)
Q16643	DBN1	Drebrin	
Q96TA1	NIBAN2	Protein Niban 2	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P51636	CAV2	Caveolin-2	scaffold/adaptor protein (PC00226)
P16403	H1-2	Histone H1.2	chromatin/chromatin-binding, or -regulatory protein (PC00077)
P78527	PRKDC	DNA-dependent protein kinase catalytic subunit	non-receptor serine/threonine protein kinase (PC00167)
Q9NS69	TOMM22	Mitochondrial import receptor subunit TOM22 homolog	primary active transporter (PC00068)
O00567	NOP56	Nucleolar protein 56	RNA metabolism protein (PC00031)
O60832	DKC1	H/ACA ribonucleoprotein complex subunit DKC1	centromere DNA-binding protein (PC00071)
P18858	LIG1	DNA ligase 1	DNA ligase (PC00012)
Q9Y520	PRRC2C	Protein PRRC2C	RNA metabolism protein (PC00031)
Q9H7N4	SCAF1	Splicing factor, arginine/serine-rich 19	RNA splicing factor (PC00148)
P16949	STMN1	Stathmin	non-motor microtubule binding protein (PC00166)

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
Q9Y2W1	THRAP3	Thyroid hormone receptor-associated protein 3	DNA metabolism protein (PC00009)
P02545	LMNA	Prelamin-A/C	
Q9Y4B5	MTCL1	Microtubule cross-linking factor 1	
Q9H3N1	TMX1	Thioredoxin-related transmembrane protein 1	
P00533	EGFR	Epidermal growth factor receptor	transmembrane signal receptor (PC00197)
Q96T23	RSF1	Remodeling and spacing factor 1	
Q9Y6G9	DYNC1LI1	Cytoplasmic dynein 1 light intermediate chain 1	microtubule or microtubule-binding cytoskeletal protein (PC00157)
P08238	HSP90AB1	Heat shock protein HSP 90-beta	Hsp90 family chaperone (PC00028)
O76021	RSL1D1	Ribosomal L1 domain-containing protein 1	ribosomal protein (PC00202)
O43169	CYB5B	Cytochrome b5 type B	oxidoreductase (PC00176)
Q9C0C2	TNKS1BP1	182 kDa tankyrase-1-binding protein	

UniProt KB ID	Gene ID	Gene Name	PANTHER Protein class
P27824	CANX	Calnexin	chaperone (PC00072)
Q9UBH6	XPR1	Xenotropic and polytropic retrovirus receptor 1	secondary carrier transporter (PC00258)
P46821	MAP1B	Microtubule-associated protein 1B	non-motor microtubule binding protein (PC00166)
Q14247	CTTN	Src substrate cortactin	scaffold/adaptor protein (PC00226)
Q9NTI5	PDS5B	Sister chromatid cohesion protein PDS5 homolog B	chromatin/chromatin-binding, or -regulatory protein (PC00077)
P10412	H1-4	Histone H1.4	chromatin/chromatin-binding, or -regulatory protein (PC00077)
Q14684	RRP1B	Ribosomal RNA processing protein 1 homolog B	
Q9BX95	SPP1	Sphingosine-1-phosphate phosphatase 1	phosphatase (PC00181)

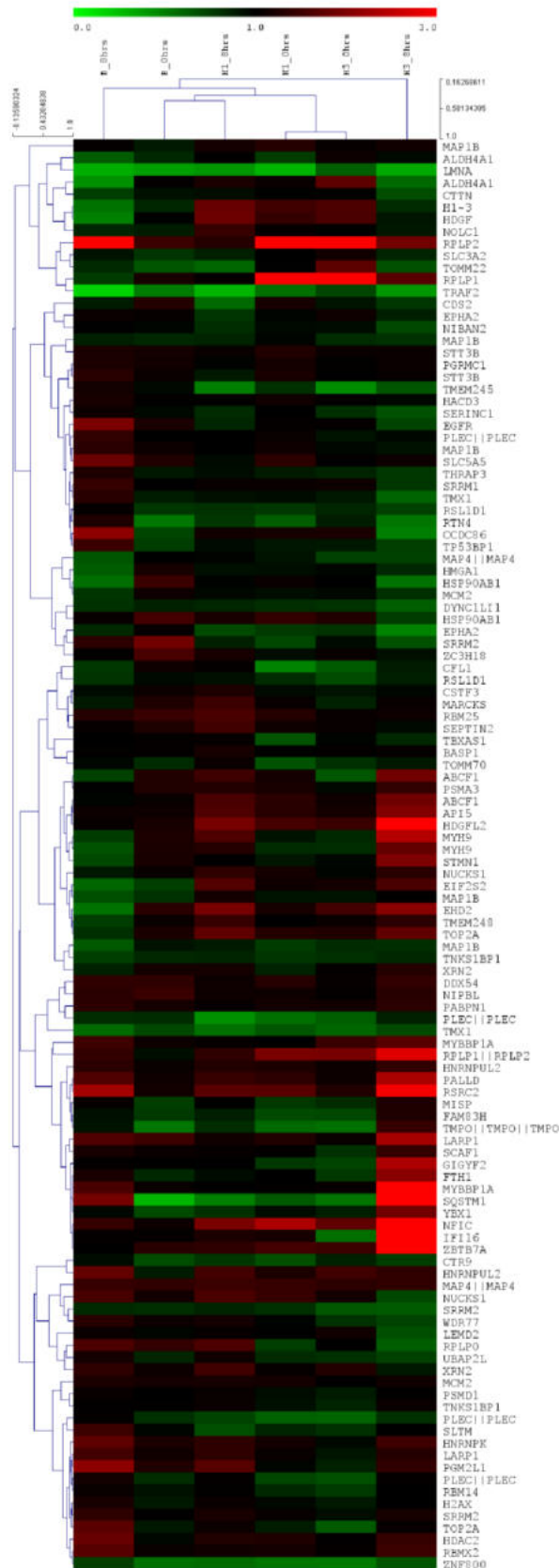


Figure 36: Gradient heat map depicting fold change in expression of phosphoproteins at multiple time-points for different strains of influenza virus.

5.13. Results for Gene Ontology (GO) Enrichment Analysis of the Differentially Expressed Proteins

GO enrichment analysis is widely used to identify the biological roles and functions of specific gene and its products. All the differentially expressed phosphoproteins were mapped to their enriched GO terms based on the functional annotation from the DAVID database. The differentially expressed proteins were categorized into three classes, including biological process, molecular function, and cellular component, as shown in the figures below. The Bar charts represent classification of significantly differentially expressed genes (DEGs) in gene ontology (GO) database. Categories are indicated using the number of genes present in each category and the number of genes identified in the GO library ($p < 0.05$).

The vertical axis represents 10 top ranks GOs with the smallest p-values and the GO terms and the horizontal axis represents the enrichment score.

Cnet plots illustrate enriched KEGG database pathways. The circular axis represents the enriched pathway categories and the enriched gene involved in them. The size and colour of dots represent the gene number and the range of p-values, respectively. Rich factor is the ratio of differentially expressed gene number enriched in the pathway to the total gene number in a certain pathway.

Figure 37: GO ontology result for differentially expressed genes in A549 cells infected with Influenza B at 0 hr. (A) the hub of genes expressed in terms of cellular component (CC) (B) the hub of genes expressed in terms of biological process (BP)

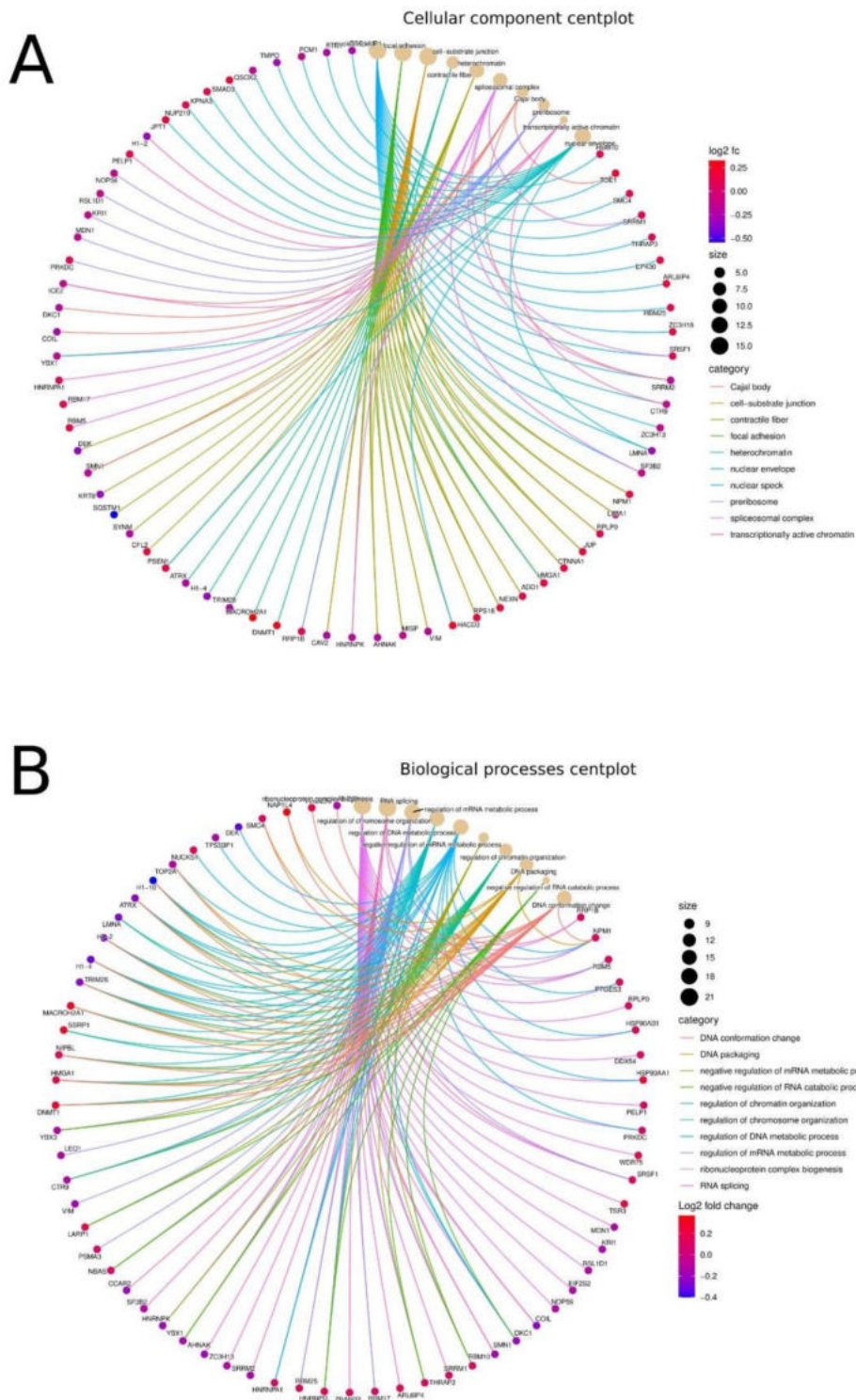


Figure 38: GO ontology result for differentially expressed genes in A549 cells infected with Influenza B at 0 hr. (A) the hub of genes expressed in terms of molecular function (MF), and (B) summarization of all the genes involved in BP, CC and MF.

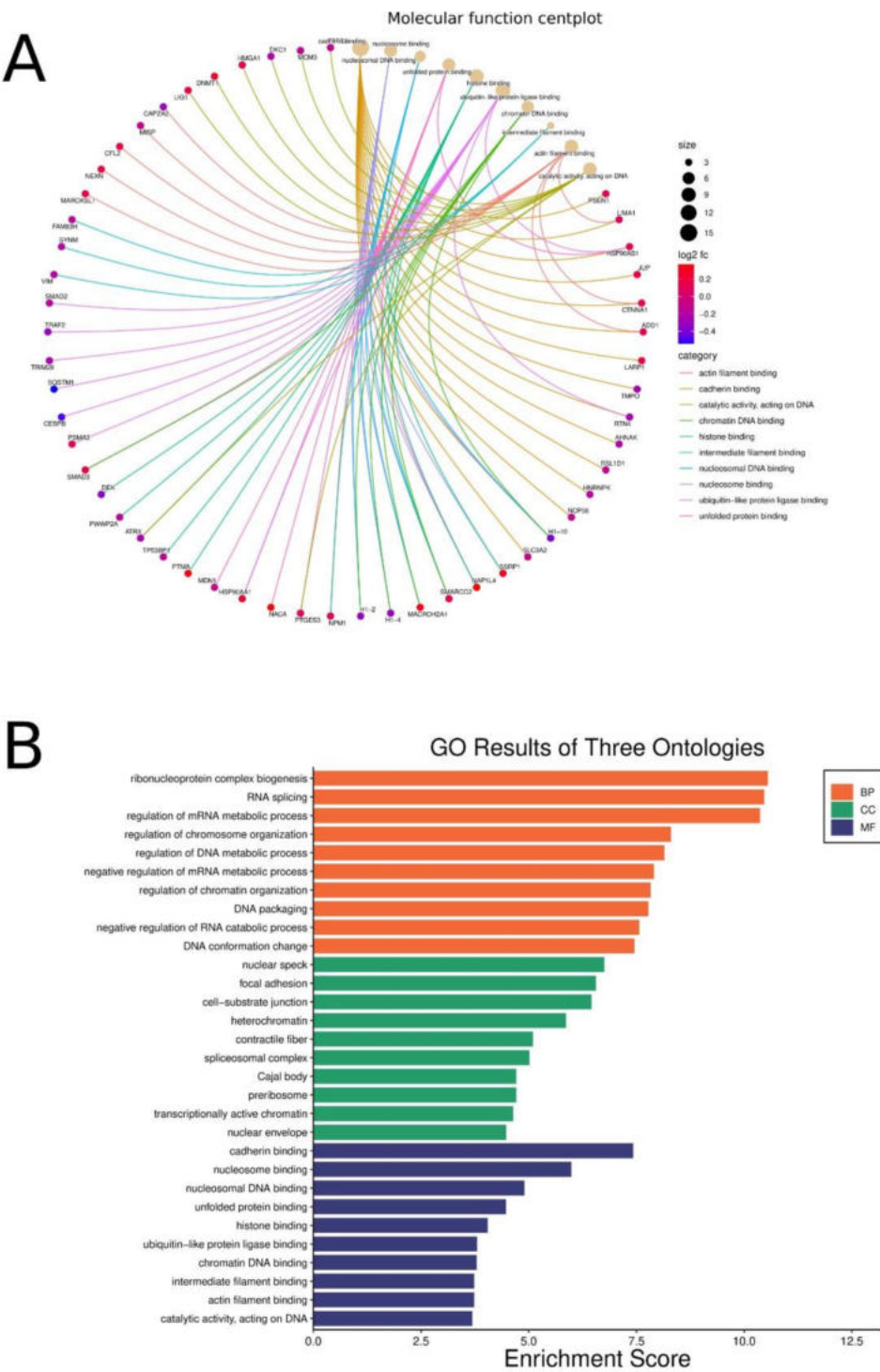


Figure 39:GO ontology result for differentially expressed genes in A549 cells infected with Influenza B at 8 hrs. (A) the hub of genes expressed in terms of cellular component (CC), (B) the hub of genes expressed in terms of biological process (BP)

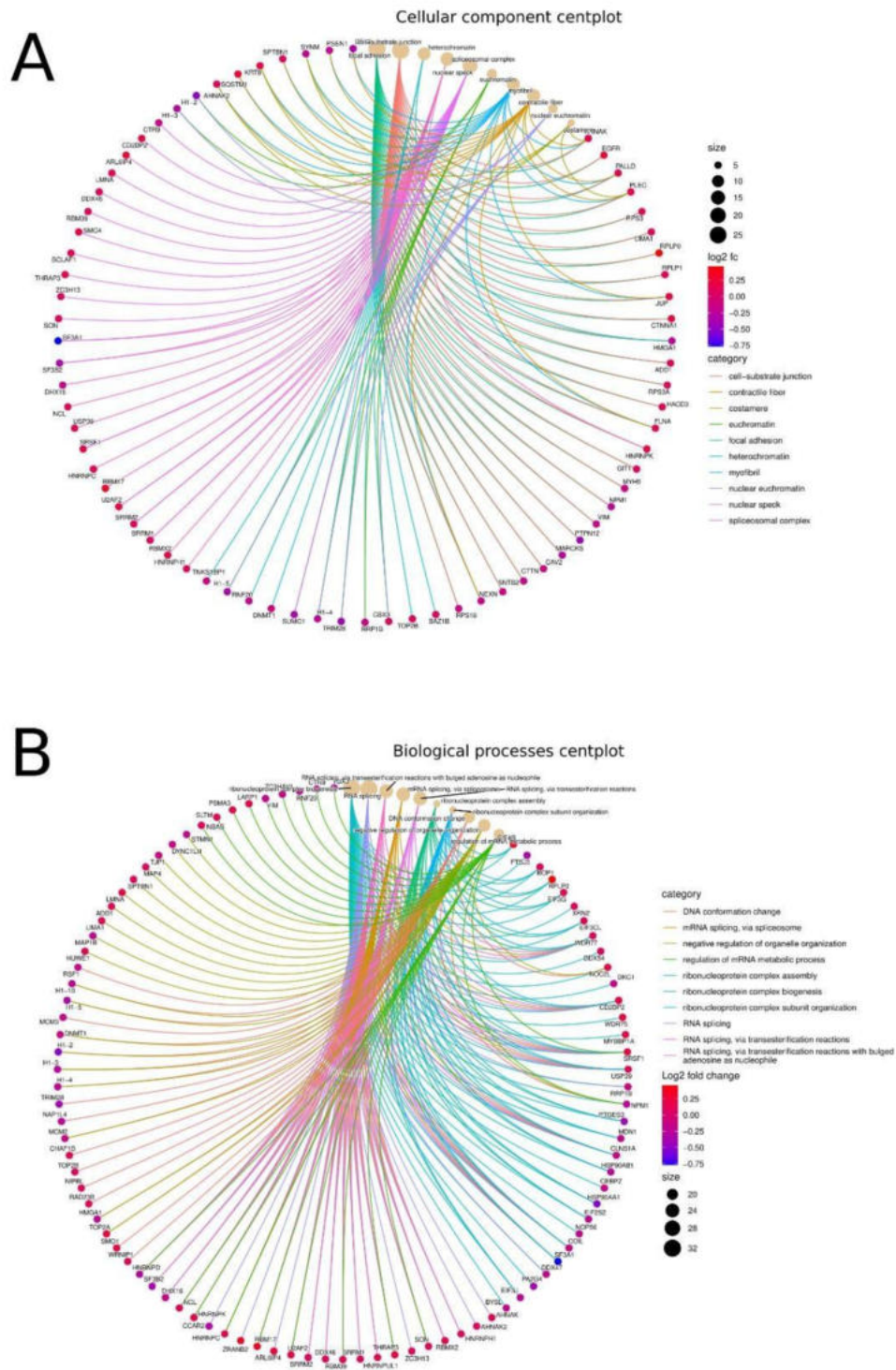


Figure 40: GO ontology result for differentially expressed genes in A549 cells infected with Influenza B at 8 hrs. (A) the hub of genes expressed in terms of molecular function (MF), and (B) summarization of all the genes involved in BP, CC and MF.

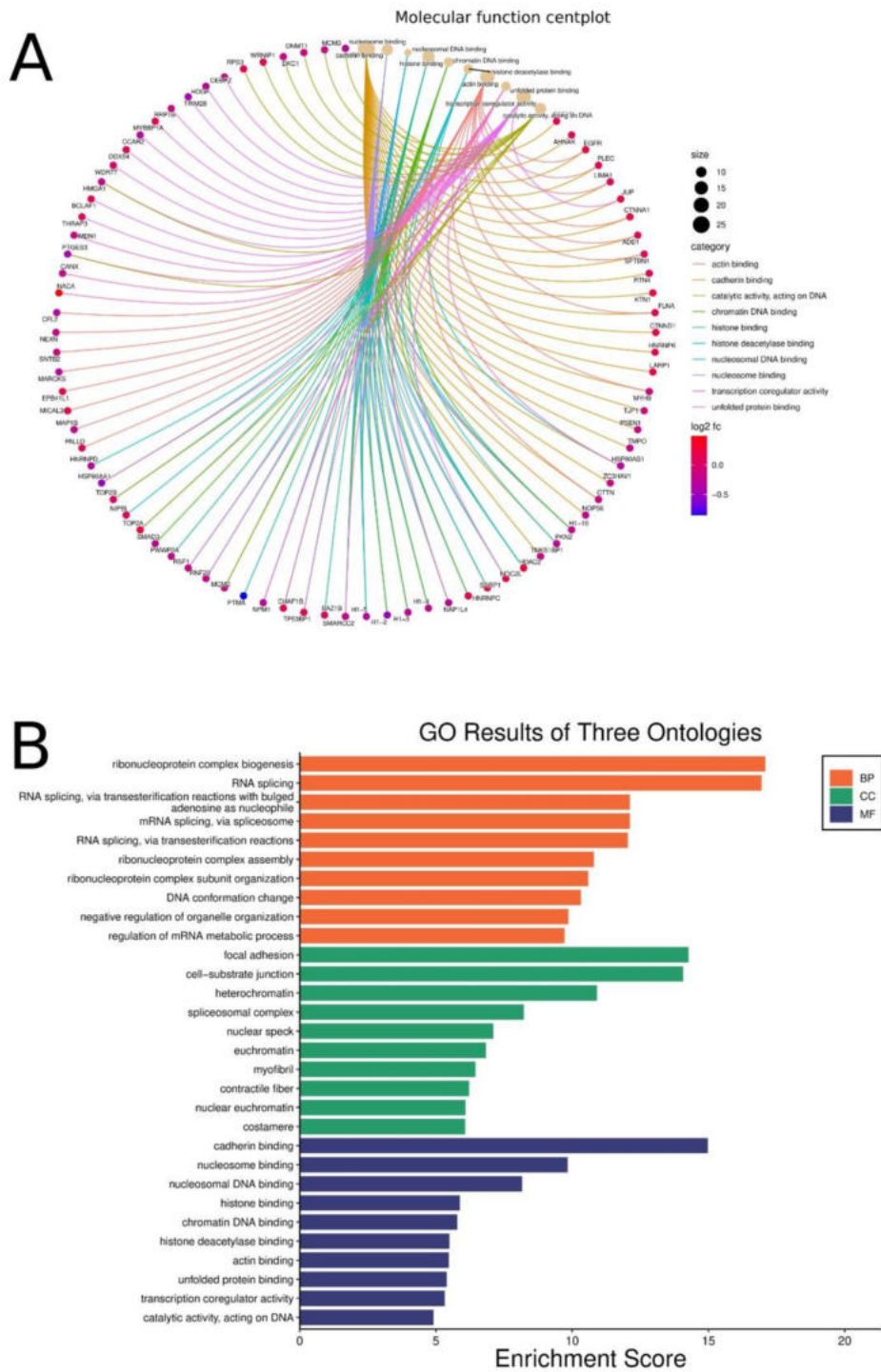


Figure 41: GO ontology result for differentially expressed genes in A549 cells infected with Influenza A – pH1N1 at 0 hr. (A) the hub of genes expressed in terms of cellular component (CC), (B) the hub of genes expressed in terms of biological process (BP)

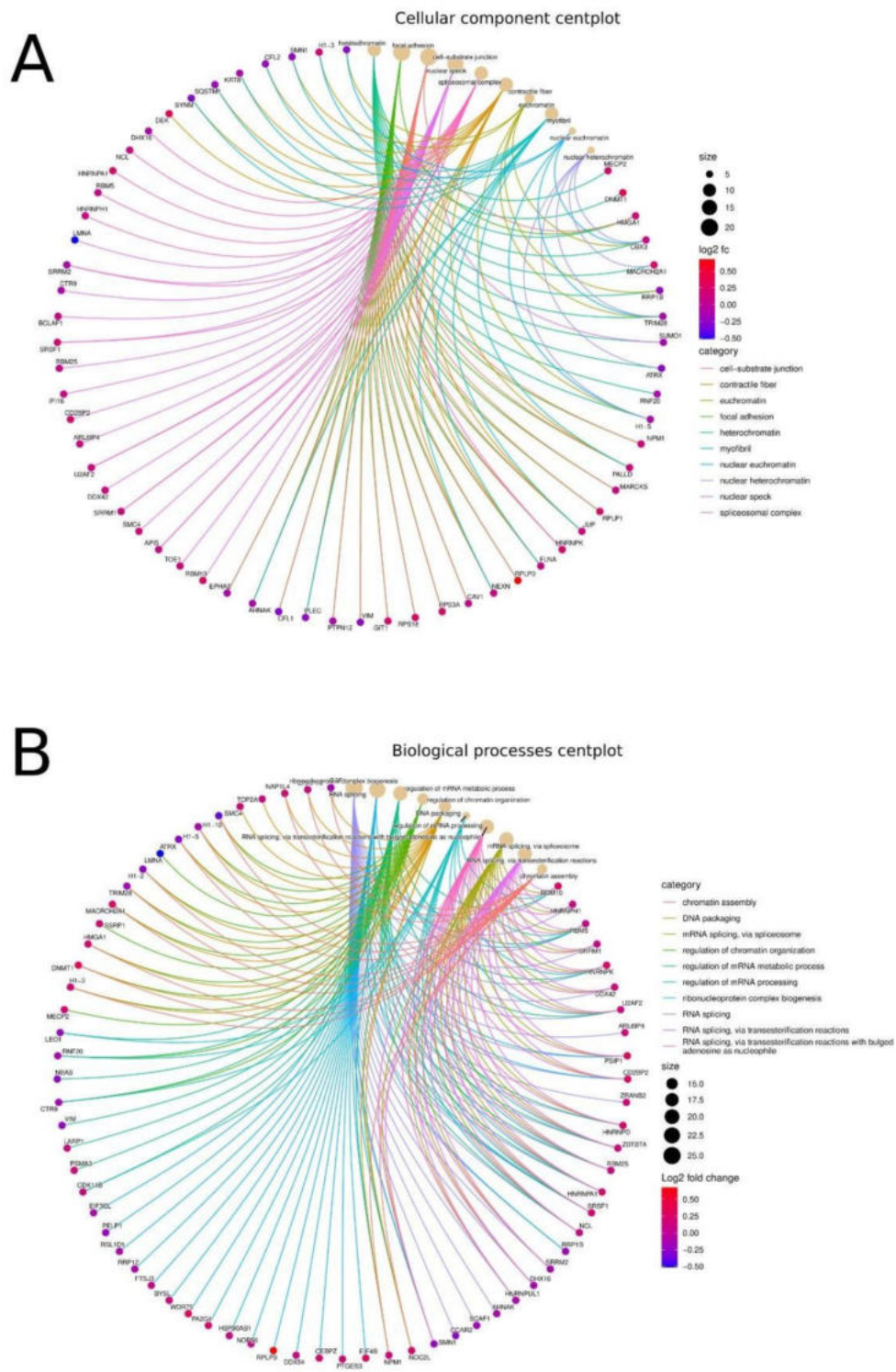


Figure 42: GO ontology result for differentially expressed genes in A549 cells infected with Influenza A – pH1N1 at 0 hr. (A) the hub of genes expressed in terms of molecular function (MF), and (B) summarization of all the genes involved in BP, CC and MF.

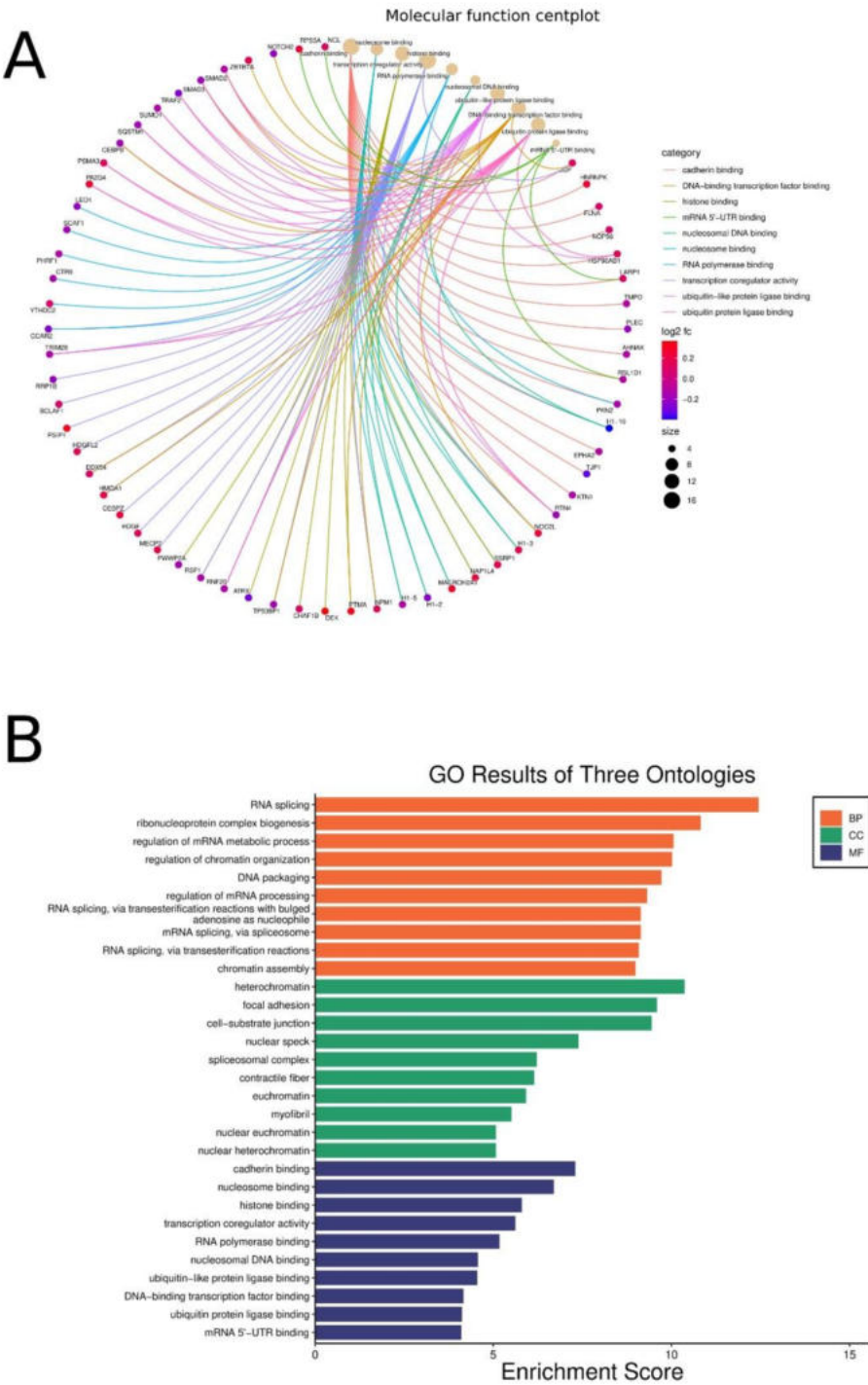


Figure 43: GO ontology result for differentially expressed genes in A549 cells infected with Influenza A – pH1N1 at 8 hrs. (A) the hub of genes expressed in terms of cellular component (CC), (B) The hub of genes expressed in terms of biological process (BP)

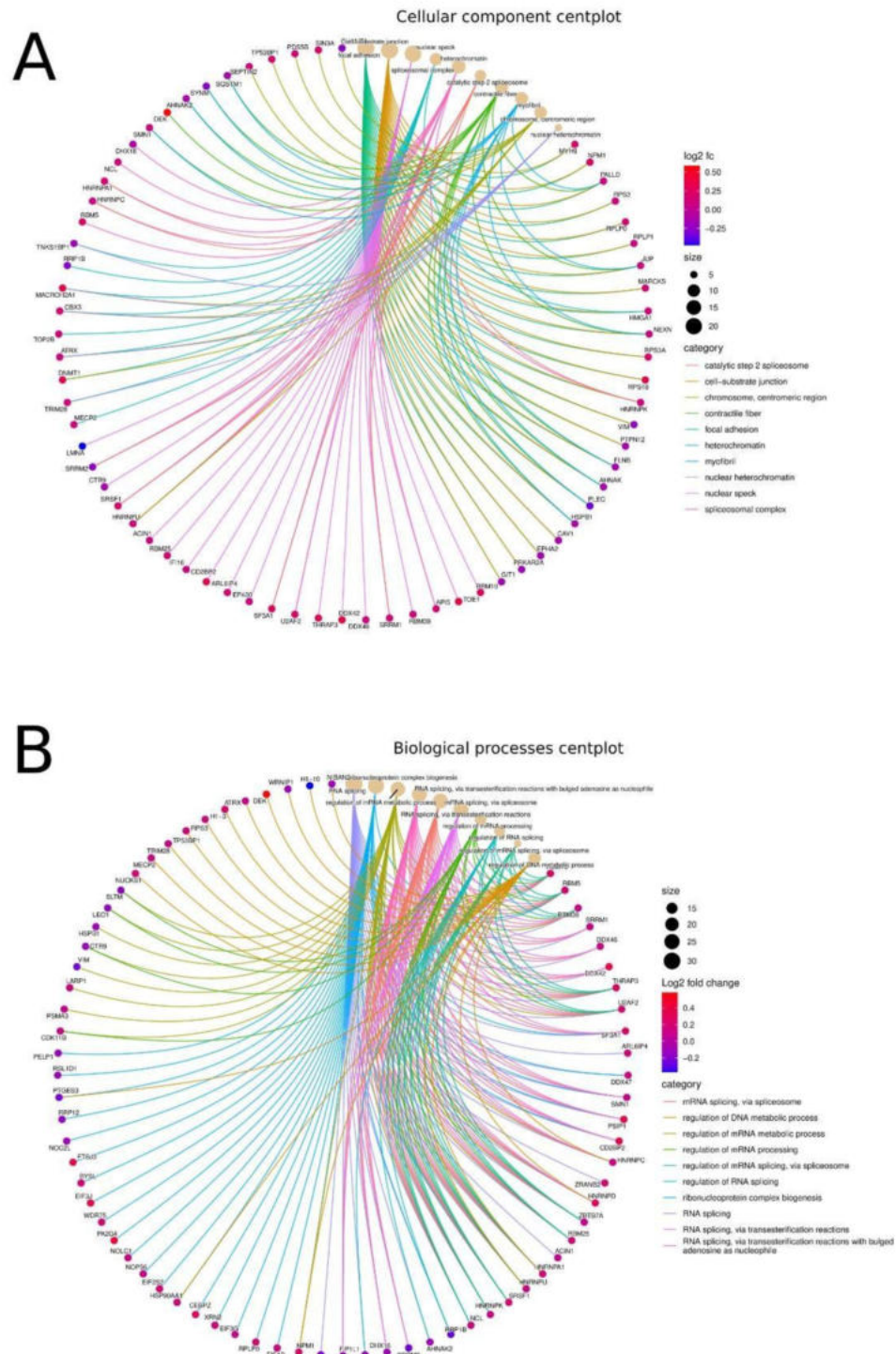


Figure 44: GO ontology result for differentially expressed genes in A549 cells infected with Influenza A – pH1N1 at 8 hrs. (A) the hub of genes expressed in terms of molecular function (MF), and (B) summarization of all the genes involved in BP, CC and MF.

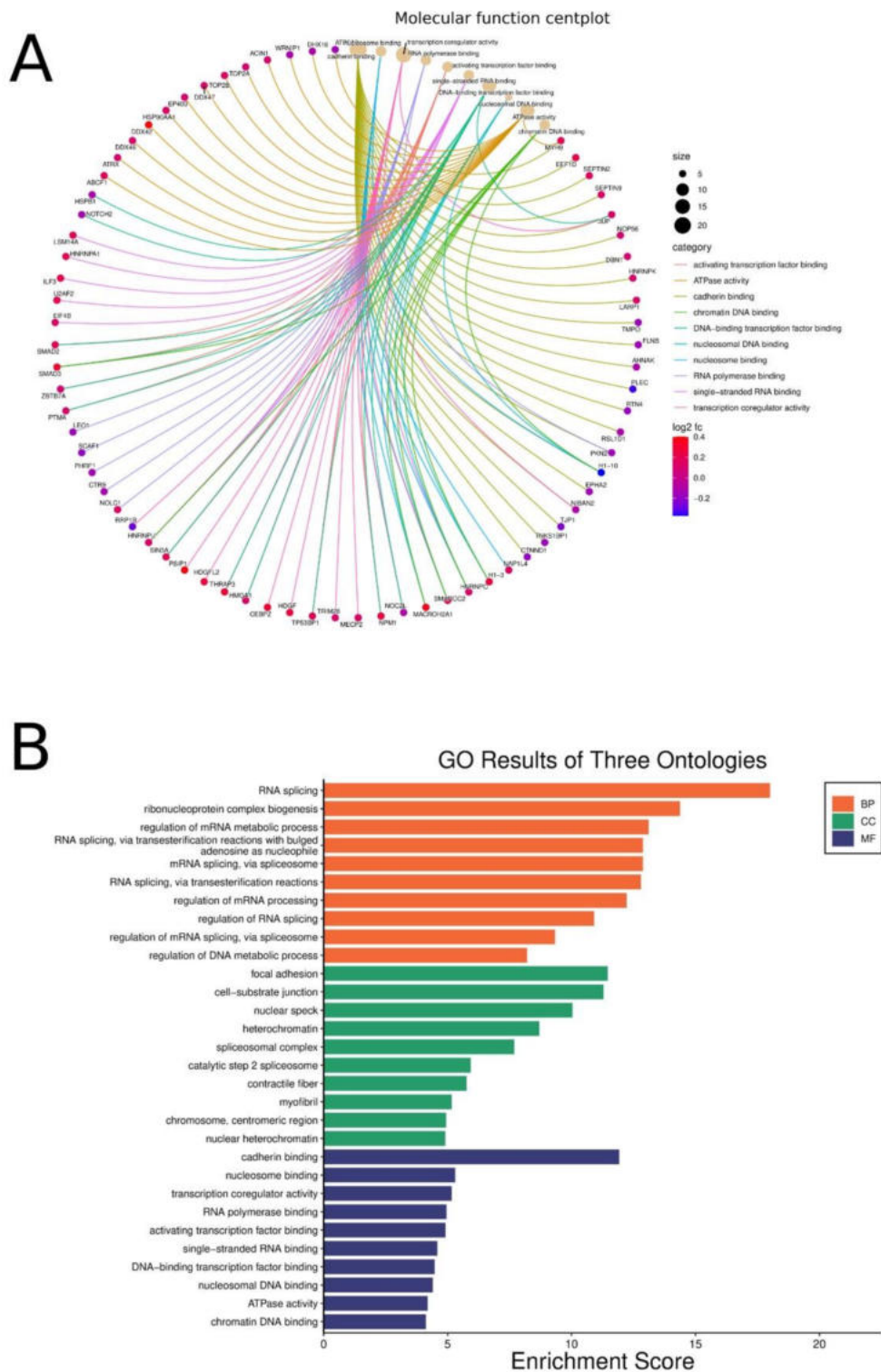


Figure 45: GO ontology result for differentially expressed genes in A549 cells infected with Influenza A – H3N2 at 0 hr. (A) the hub of genes expressed in terms of cellular component (CC), (B) the hub of genes expressed in terms of biological process (BP)

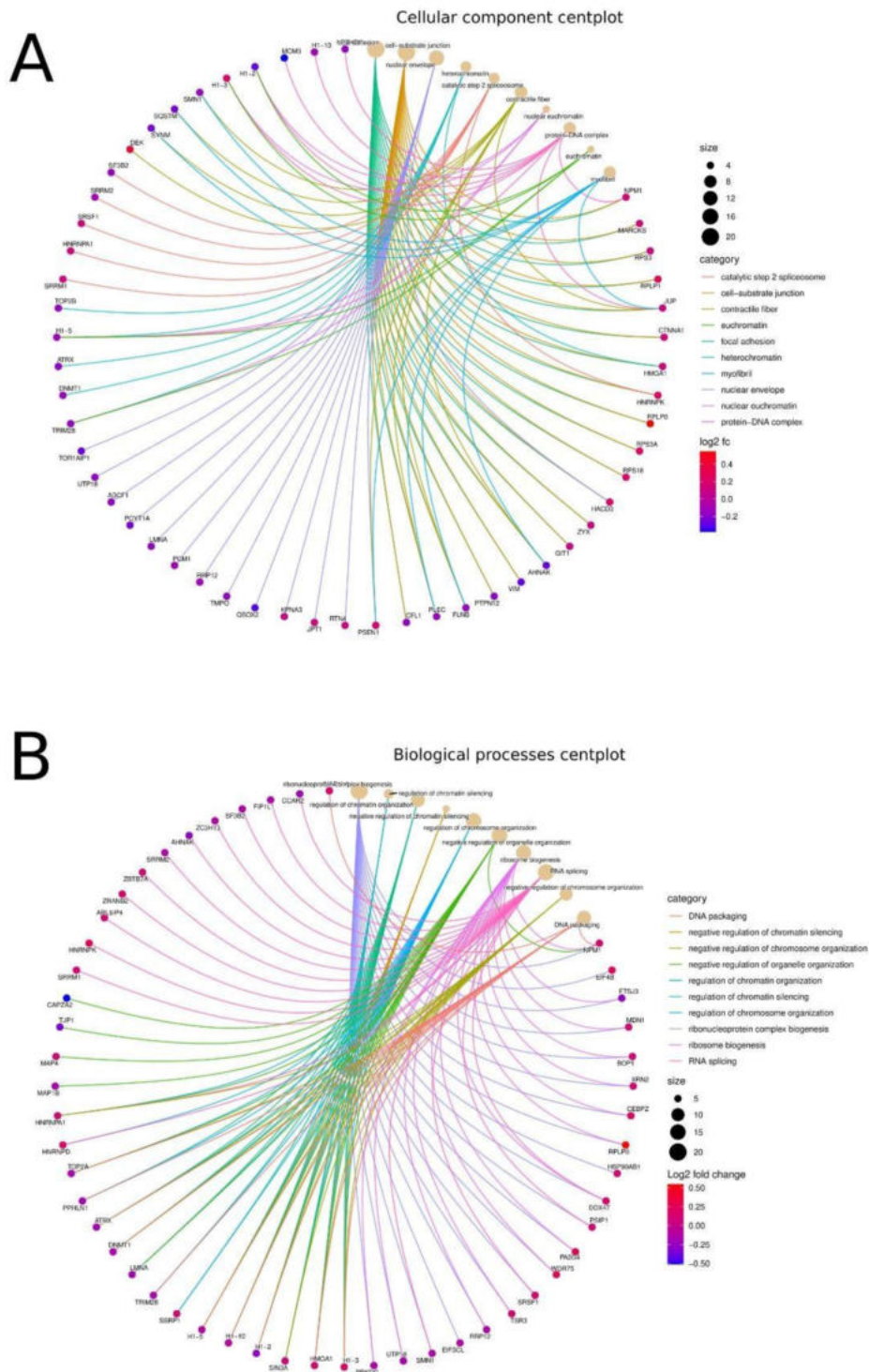


Figure 46: GO ontology result for differentially expressed genes in A549 cells infected with Influenza A – H3N2 at 0 hr. (A) the hub of genes expressed in terms of molecular function (MF), and (B) summarization of all the genes involved in BP, CC and MF.

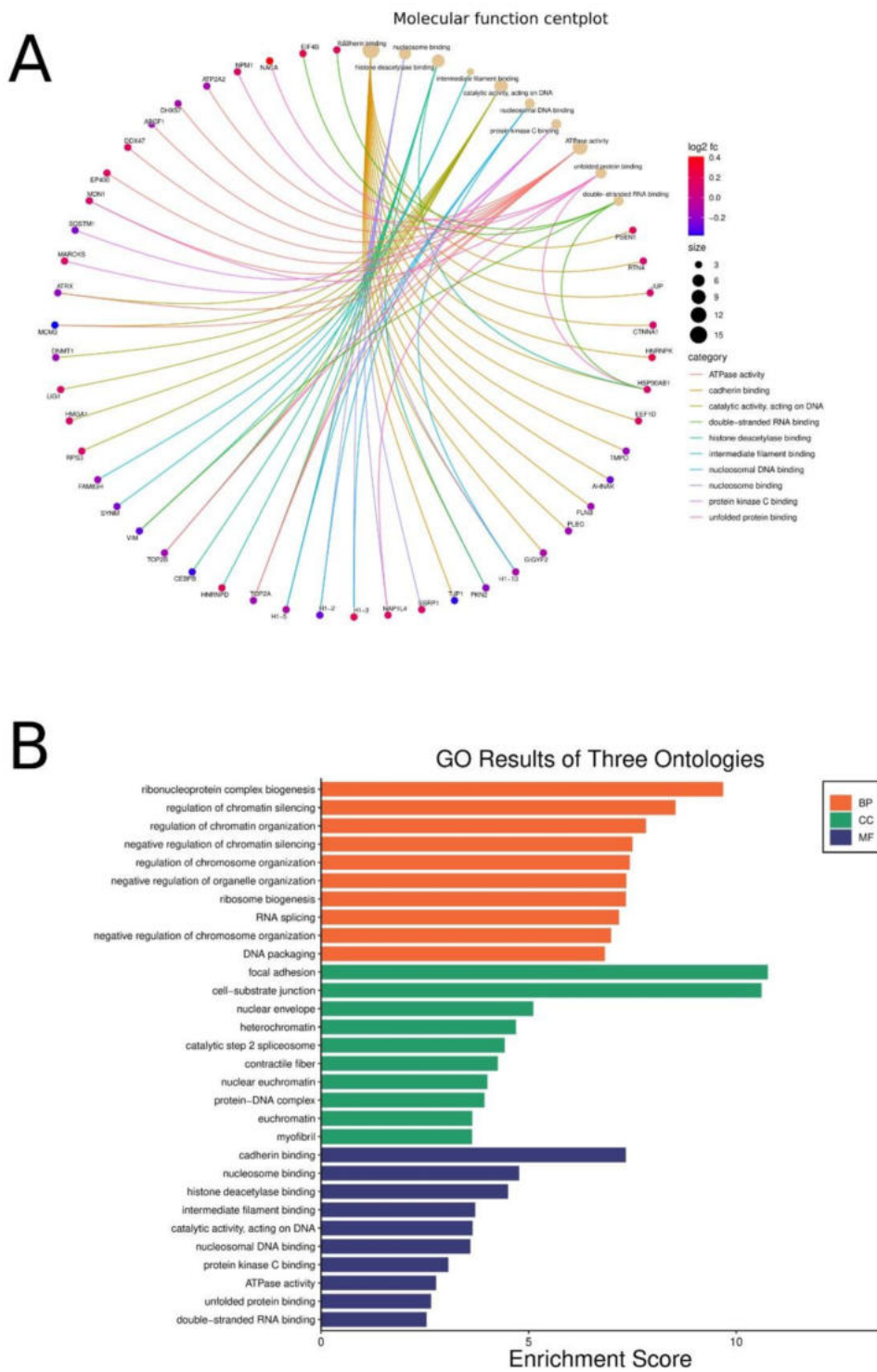


Figure 47: GO ontology result for differentially expressed genes in A549 cells infected with Influenza A – H3N2 at 8 hrs. (A) the hub of genes expressed in terms of cellular component (CC), (B) the hub of genes expressed in terms of biological process (BP)

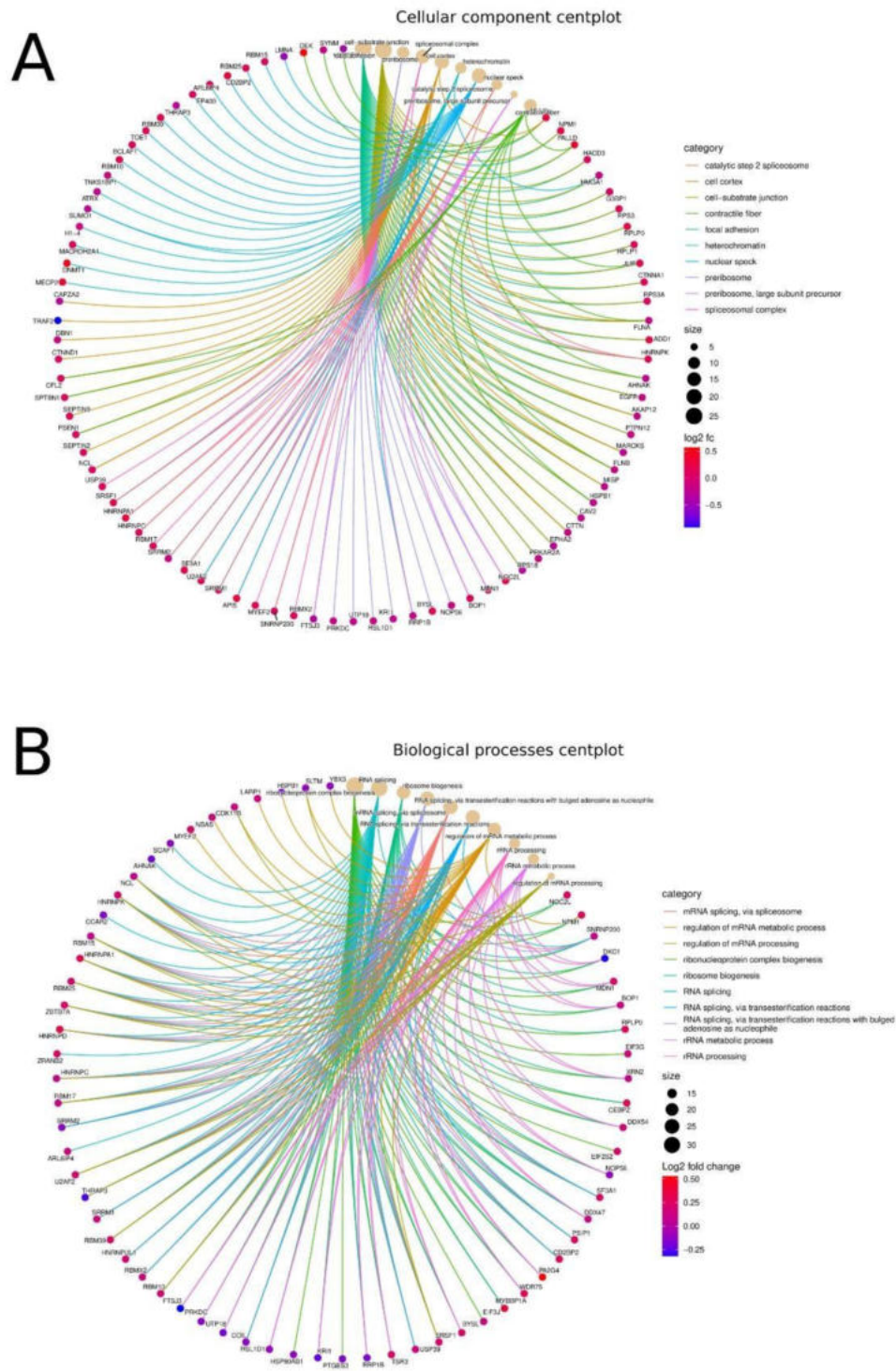


Figure 48: GO ontology result for differentially expressed genes in A549 cells infected with Influenza A – H3N2 at 8 hrs. (A) the hub of genes expressed in terms of molecular function (MF), and (B) summarization of all the genes involved in BP, CC and MF.

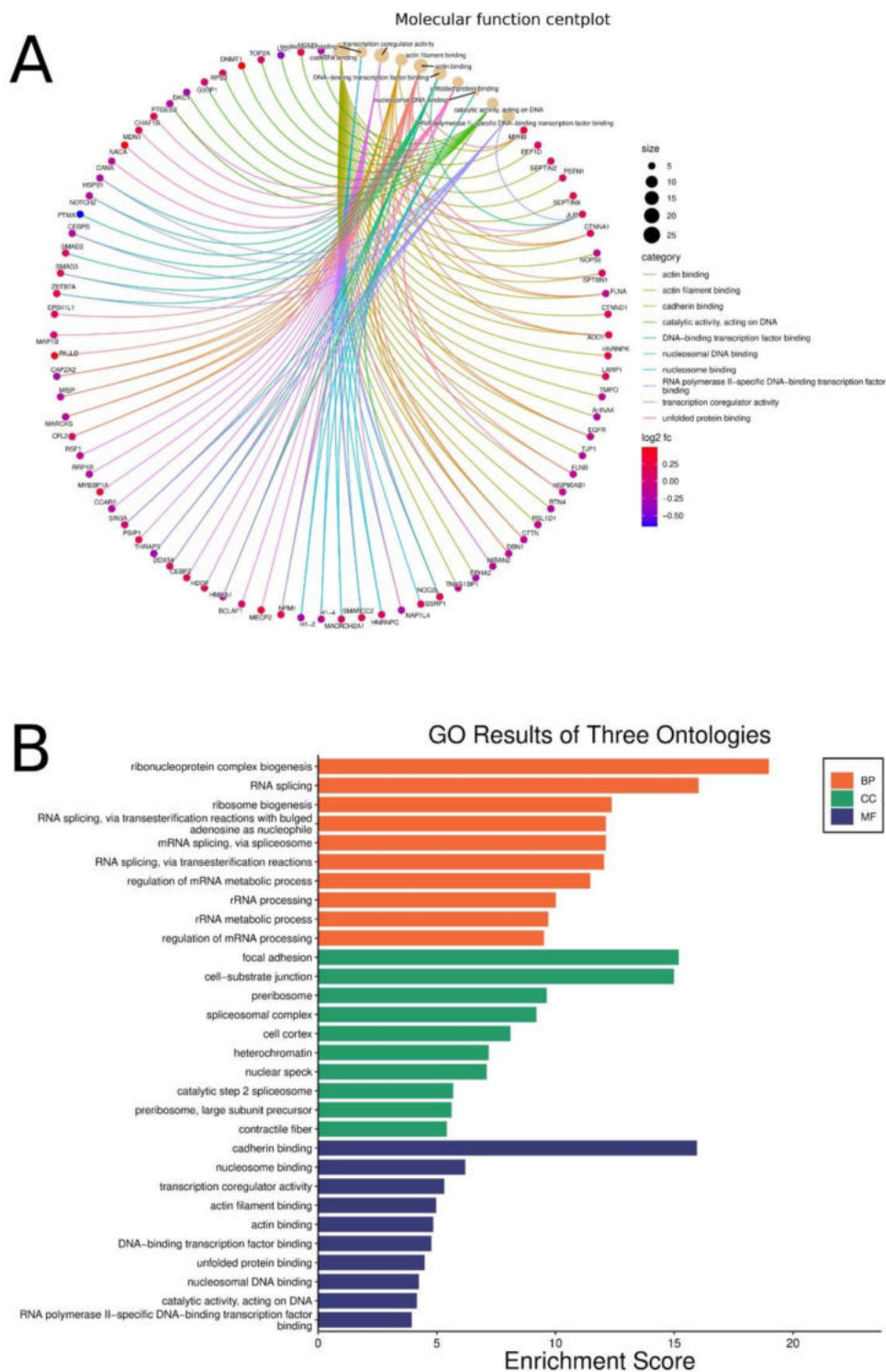


Figure 49: KEGG pathways for A549 cells infected with Influenza B at 0 hr. A – Cell cycle B – Endocytosis C – Fluid Shear stress and Atherosclerosis D – IL17 signaling pathway

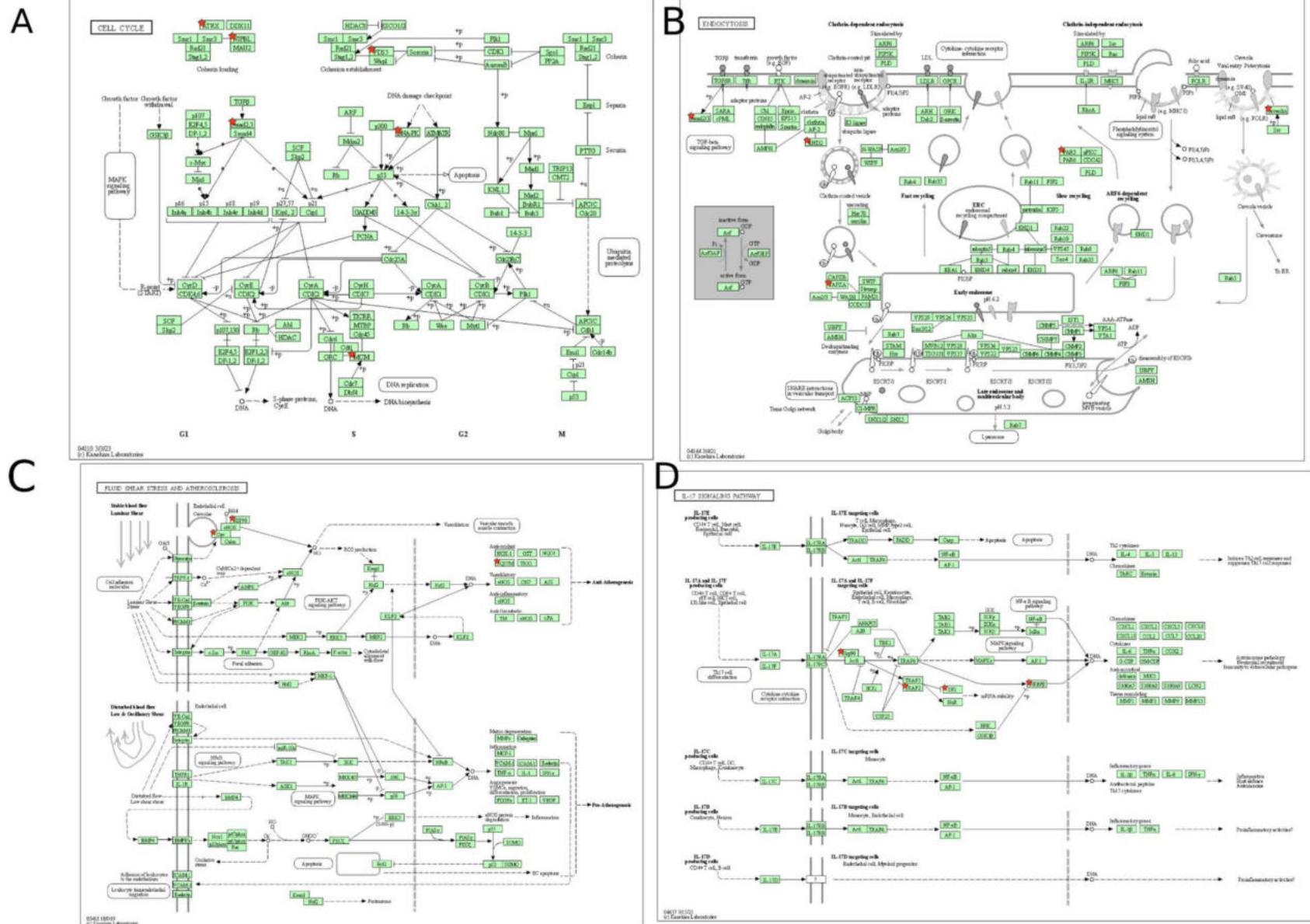


Figure 50: KEGG pathways for A549 cells infected with Influenza B at 0 hr. A – Necroptosis B – Protein processing in Endoplasmic Reticulum C – Th17 cell differentiation D – Spliceosome

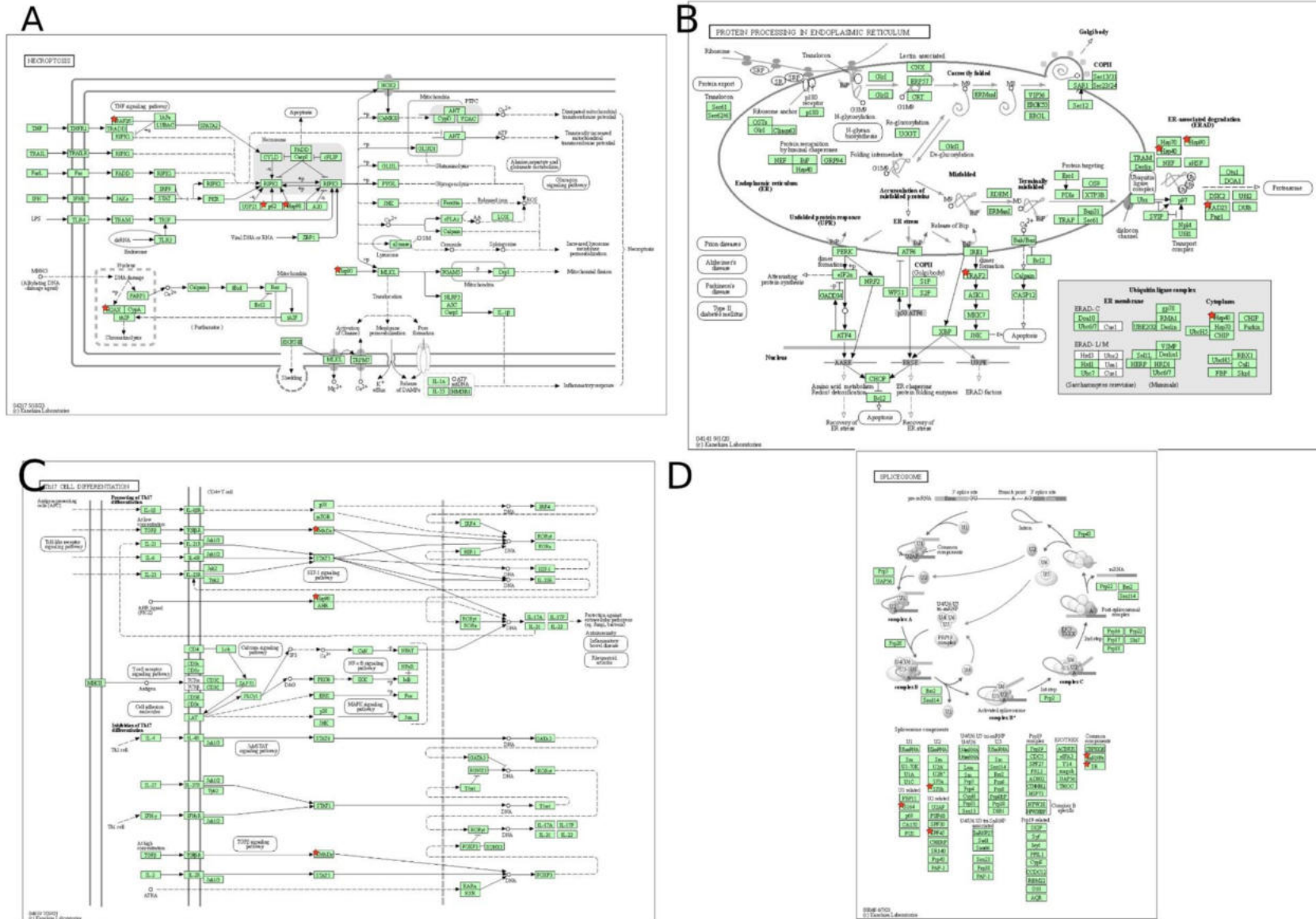


Figure 51: KEGG pathways for A549 cells infected with Influenza B at 8 hrs. A – Adherens junction B – Coronavirus disease – COVID 19 C – Cell cycle

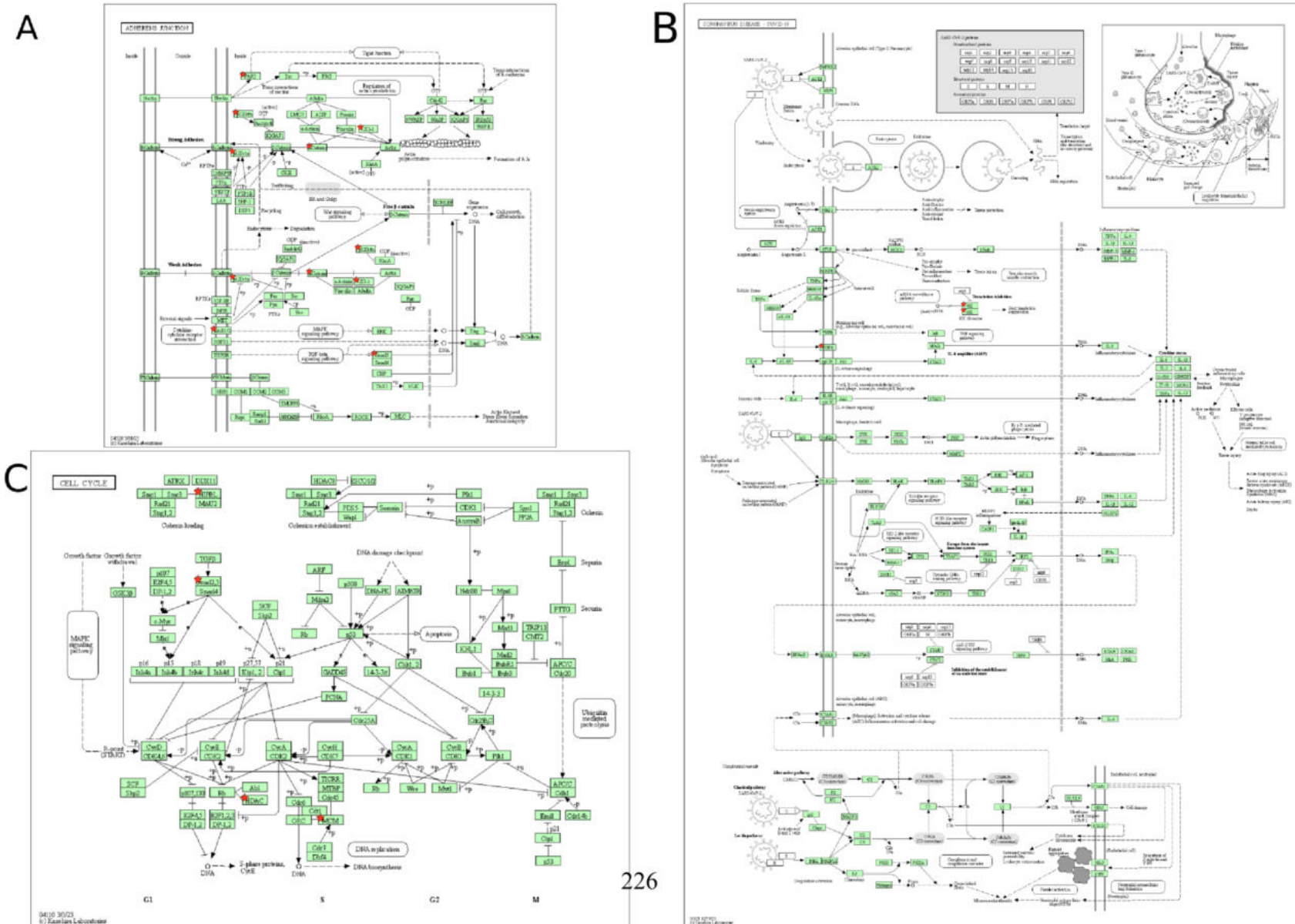


Figure 52: KEGG pathways for A549 cells infected with Influenza B at 8 hrs. A – Pathogenic *Escherichia coli* infection B – *Salmonella* infection C – DNA replication

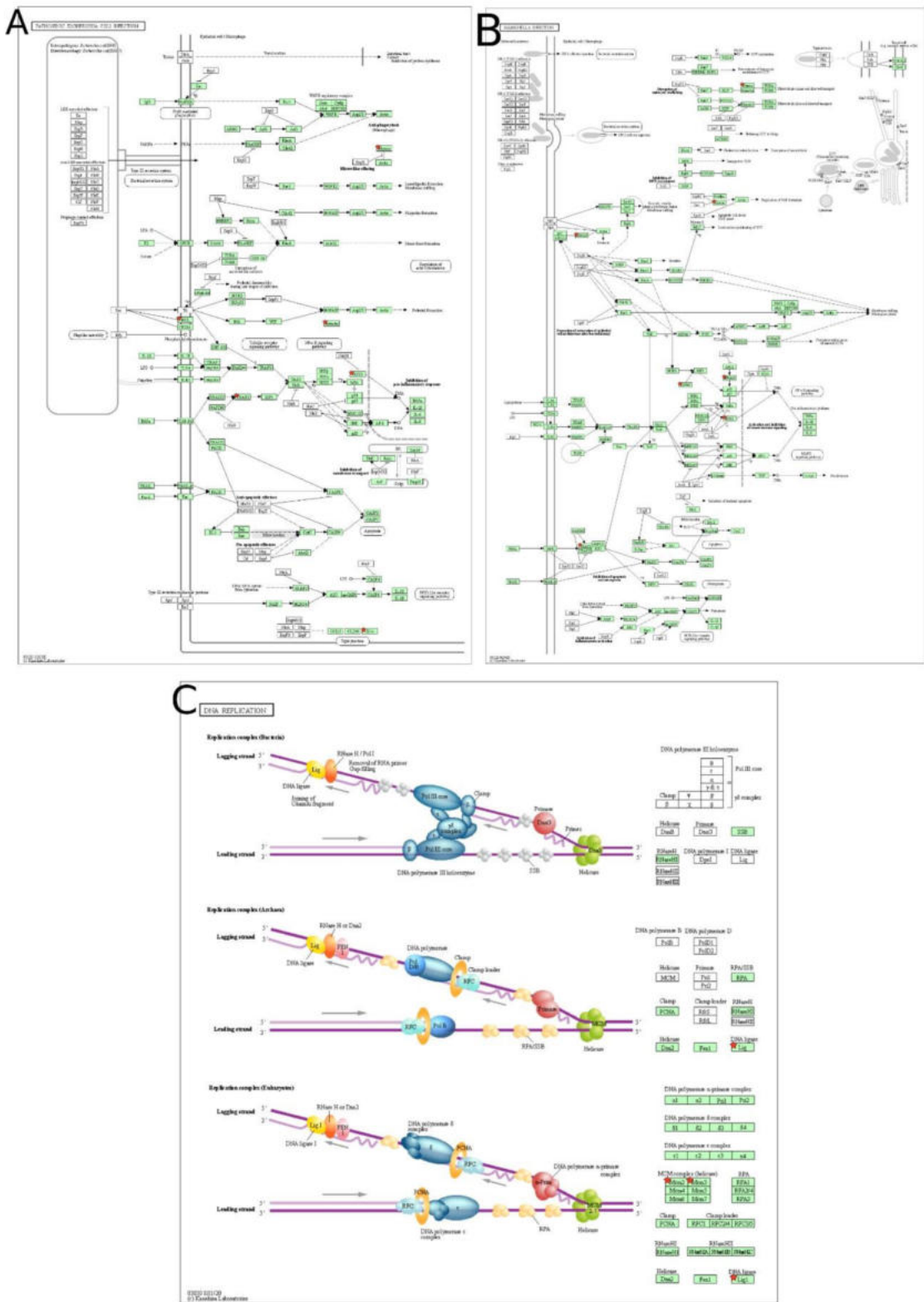


Figure 53: KEGG pathways for A549 cells infected with Influenza B at 8 hrs. A – Tight junction B – Spliceosome C – Ribosome Biogenesis in eukaryotes

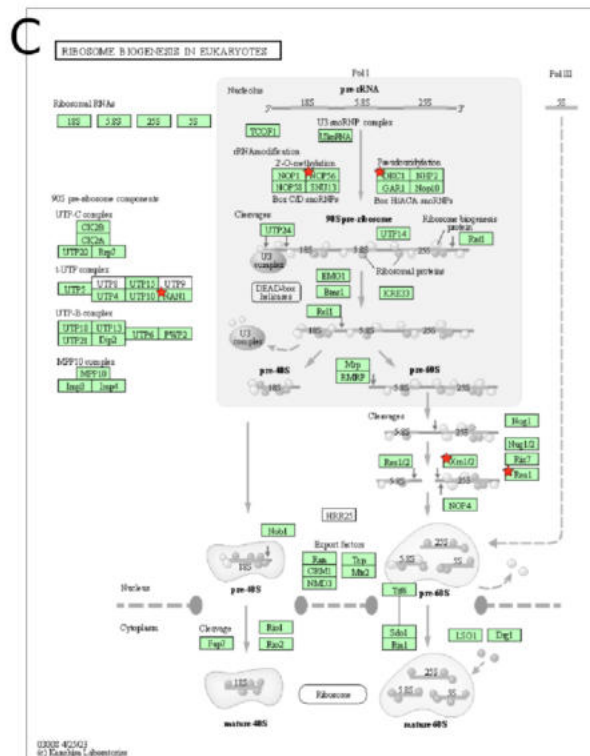
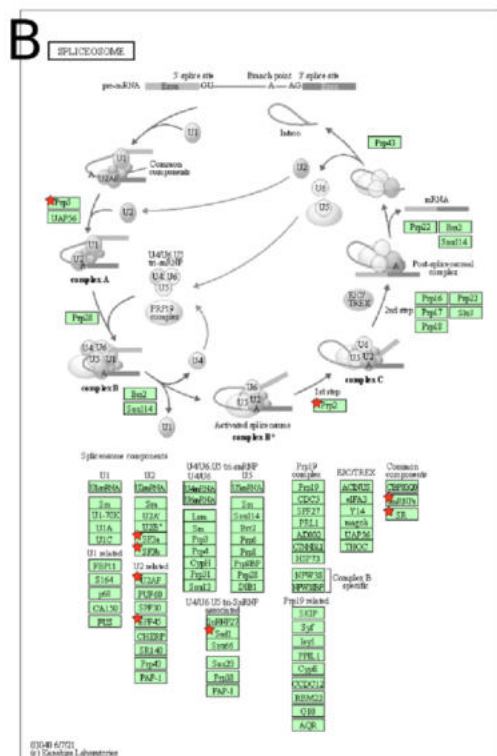
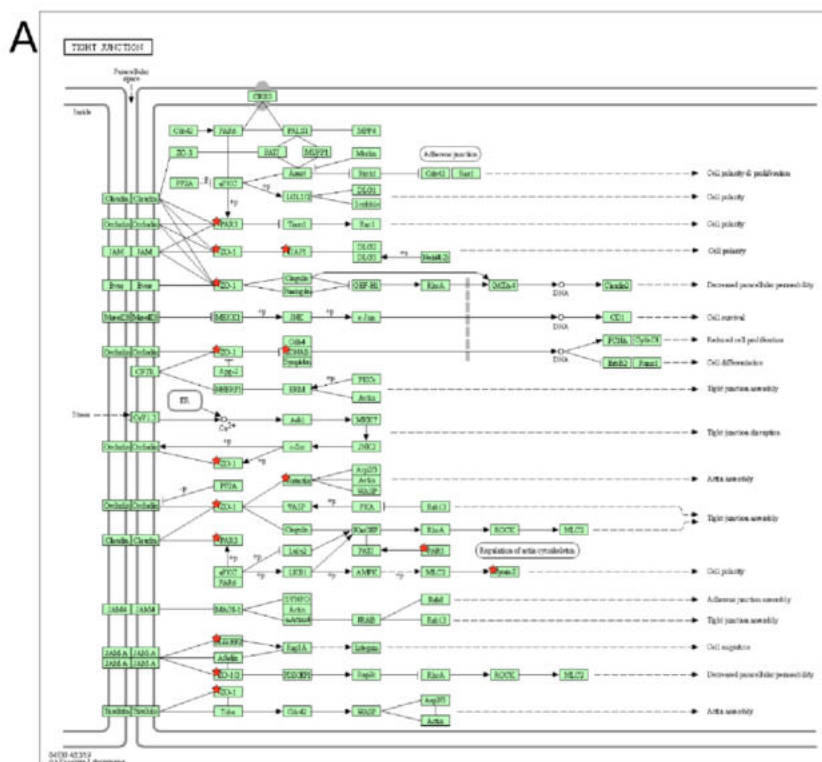


Figure 54: KEGG pathways for A549 cells infected with Influenza B at 8 hrs. A – Fluid Shear stress and Atherosclerosis B – IL17 signaling pathway C – Protein processing in Endoplasmic Reticulum D – Ribosome

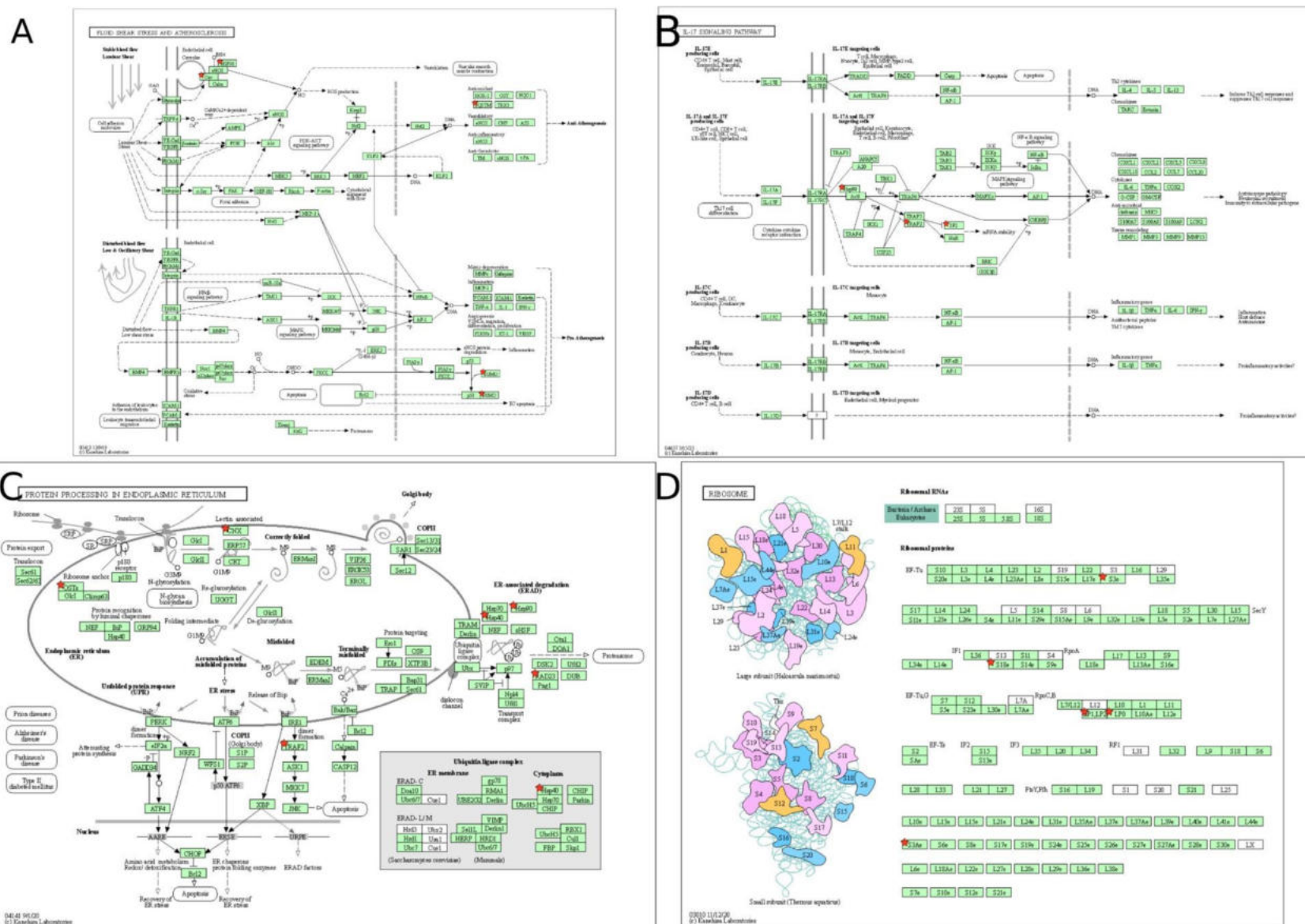


Figure 55: KEGG pathways for A549 cells infected with Influenza A - pH1N1 at 0 hr. A – Endocytosis B – IL17 signaling pathway C – NOD like receptor signaling pathway D – Protein processing in Endoplasmic Reticulum

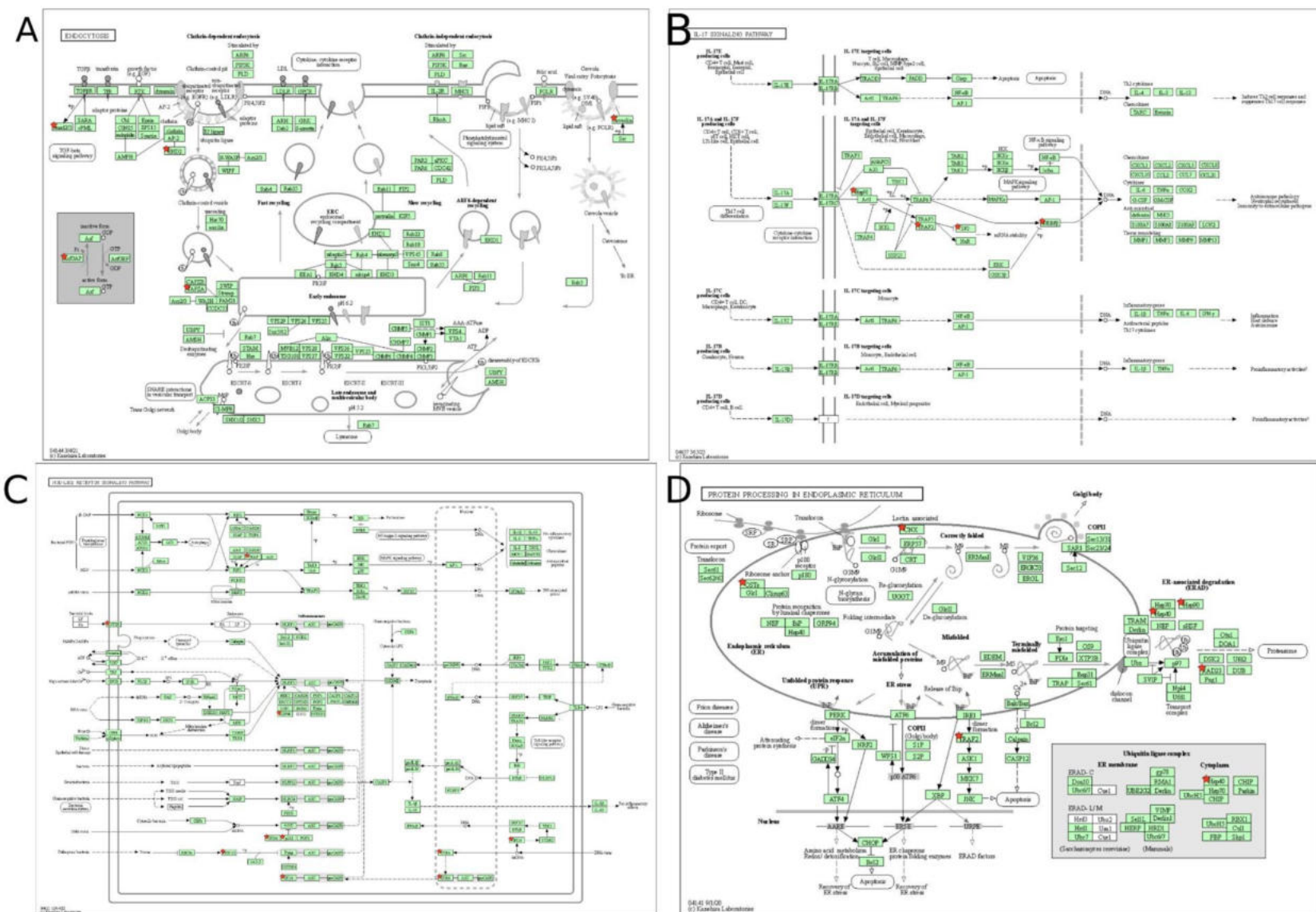
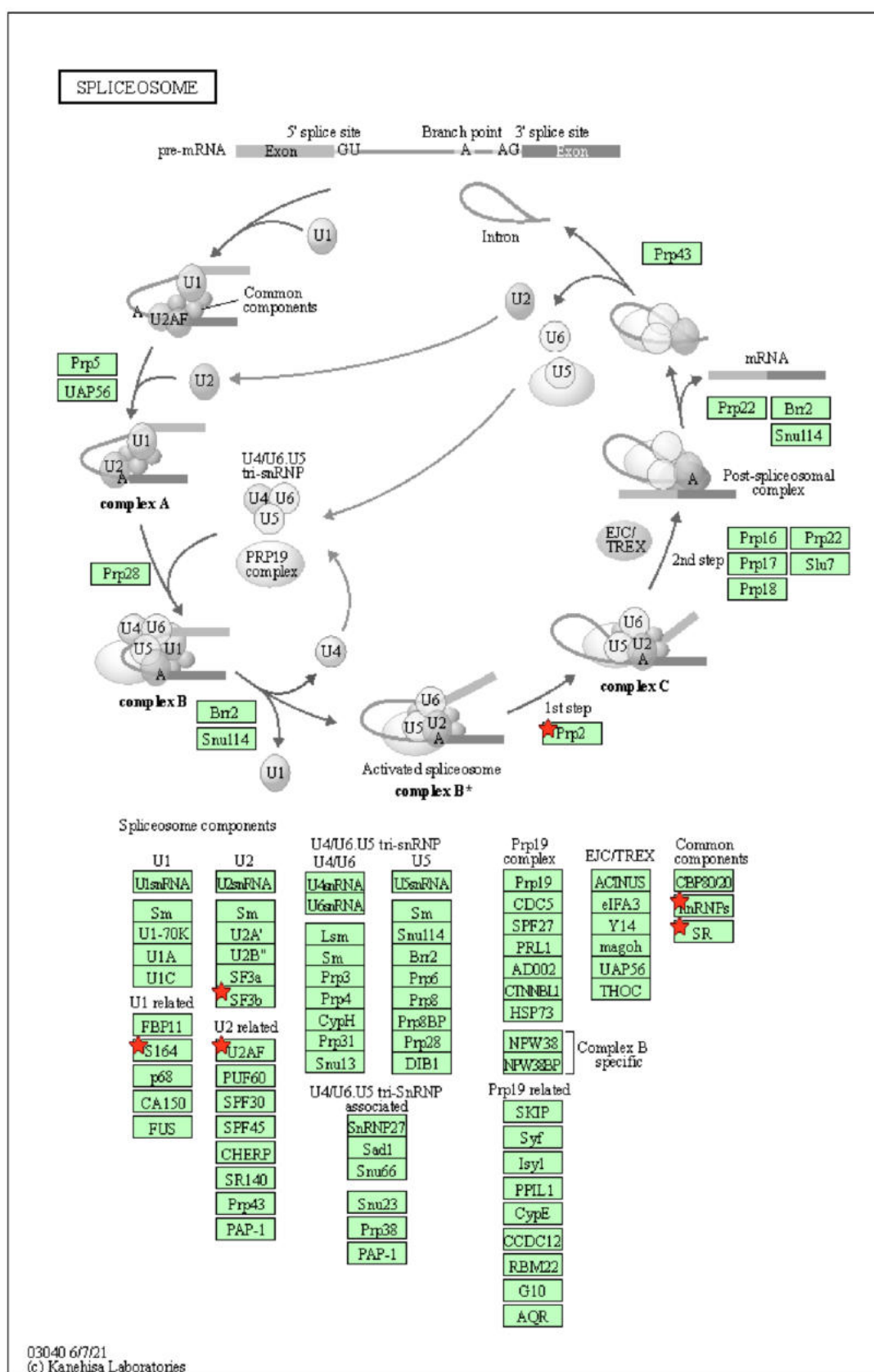


Figure 56: KEGG pathways for A549 cells infected with Influenza A - pH1N1 at 0 hr. A – Spliceosome



B – Spliceosome



B – Adherens junction

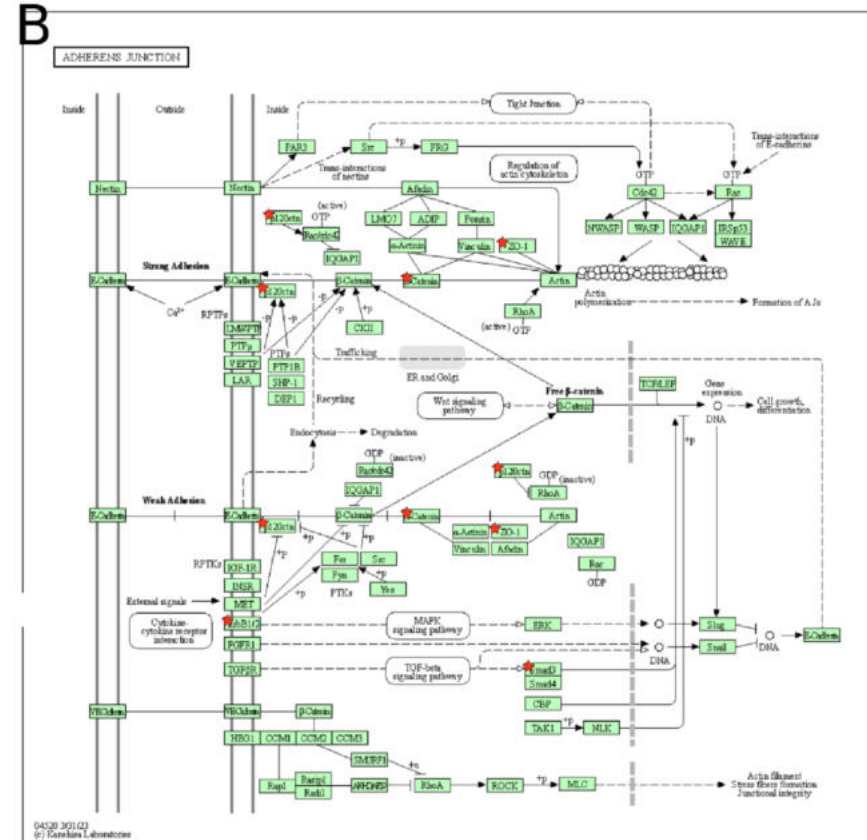
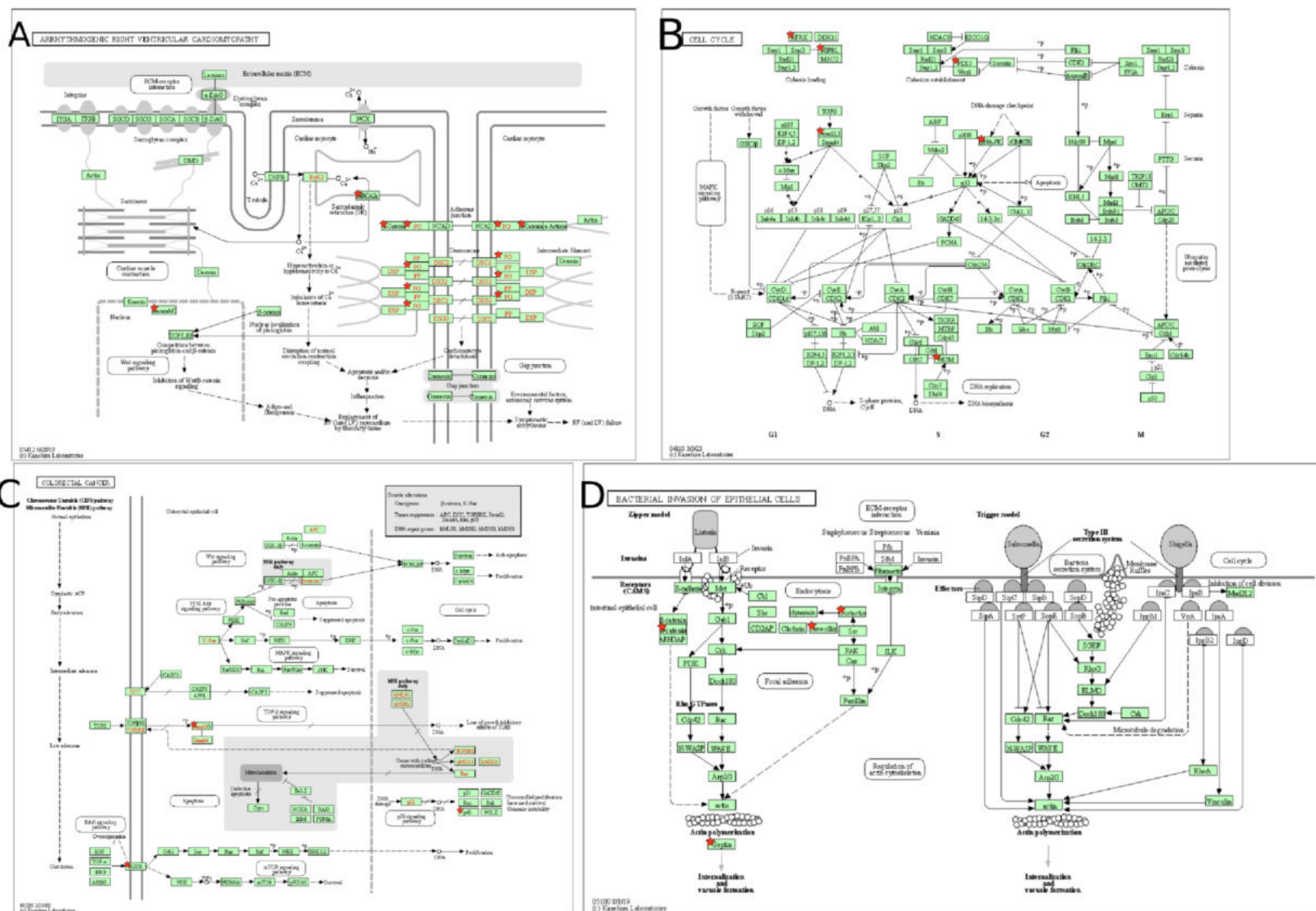


Figure 61: KEGG pathways for A549 cells infected with Influenza A – H3N2 at 8 hrs. A – Arrhythmogenic right ventricular cardiomyopathy B – Cell cycle C – Colorectal cancer D – Bacterial invasion of epithelial cells



- Pathogenic *Escherichia coli* infection

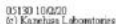


Figure 63: KEGG pathways for A549 cells infected with Influenza A – H3N2 at 8 hrs. A
– *Salmonella* infection

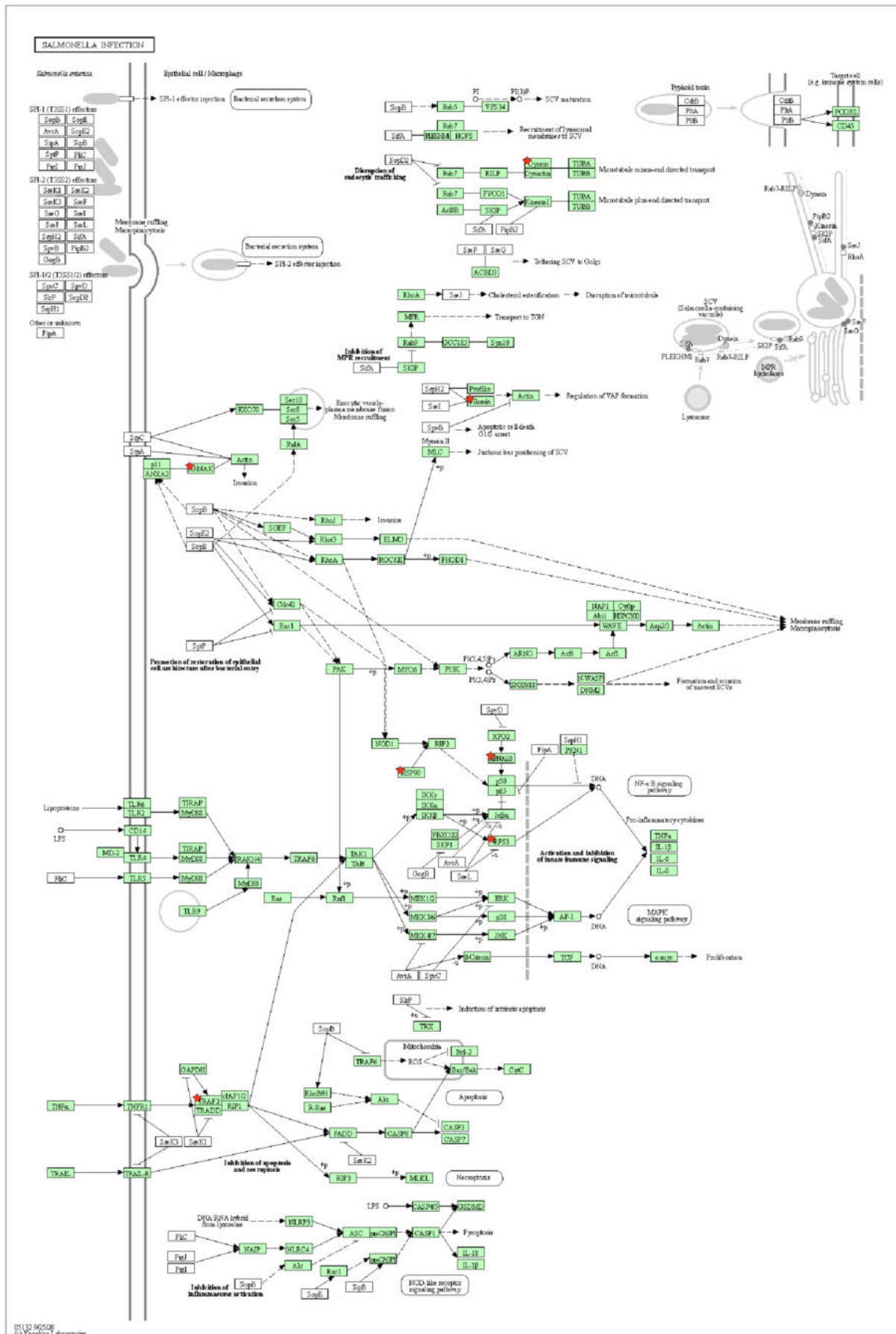


Figure 64: KEGG pathways for A549 cells infected with Influenza A – H3N2 at 8 hrs. A – Proteoglycans in cancer

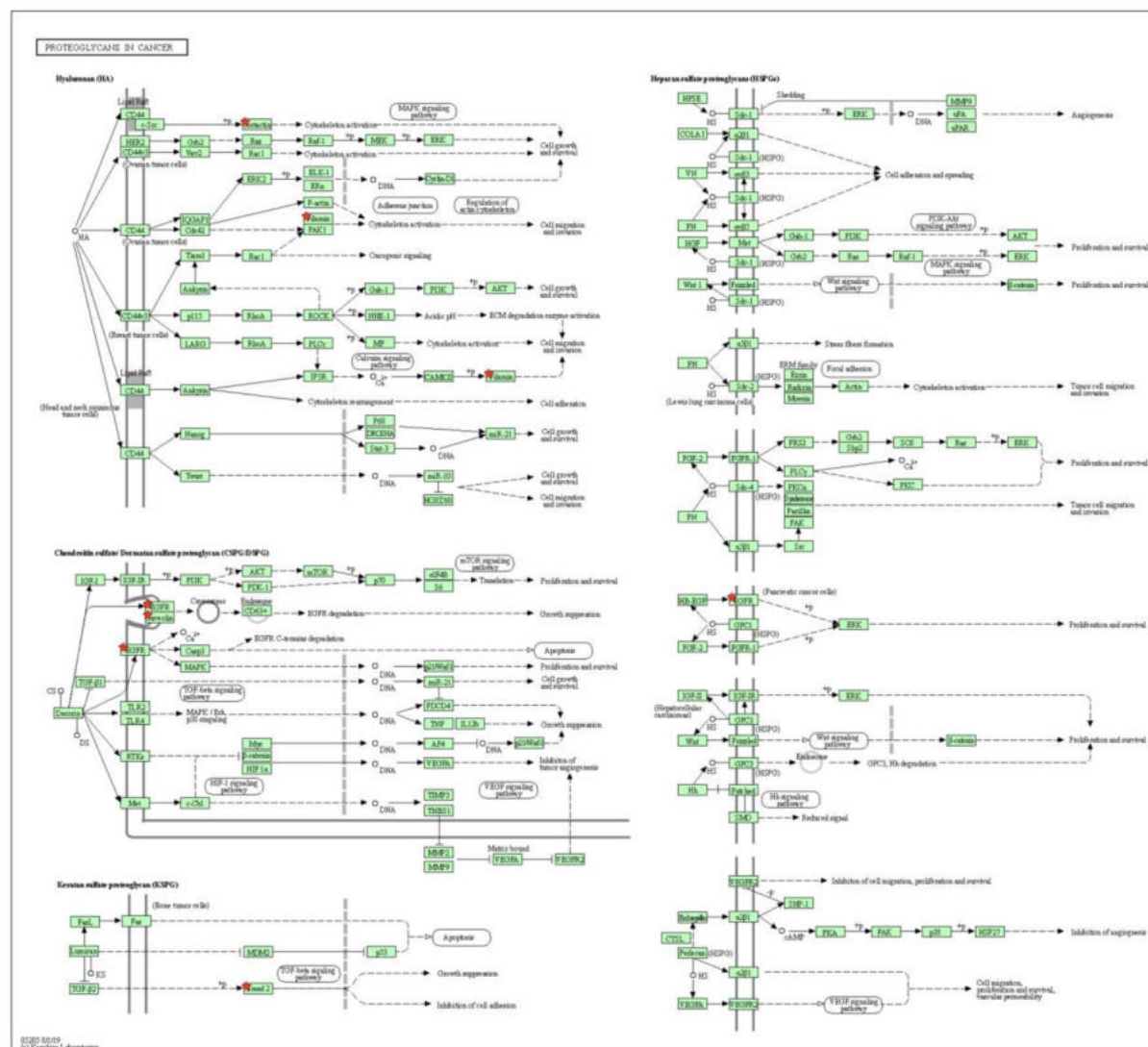


Figure 65: KEGG pathways for A549 cells infected with Influenza A – H3N2 at 8 hrs. A – Spliceosome B – Ribosome biogenesis in eukaryotes C – Pancreatic cancer D – IL 17 signaling pathway E – Gastric cancer

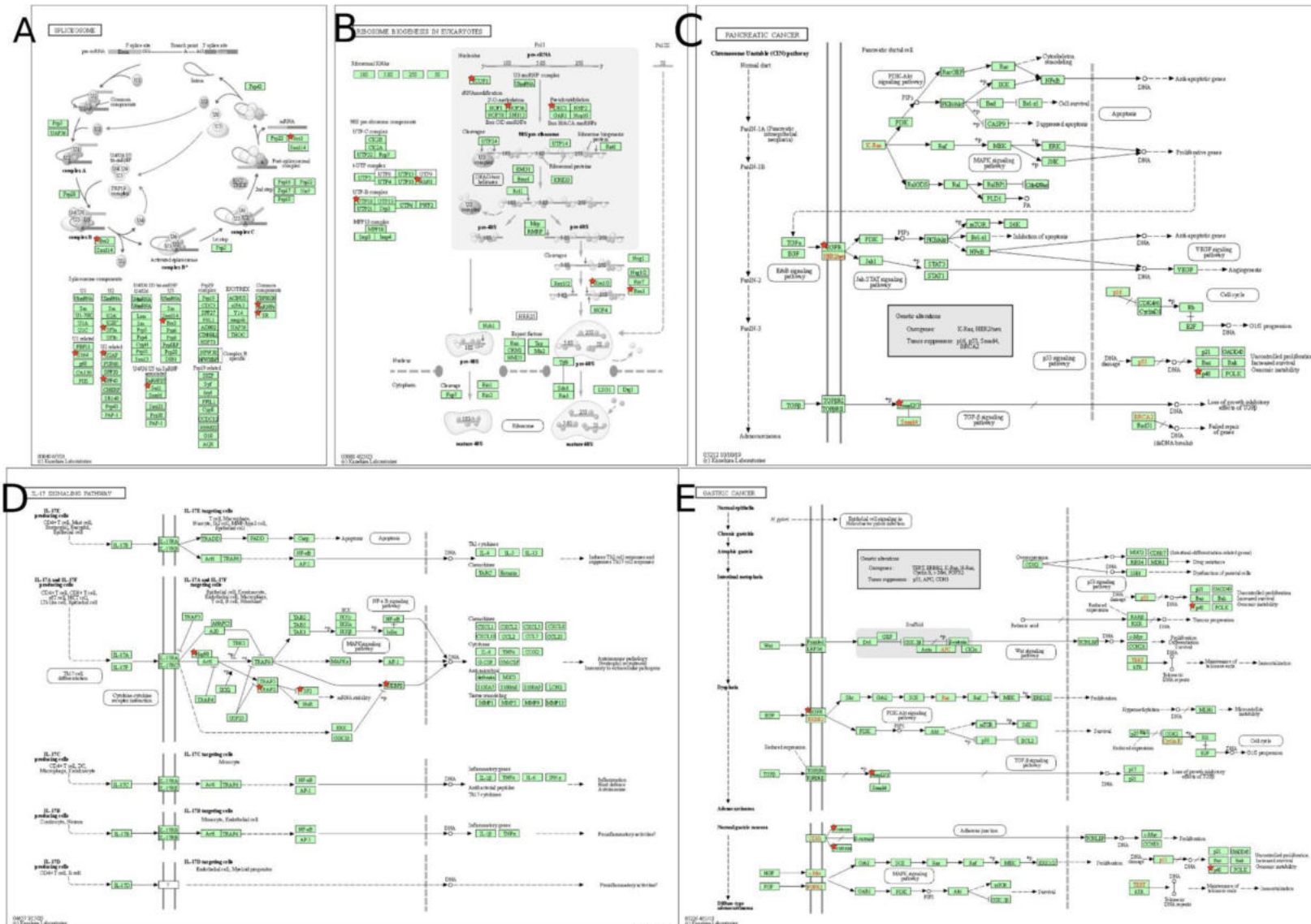
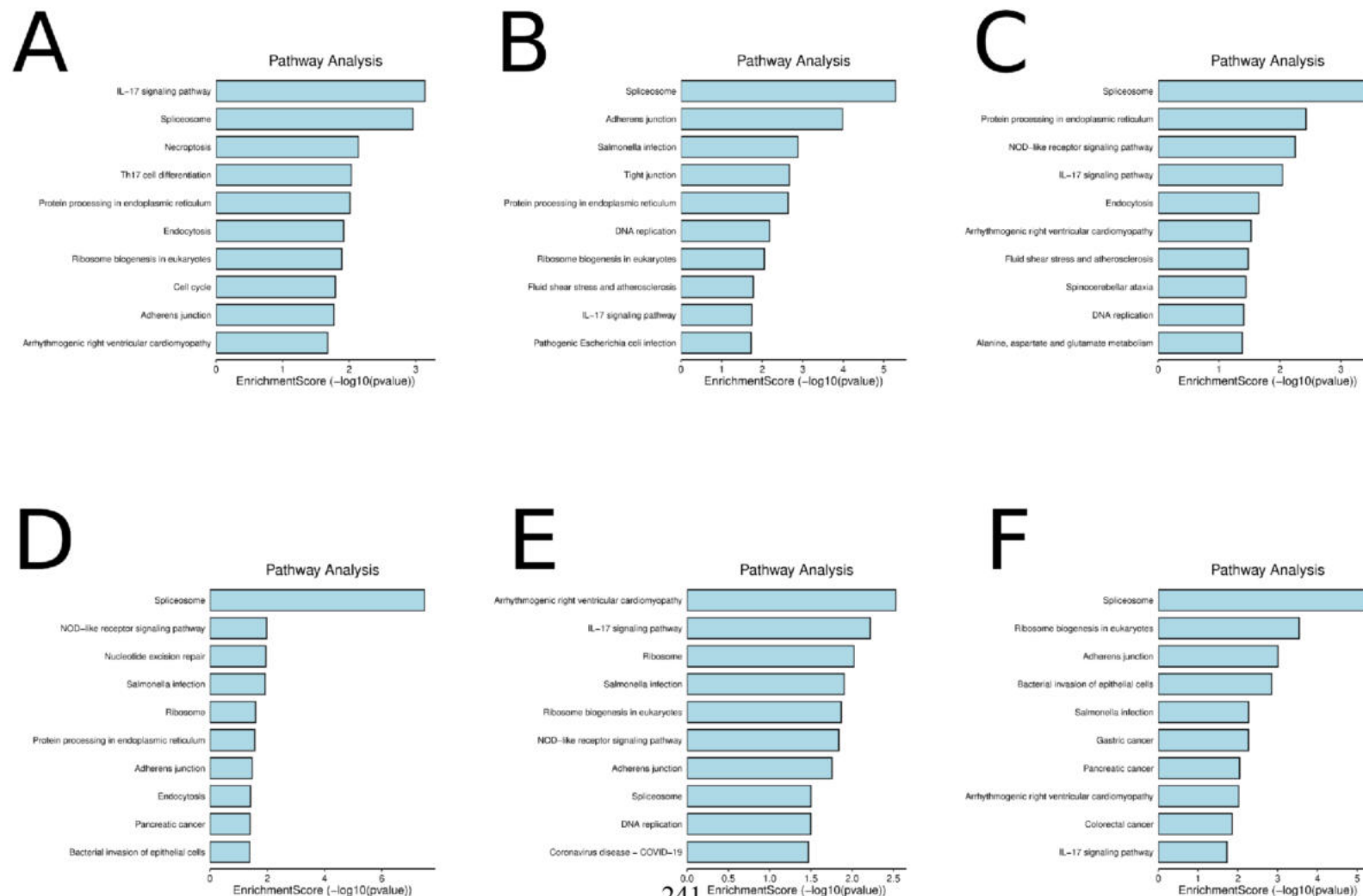


Figure 66: Summarization of enriched pathways from KEGG database for differentially expressed genes. (A) – enriched pathways after infection with Influenza B at 0 hr (B) - enriched pathways after infection with Influenza B at 8 hrs (C) – enriched pathways after infection with pH1N1 at 0 hr (D) – enriched pathways after infection with pH1N1 at 8 hrs (E) – enriched pathways after infection with H3N2 at 0 hr (F) – enriched pathways after infection with H3N2 at 8 hrs



5.14. STRING Database Analysis for A549 cells infected with Influenza B at different time – points.

The protein–protein interaction network analysis was constructed using the STRING database for the differentially expressed phosphoproteins in A549 cells.

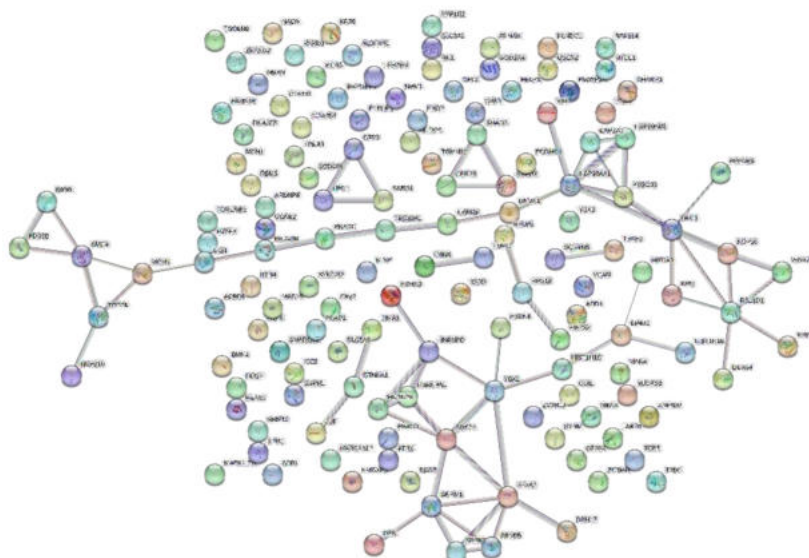


Figure 67: Protein-protein interactions using STRING tool at 0 hr-time interval of the influenza B infection with highest confidence (0.900). The line colour indicates the type of interaction evidence, and the line thickness indicates the strength of data support.

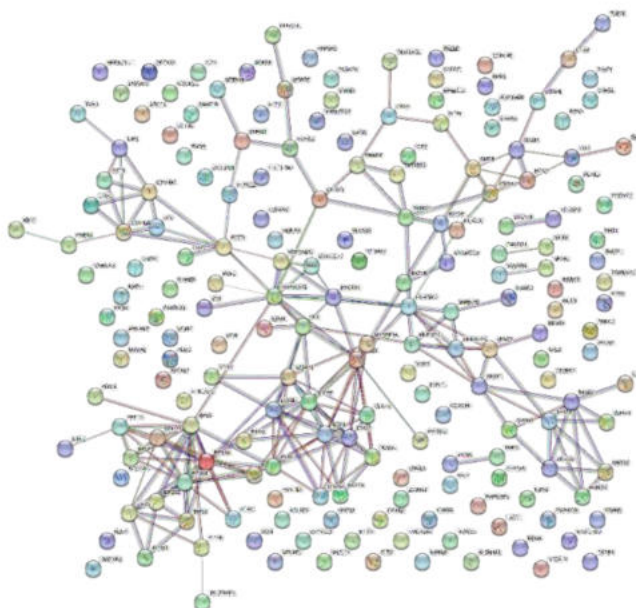


Figure 68: Protein-protein interactions using STRING tool at 8 hrs-time interval of the influenza B infection with highest confidence (0.900). The line colour indicates the type of interaction evidence, and the line thickness indicates the strength of data support.

5.15. STRING Database Analysis for A549 cells infected with Influenza A – pH1N1 at different time – points

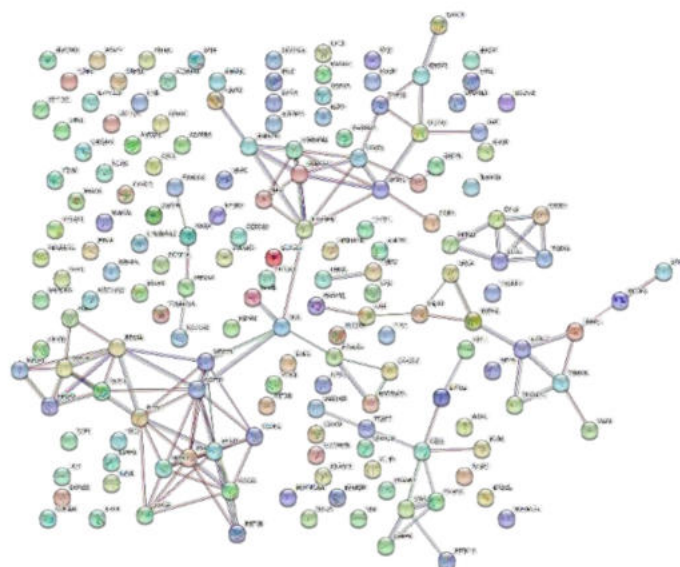


Figure 69: Protein-protein interactions using STRING tool at 0 hr time interval of the influenza A – pH1N1 infection with highest confidence (0.900). The line colour indicates the type of interaction evidence, and the line thickness indicates the strength of data support.

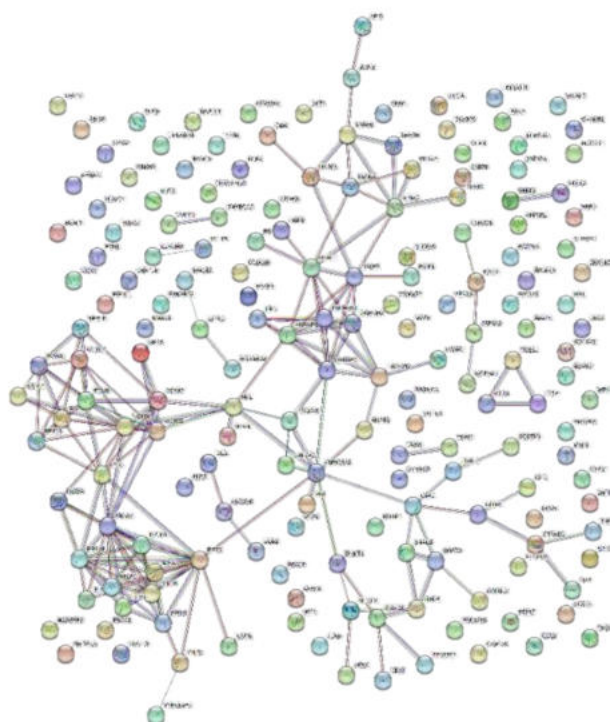


Figure 70: Protein-protein interactions using STRING tool at 8 hrs-time interval of the influenza A – pH1N1 infection with highest confidence (0.900). The line colour indicates the type of interaction evidence, and the line thickness indicates the strength of data support.

5.16. STRING Database Analysis for A549 cells infected with Influenza A – H3N2 at different time – points

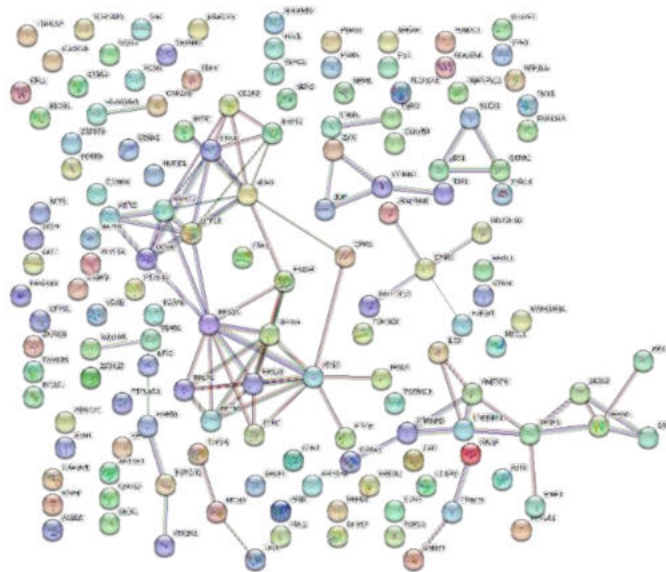


Figure 71: Protein-protein interactions using STRING tool at 0 hr-time interval of the influenza A – H3N2 infection with highest confidence (0.900). The line color indicates the type of interaction evidence, and the line thickness indicates the strength of data support.

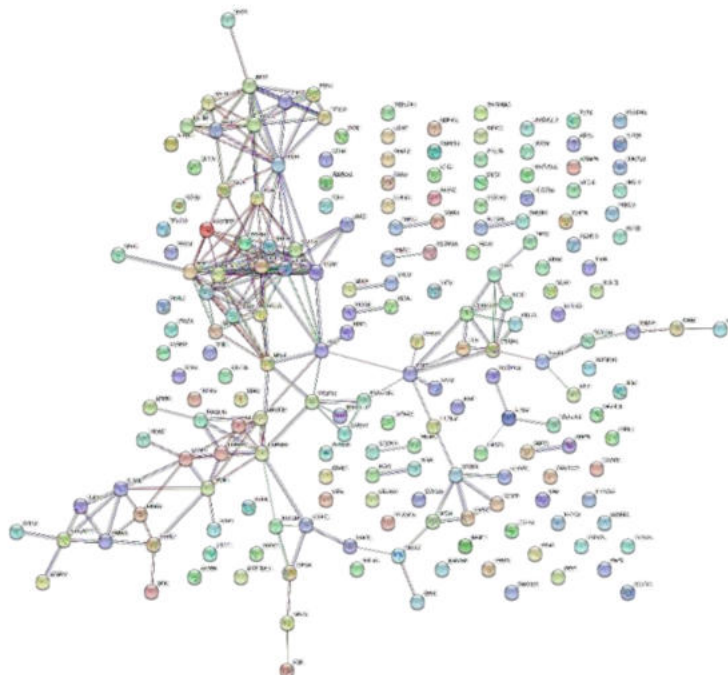


Figure 72: Protein-protein interactions using STRING tool at 8 hrs-time interval of the influenza A – H3N2 infection with highest confidence (0.900). The line colour indicates the type of interaction evidence, and the line thickness indicates the strength of data support.

CHAPTER 5:

DISCUSSION

6. Gene Expression Analysis:

The first line of defence against infectious pathogens depends heavily on innate immune responses. Understanding the function and interaction of these systems is essential to comprehending the short- and long-term impacts of viral infections. The interferon system and its up- and downstream genes and proteins form a crucial first-line defence against viral infections. (97) These innate reactions, in addition to the many components of the adaptive immune response system, are also very crucial in the process of developing efficient vaccines. (98) Studies have shown that, innate and adaptive immune responses are both significantly involved during influenza virus infections. (53,54,99,100) After an infection, the immune system becomes active and releases cytokines. These cytokines, in turn, activate multiple pathways, which in turn inhibit viral entrance and reproduction at various stages. The antiviral response can be negatively affected when key components of the innate immune system are missing. On the other hand, excessive stimulation of the innate immune system can lead to immunopathology, which has the potential to result in death. This exemplifies the precarious balancing act that must be performed between offering protection and preventing immunopathology. This work used the human lung carcinoma (A549) cell line to undertake gene expression analyses of cytokines that were generated by several influenza viral infections at 4 hrs and 24 hrs. (99,101) Infected cells create more than 15 different cytokines, yet they do not produce any chemokines. In this section, we will be focusing on particular cytokines that play key functions and are of major importance, such as type 1 interferons, tumour necrosis factor, interleukin 8, and interleukin. (102)

6.1. IL - 8

In human airway epithelial cells, viral infection can induce the expression of interleukin-8, which in turn can lead to an increase in the amount of interleukin-8 that is synthesised by the cells. The gene expression of IL-8 exhibited an increase of 1.2, 3.7 and 1 fold after 24 hrs in pH1N1, H3N2 and B respectively. The major site of influenza infection is the epithelial cell. It is anticipated that the generation of interleukin-8 will play a part in the pathophysiology of virus-induced airway inflammation due to the fact that the epithelial layer is the location of the most intense inflammation in an airway that has been infected with influenza. Interleukin-8 gene expression is induced by viral infection, although the mechanism is unknown. IL8 gene expression is known to be stimulated by interleukin I. It is possible that the production of interleukin-8 by the epithelium occurs at an early stage in the progression of virus-induced airway illness. (103)

6.2. IL - 6

The gene expression of IL-6 increased by 4, 8 and 3 folds after 24 hrs in pH1N1, H3N2 and B respectively. IL-6 has long been known of as a proinflammatory cytokine and an indicator of inflammation. It has been demonstrated that IL-6 is an essential factor in protecting the host from the mortality caused by H1N1 by preventing the death of virus-induced neutrophil cells. According to the findings of another study, IL-6 is necessary not only to increase viral clearance and survival but also to reduce the severity of lung inflammation that is brought on by influenza. In addition, IL-6 is essential for virally specific memory CD4 T cells to be remembered in secondary infections, although this is not the case for CD8 T cells. This is because IL-6 restricts the function of virus-specific regulatory T cells (Tregs), which promotes the elimination of the virus and the continued existence of the host. Despite the fact that IL-6 has been linked to poor clinical outcomes, in vitro studies suggest it may promote significant defensive responses that aid in disease resolution. (102)

6.3. Interferons

Interferons, also known as IFNs, are a family of cytokines that have been researched extensively and play a key part in innate immunity. They do this by inducing a "antiviral state" in infected cells as well as cells that are adjacent. The fundamental function of type I IFN signalling is to stimulate the production of ISG-encoded proteins, which possess important antiviral capabilities and are able to inhibit viral replication in infected epithelial cells. This is the primary function of type I IFN signalling. IFN- α can bind to the IFN- α receptor (IFN- α R) and trigger a signalling cascade via the JAK-STAT pathway, which leads to ISG transcription. ISG-encoded proteins have powerful antiviral properties, preventing viral multiplication within infected epithelial cells. A lack of IFN-R inhibits the production of ISGs in response to an infection with the H3N2 influenza virus. In the same manner, infections caused by H1N1 are characterised by a decreased expression of antiviral genes and an enhanced permissiveness for the generation of viral proteins. IFN is responsible for the induction of PKR, which is a key regulatory component in a variety of apoptotic pathways. A wide array of infected cell types have been shown to exhibit activated PKR. These infections were caused by the influenza virus. As a consequence of PKR activation, the phosphorylation of the eukaryotic initiation factor (eIF)-2, the activation of the nuclear factor (NF)- κ B, and the transcriptional stimulation of many pro-apoptotic genes take place. These genes include those that code for Fas, p53, and Bax. (102,104)

6.4. TNF- α .

The quintessential proinflammatory cytokine that lies at the "heart of the influenza cytokine storm" is known as TNF- α . It would appear that the expression of TNF in lung epithelial cells, which are the principal targets of influenza virus infection, is essential for the regulation of influenza virus infection in the respiratory tract of the host. Because of its ability to recruit various host cells, such as monocytes and T and B lymphocytes, to infection sites, TNF- α plays an important part in the process of eradicating influenza virus infection in the respiratory tract before the secondary immune response is initiated. This is because of its ability to recruit these cells. After twenty-four hours, there was a rise in pH1N1 that was eighteen times greater than before. The H3N2 virus showed a tenfold increase over this time period. Anti-TNF medication can lower the severity of weight loss and sickness following exposure to the influenza A H3N2 virus, suggesting that it could be a promising therapeutic target. Anti-TNF therapy can diminish the severity of weight loss and illness after exposure to the virus. According to the findings, not only does TNF have an effect on the homeostasis of the local microenvironment, but it also has widespread systemic effects once it enters circulation. As a consequence of this, anti-TNF medications might be an option worth considering for the treatment of severe influenza. In influenza-infected cells, the generation of cytokines and the formation of inflammatory cell infiltrates can be inhibited by using TNF-neutralizing monoclonal antibodies or soluble TNF receptor fusion proteins, as was demonstrated in a number of investigations. (105)

6.5. CCL5

Chemotactic factors, such as CCL5, are produced, which pull leukocytes and monocytes into the alveolar lumen. Concurrently, adhesion molecules are released more frequently near the site of the infection. Leukocytes are able to activate chemotaxis, release cytokines, and proliferate with the assistance of the beta chemokines CCL5, which bind to the CCR5 receptor. If these symptoms continue to worsen, it is possible that they will cause significant tissue damage, which will in turn reduce the amount of oxygen exchanged and cause respiratory distress. Increased levels of both SAMHD1 and CCL5, which is also known by its acronym RANTES, have been repeatedly related to the suppression of IAV replication. If these innate signals during severe influenza could be understood better, it could be possible to find new antiviral targets and protective indicators. (106)

Direct infection of lung epithelial, endothelial, and innate immune cells causes a "primary cytokine storm" that inhibits viral spread and replication, recruits and activates effector cells capable of more thorough virus and virally-infected cell elimination, and stimulates various

cell types that can contribute to lung tissue restoration. A second wave of cytokines is secreted by these cells, which continue the virus clearance process, reduce inflammation, and try to restore lung function. Few of the secondary cytokines expressed are type 2 IFNs and IL10.

6.6. IFN- α

IFN- α is the only type II IFN that is created in the body after an influenza infection. The interaction of IFN- with type II IFN receptors (IFN-R1 and IFN-R2) results in the formation of STAT1-STAT1 homodimers, which in turn increase the production of ISGs via the conventional JAK-STAT pathway. IFN- is a potent antiviral cytokine that plays a number of different activities in the immune system. These tasks include enhancing the cytotoxicity of immune cells, promoting DC activation, and producing antibodies in B cells. T cells and natural killer (NK) cells are principally responsible for producing IFN- in the body following an influenza infection. The so-called "primary cytokine storm" results in the production of IL-18, which in turn leads to the maturation of CD8 and NK cells that are highly activated in response to antigens unique to those cells, which then results in the production of IFN-, which assists in the regulation of viruses and other immune activities. In conclusion, the production of IFN- by T cells and NK cells is associated with a wide range of effects following influenza infection. These effects include an improvement in host disease outcomes, an increase in viral clearance, and a strengthening of both cellular and humoral immune responses. (102)

6.7. IL-10

IL-10 is an anti-inflammatory cytokine that, when present in the body during bacterial or viral infections, inhibits both the innate and adaptive immune responses. During an influenza infection, particularly during the adaptive immune response, there is an abundance of IL-10. Effector CD4 and CD8 T cells that contain significant amounts of the transcription factors T-bet and IFN- γ are the primary cells responsible for the production of IL-10. In lungs that have been infected with H1N1, the levels of IL-10 and IFN- γ are significantly reduced when CD4 and CD8 T cells are neutralised. Consistent with its immunosuppressive actions, increased quantities of a variety of proinflammatory cytokines, including as IL-17A, IL-17F, IFN- γ , and TNF- α , are seen in animals that are deficient in IL-10 or who have been administered an antibody to disrupt the signalling of the IL-10 receptor. In human influenza infections, elevated levels of the cytokine IL-10 have been associated to both severe lung inflammation and fatalities. On the other hand, high levels of IL-10 may be an indication that the host is making an effort to reduce the inflammatory damage caused by other elements of the cytokine storm. When seen as a whole, IL-10 seems to have a time-dependent regulatory role and may

operate as a warning indication of a negative reaction. This is apparent when the protein is examined in its whole. (107)

6.8. IFITM

IFITM proteins are one type of protective factor that can help minimise the amount of host cell invasion caused by enveloped viruses. At 24 hpi, the fold change in IFITM1 was 1.02, 76.39, 67.77, and 77.96 respectively, compared to 1.13, 56.22, 35.24, and 17.33 at 4 hpi for the control, pH1N1, and H3N2 viruses, respectively. We may deduce from this that there was an increase of the IFITM 1 gene upon infection. At 24 hpi, there was a greater fold change. It is hypothesised that IFITM1, due to the fact that it is surface-localized on the cell, plays an essential part in preventing viruses from entering the cell by direct fusion with the plasma membrane. IFITM1 possesses a unique method of action against IAV at the plasma membrane, one that is independent of its expression within the endocytic compartments of the cell. It is not apparent how IFITM1 provides antiviral action against IAV; nevertheless, past research suggests that the activity of IFITM1 against IAV needs its expression on the cell surface.

It is also likely that the capacity of each IFITM to restrict IAV infection is dependent on the cell type. This may be because different cell types contain low basal expression levels of certain IFITM proteins. In the study, for instance, IFITM1 knockdown did not increase the infectivity of IAV in HeLa cells. Furthermore, antiviral activity of endogenous IFITM1 was shown only after IFN- stimulation to boost IFITM expression levels in A549 cells prior to treatment with IFITM1-specific siRNA and IAV infection. This was done before the cells were subjected to IAV infection. (108) The fold change in IFITM2 was 1.04, 9.81, 3.82, 1.34 for at 4 hpi in control, pH1N1, H3N2 and B while for 24 hpi it was 1.02, 242.68, 462.08, 23.42 respectively. From this data we can conclude that IFITM2 is highly upregulated at 24 hpi compared to 4 hpi. IFITMs could enlarge acidic compartments in cells. IFITM-2 increases the number and size of acidic compartments and different IFITMs localize to diverse membrane regions, most likely by binding to different biological components, and change the membrane structure, preventing viral membrane fusion. (109) In addition, IFITM2 are endocytosed from the cell surface and allowed to accumulate in endosomal and endo-lysosomal membranes under steady-state circumstances due to a tyrosine-based internalisation motif that is recognised by the endocytic adaptor AP-2 and regulated by phosphorylation. This allows IFITM2 to be taken up from the cell surface. As a direct consequence of this, IFITM2 are mostly located in early endosomes and late endosomes, respectively, although phosphorylated protein pools continue to be detected in the plasma membrane. IFITM1 is frequently located

at or near the plasma membrane, which may be the reason why the antiviral selectivity of the IFITM proteins does not overlap. On the other hand, IFITM2 is a more potent inhibitor of endocytic viruses like IAV and Zika virus. (110)

The gene expression of IFITM3 was upregulated by 1.04, 8.05, 6.68, 7.11 33 folds at 4 hpi in control, pH1N1, H3N2 and B while for 24 hpi it was 1.02, 5455.21, 673.87, 688.89 respectively. This concludes that IFITM3 is highly expressed in infected cells. At basal levels, IFITM3 expression is elevated, that contribute to viral resistance further enhancing cellular defence. IFITM3 depletion completely restored IAV HA-mediated infection, indicating that IFITM 3 more effectively limits IAV. Furthermore, IFITM3 colocalizes significantly with markers of late endosomes and lysosomes, but poorly with an early endosome marker. (111) IFITM3 is responsible for the enhanced viral suppression that occurs when the virus enters the cell via Rab7-positive late endosomes or lysosomes. The fact that mutation of the IFITM3 endocytic signal leads in its cell surface accumulation and gains a role to restrict the infection of human parainfluenza virus 3 (HPIV-3), which enters cells via direct fusion with the plasma membrane, exemplifies this rule. This finding was made possible by the fact that HPIV-3 enters cells by direct fusion with the plasma membrane. Another illustration of this would be the fact that the sensitivity of IAV to IFITM3 appears to be reliant on the pH value at which the IAV hemagglutinin initiates membrane fusion and, as a consequence, the endocytic compartments in which membrane fusion takes place. However, there are just a few instances in which this rule is broken. For instance, it is not known how Moloney leukaemia virus (MLV) and Sendai virus, all of which fuse at the cell plasma membrane, as well as Lassa fever virus (LASV) and lymphocytic choriomeningitis virus (LCMV), both of which fuse at Rab7-positive late endosomes, circumvent IFITM1 and IFITM3 restrictions, respectively. These viruses both fuse at Rab7-positive late endosomes. (112)

7. Proteomics:

Understanding the status of a biological system requires not only the analysis of gene expression data, but also the measurement of the amount of protein that is now present because this measurement provides crucial information that is not easily identifiable from gene expression data. (113) Because proteomic techniques are capable of detecting proteins and peptides in a manner that is unique to them, they are extremely useful for doing comprehensive biomarker research. These methods offer a simplified alternative to the complicated methodologies that have

traditionally been used, which enables researchers to explore biomarkers in a manner that is both comprehensive and effective.

Proteomic techniques have emerged as a powerful way for getting a more in-depth understanding of biological processes. These approaches involve doing an analysis of the structural makeup of cellular proteins as well as the functional connections that exist between these molecules. (114) These techniques are used to study the proteome, which is the complete set of proteins found in a cell. This strategy provides complete insights into the proteins that are uniquely expressed under certain situations, offering light on the particular signalling modifications. (115) An analysis of the differential expression of proteins and phosphoproteins can be extremely useful when attempting to identify the molecular characteristics of a particular disease state and to provide support for the development of biomarkers. (116) Numerous research have used proteomic techniques to explore changes in different cells during disease states or in response to infections. These investigations have led to the identification of unique insights through the utilisation of tandem mass tags.

In this investigation, we used a proteomics-based tandem mass spectrometry strategy to discover proteins and phosphoproteins whose levels of expression varied significantly across the A549 cells infected with different strains of influenza virus. We wanted to understand the underlying biological mechanisms and uncover significantly enriched pathways linked with the differentially expressed proteins, so we looked at the whole proteome profile along with the phosphoprotein profile of A549 cells infected with different strains of influenza virus. The objective of this study was to identify novel avenues for the development of anti-influenza therapies and to gain fresh insights into the intricate interactions between the influenza virus and its host.

8. Phosphoproteomics:

Researching the biochemical profiles of cells and identifying the hallmark phosphoproteins inside them allows us to better understand the functions of metabolic networks within the cell. For the samples of A549 cells infected with different strains of influenza virus at different time-points, LC-MS/MS analysis generated a dataset of 2510 proteins and 465 phosphoproteins. Filtering these proteins based on the organism *Homo sapiens*, we identified 113 upregulated and 158 downregulated proteins in influenza B virus infected-A549 cells at 0 hpi, 272 upregulated and 429 downregulated proteins in influenza B virus infected-A549 cells at 8 hpi, 178 upregulated and 177 downregulated proteins in influenza A – pH1N1 virus infected-A549 cells at 0 hpi, 291 upregulated and 210 downregulated proteins in influenza A – pH1N1 virus infected-A549 cells at 8 hpi, 104 upregulated and 87 downregulated proteins in influenza A – H3N2 virus infected-A549 cells at 0

hpi, 289 upregulated and 302 downregulated proteins in influenza A – H3N2 virus infected-A549 cells at 8 hpi, with log-fold changes ranging between 0.8 and 1.25.

Whereas, we identified 85 upregulated and 89 downregulated phosphoproteins in influenza B virus infected-A549 cells at 0 hpi, 143 upregulated and 122 downregulated phosphoproteins in influenza B virus infected-A549 cells at 8 hpi, 98 upregulated and 95 downregulated phosphoproteins in influenza A – pH1N1 virus infected-A549 cells at 0 hpi, 138 upregulated and 82 downregulated phosphoproteins in influenza A – pH1N1 virus infected-A549 cells at 8 h.p.i., 81 upregulated and 86 downregulated phosphoproteins in influenza A – H3N2 virus infected-A549 cells at 0 hpi, 147 upregulated and 105 downregulated phosphoproteins in influenza A – H3N2 virus infected-A549 cells at 8 hpi with log-fold changes ranging between 0.8 and 1.25.

8.1. GO enrichment using DAVID database:

GO terms pertaining to biological processes, cellular components, and molecular functions were used to identify the functional enrichment of proteins. The most significantly enriched biological processes were associated with cellular process i.e., ribonucleoprotein complex biogenesis, RNA splicing, regulation of mRNA metabolic process, RNA splicing - via transesterification reactions with bulged adenosine as nucleophile, negative regulation of organelle organization, regulation of mRNA metabolic process and regulation of chromosome organization (GO:0022613, GO:0008380, GO:1903311, GO:0000377, GO:0010639, GO:1903311, GO:0033044) for cells infected with Influenza A – pH1N1, H3N2 and Influenza B at 0 hpi and 8 hpi. In terms of molecular function, the enriched categories were found to be cadherin binding, transcription coregulator activity (GO:0045296, GO:0003712) for cells infected with all the influenza virus post 0 hpi and 8 hpi along with ATPase activity (GO:0016887) for H3N2 at 0 hpi, showing the active induction of a pro-oxidant situation within the host cell in order to aid the progression of viral pathogenesis and proliferation. (117) In the case of cellular component, most of the differential genes were found to be focal adhesion, cell-substrate junction, heterochromatin, spliceosomal complex and nuclear speck (GO:0005925, GO:0030055, GO:0000792, GO:0005681, GO:0016607), respectively.

8.2. Pathway Analysis using KEGG:

Using KEGG pathways, an analysis of the biological relevance of differentially expressed proteins in enriched pathways was carried out. Many significantly enriched pathways were identified, namely, ID: hsa03040 – Spliceosome, ID: hsa04520 - Adherens junction, ID: hsa05132 - Salmonella infection, ID: hsa04530 - Tight junction, ID: hsa0414 - Protein

processing in endoplasmic reticulum, ID: hsa03030 - DNA replication, ID: hsa03008 - Ribosome biogenesis in eukaryotes, ID: hsa05418 - Fluid shear stress and atherosclerosis, ID: hsa04657 - IL-17 signalling pathway, ID: hsa05130 - Pathogenic Escherichia coli infection, ID: hsa03013 - Nucleocytoplasmic transport, ID: hsa03010 – Ribosome, ID: hsa05171 - Coronavirus disease - COVID-19, ID: hsa05120 - Epithelial cell signalling in Helicobacter pylori infection, ID: hsa04110 - Cell cycle, ID: hsa05206 - MicroRNAs in cancer, and ID: hsa04621 - NOD-like receptor signalling pathway

It was discovered that the top three pathways hits that were significantly enriched were found to be IL-17 signalling pathway, Spliceosome and Necroptosis involving genes, HSP90AB1, HSP90AA1, SRSF1, CEBPB, TRAF2, RBM17, RBM25, HNRNPA1, SRSF1, HNRNPK, SF3B2, MACROH2A1, SQSTM1 for cells infected with influenza B at 0 hr. Whereas, Spliceosome, Adherens junction, Salmonella infection involving genes DDX46, U2AF2, RBM17, HNRNPC, SRSF1, USP39, HNRNPK, DHX16, SF3B2, SF3A1, EGFR, PARD3, CTNNA1, CTNND1, TJP1, SMAD3, AHNAK, AHNAK2, RPS3, KPNA3, FLNA, HSP90AB1, DYNC1LI1, HSP90AA1, TRAF2 for cells infected with influenza B at 8 hrs. Spliceosome, Protein processing in endoplasmic reticulum, NOD-like receptor signalling pathway involving genes HNRNPK, DDX42, U2AF2, RBM25, HNRNPA1, SRSF1, DHX16, CANX, HSP90AB1, RAD23B, STT3B, DNAJC5, TRAF2, HSP90AB1, IFI16, TP53BP1, PKN2, TRAF2, ANTXR1 for cells infected with influenza A – pH1N1 at 0 hr. Whereas, Spliceosome, NOD-like receptor signalling pathway, Nucleotide excision repair involving genes DDX46, DDX42, U2AF2, SF3A1, HNRNPC, RBM25, ACIN1, HNRNPA1, HNRNPU, SRSF1, HNRNPK, DHX16, TP53BP1, HSP90AA1, IFI16, PKN2, TRAF2, ANTXR1, LIG1, RAD23B, DDB2 for cells infected with influenza A – pH1N1 at 8 hr. Arrhythmogenic right ventricular cardiomyopathy, IL-17 signalling pathway, Ribosome involving genes JUP, CTNNA1, LMNA, ATP2A2, HSP90AB1, SRSF1, CEBPB, TRAF2, RPS3, RPLP1, RPLP0, RPS3A, RPS18 for cells infected with influenza A – H3N2 at 0 hr. Whereas, Spliceosome, Ribosome biogenesis in eukaryotes, Adherens junction involving genes SNRNP200, U2AF2, SF3A1, RBM17, HNRNPC, RBM25, HNRNPA1, SRSF1, USP39, HNRNPK, DKC1, MDN1, TCOF1, XRN2, NOP56, WDR75, UTP18, CTNNA1, SMAD3, CTNND1, EGFR, TJP1 for cells infected with influenza A – H3N2 at 8 hr respectively.

8.3. Phosphoproteins Analysis:

Among all the pathways the genes encoding phosphoproteins which were found to be majorly expressed during the infection state were found to be Heat-shock proteins (HSPs), TNF

receptor-associated factor 2 (TRAF2), Serine/arginine-rich splicing factor 1 (SRSF1), Heterogeneous nuclear ribonucleoprotein K – HNRPK, RNA-binding protein (RBM25), Ribosomal Protein Lateral Stalk Subunit P1 (RPLP1), Small Nuclear Ribonucleoprotein U5 Subunit 200 (SNRNP200), DEAH-Box Helicase 16 (DHX16), etc. All these genes encoding the majorly expressed phosphoproteins are discussed in details below.

8.3.1. Heat-shock proteins (HSPs)

HSP90 gene was found to be differentially expressed in the cells infected with all the three strains at 0 hpi as well 8 hpi except for H3N2 at 8 hpi. As molecular chaperones, stress proteins (SPs), which also include heat-shock proteins (HSPs), RNA chaperones, and ER-associated stress proteins, are essential for the preservation of cellular equilibrium. HSPs have several important activities, some of which include preventing cells from being damaged by damaging stress, assisting in the correct folding of polypeptides that have been misfolded or unfolded, and enabling the presentation of immune and inflammatory cytokines. (118) On the other hand, they may also serve a dual purpose, since they may work along with a variety of viruses and cancer cells to ensure their continued existence. (119,120)

Hsp90 is a chaperone that is extremely prevalent and can be found in all eukaryotic cells. It plays an essential part in the regulation of significant signalling pathways that are located inside these cells. It is an ATP-dependent chaperone that comes in a variety of isoforms, some of which are found in the cytoplasm, such as Hsp90, Hsp90-A2, and Hsp90. Other isoforms include Grp94, which is found in the endoplasmic reticulum (ER), and TRAP1, which is found in the mitochondria. (120) Humans have the highest frequency of Hsp90 and Hsp90, among these heat shock proteins. An ATPase-dependent hydrolytic domain is located in the N-terminal area of Hsp90, a middle linker region is located in the middle of the protein, and a dimerization domain is located in the C-terminal portion of the protein. Hsp90, much like other heat shock proteins, attaches to non-native substrate peptides in order to stop the aggregation and destruction of those peptides. When Hsp90 attaches to ATP, the N-terminal domain briefly transforms into a dimer state so that it can perform its function. After the dimerization process has been finished, the substrate peptide will be able to attach to Hsp90 thanks to this. The subsequent hydrolysis of ATP, which results in the release of energy, produces a conformational shift in the N-terminal domain. This change makes it simpler for the substrate to fold into the correct orientation. (118,121)

Hsp90 is engaged in several different cellular processes in addition to its job as a chaperone. These processes include the maintenance of telomeres, apoptosis, and the advancement of the cell cycle. (122) Importantly, Hsp90 has connections with the assembly of RNA polymerase and the nuclear import of specific single-stranded RNA viruses, like the PB2 protein found in the influenza virus. In addition to this, it is necessary for the correct folding of viral capsid proteins and the assembly of viral particles during the process of viral replication. (123)

During an influenza virus infection, Hsp90 creates a complex with the viral RdRp subunits, specifically polymerase basic protein-1 (PB1) and polymerase basic protein-2 (PB2). This interaction occurs during the progression of a flu infection. Following this, each component of this complex moves into the nucleus of the cell. Hsp90 is responsible for ensuring that both PB1 and PB2 remain stable during transport in this procedure. After entering the nucleus, Hsp90 detaches from the Hsp90/PB1/PB2 complex and forms a new functional complex with polymerase acidic protein (PA). During the phase of influenza RdRp nuclear import, the acetylation state of Hsp90 is rigorously regulated by histone deacetylases 6/8 (also known as HDAC6/8), according to researchers. It has been reported that HDAC6/8 inhibitors effectively inhibit virus replication by limiting the nuclear import of the viral polymerase and, consequently, by limiting the nuclear import of the viral polymerase. (124)

The mTOR/p70S6K signalling pathway is responsible for the upregulation of Hsp90 expression that occurs as influenza infections advance. Hsp90 also plays a crucial function by binding to and stabilising influenza neuraminidase (NA), a major surface glycoprotein involved in the discharge of newly produced virus particles (virions) from infected cells. This is an important step in the process of releasing newly created virus particles from infected cells. (125) Overall, the high expression of Hsp90 in infected cells emphasizes its central role in coordinating cellular responses to viral infections. The elevated expression of Hsp90 during viral infections is likely driven by the need for increased protein folding and stabilization, as well as its role in coordinating various cellular processes to support the viral infection. Understanding the intricate interactions between Hsp90 and viral components could pave the way for developing novel antiviral strategies or therapeutic interventions.

8.3.2. TNF receptor-associated factor 2 (TRAF2)

TRAF2 gene was found to be differentially expressed in the cells infected with all the three strains at 0 hpi as well 8 hpi except for H3N2 at 8 hpi. TNF receptor-associated factor 2, also known as TRAF2, is a trimeric protein that is a member of the TNF receptor-associated factor family, often known as TRAFs. It is only in its oligomeric configuration that it is able to bind to receptors and interact with the other proteins that are part of the TNFR signalling cascade. TRAF2 has a one-to-one relationship with TNF receptors and, in conjunction with TRAF1, makes up a heterodimeric complex. The activation of MAPK8/JNK and NF-kappaB by TNF-alpha is essential for the host's immunological response; nevertheless, this complex is required for it to take place. In addition, TRAF2, in conjunction with TRAF1, interacts with proteins known as inhibitors of apoptosis (IAPs). TRAF2 functions as a modulator of anti-apoptotic signals coming from TNF receptors thanks to these connections. In order to directly reduce caspase activation and prevent apoptosis, TRAF2 can help IAPs be recruited. This is made possible by its interaction with TRADD, a signal transducer linked with apoptosis in the TNF receptor. The degradation of TRAF2 can be triggered by the apoptosis inhibitor BIRC2/c-IAP1, which also has ubiquitin ligase activity. This enhances the amount of apoptosis brought on by TNF. (126)

TRAF2 facilitates signal transduction from members of the TNF receptor superfamily, enabling it to play a crucial role in interactions between hosts and viruses. It must interact with other proteins involved in the TNFR signalling pathway in order to regulate the host's immunological response to viral infections.

8.3.3. Serine/arginine-rich splicing factor 1 (SRSF1)

The SRSF1 gene was found to be differentially expressed in A549 cells infected with all the three strains. The deregulation of SRSF has been associated to a number of diseases and conditions, including cancer, autoimmune diseases, fatty liver diseases, tauopathy, and the propagation of viral genes. Even though the most recent study on SRSFs has produced very few answers to the many concerns that have been raised, we are optimistic that further investigation into the physiological and pathological roles that SRSFs play will one day help us overcome these obstacles. Particularly, the SRSF1 gene is what's known as a proto-oncogene and it's frequently shown to be overexpressed in many kinds of cancer. Its role in the alternative splicing of oncogenes

and tumour suppressors, as well as its connection with the gene p53, are the sources of its oncogenic features. It is interesting to note that SRSF1 has also been linked to involvement in metabolic pathways such as the mTOR pathway. Changes in the way that mTOR signalling is regulated may be a factor in the development of psoriasis. It has been shown that SRSF1 may play a key part in the pathogenesis of psoriasis through its interactions with RIG-I, p53, and mTOR, which are all involved in the illness. This has led researchers to hypothesise that SRSF1 is responsible for the condition. As a consequence of this, SRSF1 has the potential to be a therapeutic target for future psoriasis treatments. (127–129)

In addition, current research has demonstrated that SRSF1 is also involved in the control of the immune system. In individuals with systemic lupus erythematosus (SLE), for example, it stimulates the synthesis of IL-2 in the T cells of these patients. In addition, the findings of our research show that SRSF1 interacts with RIG-I and RNA polymerase III, which reveals a previously unknown component of its role in the control of the immune system. This interaction plays a role in the transmission of type I interferon signals. (130)

8.3.4. Heterogeneous nuclear ribonucleoprotein K – HNRPK

HNRPK gene was found to be differentially expressed in the cells infected with all the three strains at 0 hpi as well 8 hpi except for H3N2 at 0 hpi. Heterogeneous nuclear ribonucleoproteins, also known as hnRNPs, are a varied set of RNA-binding proteins (RBPs) that have a variety of functions in nucleic acid metabolism. These functions include actions related to alternative splicing, the stabilisation of mRNA, and the regulation of transcription and translation. (131) There is evidence to suggest that hnRNP K has a role in the infection process caused by viruses. For example, one study found that the NSP1 protein of SARS-CoV-2 specifically caused hnRNP A2/B1 to move from the nucleus to the cytoplasm, which may have facilitated the processing of viral RNA. (132) During an infection with pig reproductive and respiratory syndrome virus (PRRSV), lowering the levels of endogenous HnRNP F shown to effectively inhibit the synthesis of viral RNA and nucleocapsid (N) protein. (133) This was demonstrated by a separate experiment. Based on these data, it seems likely that hnRNPs play some sort of part in the control of viral infection. However, additional research is required in order to acquire a complete knowledge of the underlying

mechanisms that are involved during different viral infections. Overall, the elevated levels of hnRNP during influenza infections highlight its critical role in facilitating viral replication. Understanding the mechanisms by which hnRNP promotes viral replication could provide valuable insights into the development of targeted antiviral therapies to combat influenza and other related viral infections.

Also, as we know that the complex called the spliceosome oversees the process of RNA splicing. It is principally made up of five ribonucleoprotein particles called snRNPs with the sequences U1, U2, U4, U5, and U6. In most cases, the connection between the spliceosome and the RNA that it is targeting is directed by other proteins that govern the amount of splicing that occurs at particular locations. The host proteins hnRNP K and NS1-BP have been shown to have a role in making it easier to remove the M segment intron, which is required to make the M2 mRNA, according to research by Tsai et al. The reduction of either hnRNP K or NS1-BP prevented the virus from replicating. As a result, the proportion of M2 to M1 mRNA decreased, which in turn complicated viral replication. (134)

The study's findings also showed that hnRNP K and NS1-BP both govern the splicing of M RNA through a process that is reliant on nuclear speckles. NS1-BP is absolutely necessary for recruiting M1 RNA to these nuclear speckles, where it is able to promote the splicing process as well as the export of M mRNAs. On the other hand, the buildup of unspliced M1 RNA within these subnuclear structures was caused by the knockdown of hnRNP K. Our findings have led us to propose a concept in which NS1-BP is responsible for recognising M1 RNA and directing its localization to nuclear speckles. This model is based on our data. After that, hnRNP K encourages the splicing of M1 RNA into M2 within these speckles so that it can take place. (135)

8.3.5. Heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1)

It was found that HNRNPA1 gene was differentially expressed in cells infected with Influenza B and pH1N1 at 0 hpi whereas, 8 hpi for pH1N1 and H3N2. Heterogeneous ribonucleoprotein A1 (hnRNPA1) is an RNA-binding protein that plays an important part in the life cycle of a wide variety of viruses. This role is both functional and regulatory in nature. The levels of the hnRNPA1 protein can be modified in a variety of different ways by different viruses, which can influence the protein's stability,

function, and localisation within the cell. The Sindbis virus, enteroviruses, pig endemic diarrhoea virus, and rhinovirus are some of the viruses that have been demonstrated to be more susceptible to hnRNPA1's ability to boost viral replication and survival in several investigations. On the other hand, hnRNPA1 displays a protective response in the case of other viruses, such as the hepatitis C virus and the human T-cell lymphotropic virus. The fact that hnRNPA1 is involved in viral infections highlights the significance of its role as a major regulator of gene expression in both the host and the virus. Once we have a better understanding of the nature of certain viral infections and the pathogenesis that they cause, we will be better equipped to devise antiviral intervention tactics that are both novel and successful. (136)

8.3.6. RNA Binding Motif Protein 17 (RBM17)

It was found to be differentially expressed at 0 hpi and 8 hpi in cells infected with Influenza B whereas, in cells infected with H3N2 at 8 hpi. RBM17 is an RNA-binding protein that is a component of the spliceosome complex and plays an important part in the process of mRNA splicing. To be more specific, it plays a role in the second catalytic stage of mRNA splicing, during which it assembles at intron-exon junctions and enhances splicing reactions in order to eliminate introns and link exons. RBM17 was first identified as a component of the spliceosome complex; however, it also interacts with key splicing factors such as U2AF2, SF1, and SF3B1. In addition, the team has made the interesting discovery that RBM17 interacts to Ataxin-1 in a way that is reliant on polyglutamine as well as phosphorylation. Any dysfunctions in the spliceosome or other RNA-binding proteins can have important implications for human health, ranging from cancer to neurological illnesses. (137) Proper mRNA splicing is vitally regulated for accurate gene expression, and these dysfunctions can have substantial consequences for human health. (138)

8.3.7. CCAAT/Enhancer-binding protein beta (CEBPB)

It was found to be differentially expressed at 0 h.p.i. in cells infected with Influenza B and H3N2. The Cell differentiation, motility, growth arrest, proliferation, cell death, metabolism, and immunological responses are just some of the many biological processes in which CCAAT/enhancer-binding protein beta (CEBPB) plays a role as a transcription factor. CEBPB's ability to interpret varied signals and alter cellular responses accordingly demonstrates its functional plasticity but is dependent on the

particular cell type and cellular context. The activation of cyclooxygenase-2 (CEBPD) has been observed in several acute inflammatory disorders, and its role in mobilising macrophages is crucial for improving the host's defence against bacterial infections. CEBPD's effects, however, are governed by complex processes and regulatory elements that merit in-depth study. (139)

8.3.8. RNA-binding protein (RBM25)

It was found to be differentially expressed at 0 hpi in cells infected with Influenza B and H1N1 and 8 hpi for H1N1 and H3N2. RBM25 is an RNA-binding protein that modulates the alternative pre-mRNA splicing process. It regulates the expression of BCL2L isoforms, which are critical apoptotic factors, during apoptotic cell death. (140)

8.3.9. MacroH2A.1 Histone (MACROH2A1)

The gene was found at 0 hpi only in cells infected with Influenza B. The MACROH2A1 gene is responsible for the coding of a histone variation that plays an important role in the regulation of gene expression. (141) It has been shown that it plays an active role in the spatial and functional organisation of human papillomavirus (HPV) replication foci during the productive stage of human papillomavirus (HPV) infection. The histone variation macroH2A1 was found to be exclusively linked to the HPV genome and significantly enriched in viral replication foci within differentiated cells, according to a study that was recently posted on bioRxiv. Isoforms of the MacroH2A1 gene have been shown to be responsible for essential cellular processes including transcriptional repression, repair of double-strand breaks, and replication stress response. The findings of the research also indicate that macroH2A1 has a beneficial impact on viral late transcription, and the researchers argue that this effect is done by spatially segregating replication and transcription processes within the replication foci. (142)

8.3.10. Splicing Factor 3b Subunit 2 (SF3B2)

The gene was found at 0 hpi and 8 hpi in cells infected with Influenza B. The splicing factor 3b protein complex is encoded by the SF3B2 gene, which is responsible for encoding subunit 2 of the complex. This complex is essential for pre-mRNA splicing because it recognises the branch point sequence, which is a significant function, and it also facilitates the construction and activation of spliceosomes, which is another important function. (143)

8.3.11. Sequestosome 1 (SQSTM1)

The gene was found at 0 hpi only in cells infected with Influenza B. SQSTM1 is a gene that is responsible for the production of a protein that is active in selective autophagy. Protein aggregates, invasive infections, and damaged organelles are just a few examples of the cytoplasmic cargos that are broken down by the process known as selective autophagy. Researchers found that SQSTM1 directly interacts with the infectious bursal disease virus's (IBDV) dsRNA genome, facilitating the virus's degradation by autophagy in a study that was published in the journal *Viruses*. This study provides evidence that supports the hypothesis that SQSTM1-mediated selective autophagy may successfully eliminate the pathogen genome. This research also highlights the importance of autophagy as an antiviral defence mechanism during virus infections¹. As a consequence of this, SQSTM1 plays a crucial role in the elimination of viral dsRNA by means of selective autophagy. This highlights the significance of autophagy in warding off viral infections by removing the viral genome. (144,145)

8.3.12. Ribosomal Protein Lateral Stalk Subunit P1 (RPLP1)

The gene was found at 0 hpi only in cells infected with H3N2. The 60S acidic ribosomal protein P1 is an essential component of the large ribosomal subunit, and RPLP1 is the gene that is responsible for encoding this protein. In the context of an infection caused by a flavivirus, RPLP1 and RPLP2 have been discovered to be key factors in virus translation. These factors could potentially serve as a regulatory step for the process of translating particular cellular mRNAs. Based on this discovery, it seems likely that RPLP1 is involved in the regulation of viral protein synthesis that takes place during flavivirus infection. (146,147)

8.3.13. 60s

Ribosomal proteins, also known as RPs, are principally involved in the management and control of the assembly and translation of viruses. It has been discovered that some RPs are engaged in the replication or transcription of viral genomes within the host cell, despite the fact that their quantities are quite low. One of these RPs is called RPS27, and it has a zinc finger peptide (ZFP) motif that is of the C4 type. (148,149) This motif is capable of binding not just to zinc but also to other transition metal ions, such as iron or copper. Although RPS27 is not essential for the normal functioning of

ribosomes, it does play a critical part in the life cycle of a number of different viruses by managing the replication of viral nucleic acid and the transcription of viral genes. This is because RPS27 is responsible for controlling the transcription of viral genes. The removal of replication and infectivity for a wide variety of viruses, including the Influenza A virus (IVA), is brought about by the disruption of RPS27 in host cells. This does not have any negative effects on the host. (150,151)

8.3.14. Epidermal Growth Factor Receptor (EGFR)

The gene was found in cells infected by Influenza B and H3N2 at 8 hpi. Epidermal Growth Factor Receptor, more commonly referred to as EGFR, is an integral part of the process that makes it possible for virus particles to enter cells. In most cases, viruses will attach themselves to the surface of host cells and then interact with EGFR in order to facilitate viral entry, multiplication, dissemination, and evasion from the immune surveillance of the host. Mucin expression, tissue damage, and the development of carcinogenesis are all linked to the activation of EGFR signalling that is generated by viruses. These severe complications are the result of these issues. It is important to note that the significance of EGFR during an influenza illness changes significantly depending on whether or not the virus is fatal. (152)

In addition, EGFR-regulated signalling has emerged as a critical route that affects development and homeostasis, particularly in epithelial tissues. This is particularly true for epithelial tissues. As a consequence of this, it should not come as a surprise that many different viruses have developed diverse methods that take advantage of pathways regulated by EGFR in order to enter human cells and transform them into factories manufacturing viruses.(153,154)

8.3.15. Anthrax toxin receptor 1 (ANTXR1)

The gene was found in H1N1 infected cells at 0 hpi and 8 hpi. ANTXR1, also known as the anthrax toxin receptor, was demonstrated to be the cellular receptor that mediates Seneca Valley Virus (SVV) entry into cells by the use of genome-wide loss-of-function screening. (155,156) Because ANTXR1 is also known as anthrax toxin receptor 1, this discovery was made possible. Studies in vitro and in vivo have demonstrated that ANTXR1 is required for SVV permissivity. SVV has a direct and specific connection with ANTXR1, and it is this contact that is essential for SVV to have in order for it to bind to permissive cells¹. In addition, the expression of ANTXR1 is required for

infection and is sufficient for it to occur in cell lines that have a reduced expression of antiviral IFN genes to begin with. It is crucial to highlight that ANTXR1 has also been found as a potential prognostic biomarker for hepatocellular cancer caused by the hepatitis B virus. This finding was made. (156)

8.3.16. ATPase Sarcoplasmic/Endoplasmic Reticulum Ca²⁺ Transporting 2 (ATP2A2)

At 0 hpi, the ATP2A2 gene was identified in H3N2 infected cells. The gene known as ATP2A2 is responsible for the production of the enzyme known as sarco(endo)plasmic reticulum calcium-ATPase 2 (SERCA2). This enzyme, which belongs to the ATPase enzyme family, is responsible for controlling the amount of calcium ions that have a positive charge inside of cells. Both the endoplasmic reticulum and the sarcoplasmic reticulum include SERCA2. The endoplasmic reticulum is responsible for the processing and transport of proteins, while the sarcoplasmic reticulum is responsible for the storage and release of calcium ions to assist in the contraction and relaxation of muscles. Calcium ions are involved in a wide variety of cellular functions, all of which are important for the formation and operation of normal cells, and they do this through acting as key messengers. In response to signals from other cells, SERCA2 acts as a gatekeeper for calcium ions, allowing them to enter and exit the cell with ease. (157)

Emi Sato and her team of researchers have demonstrated that silencing ATP2A2 in keratinocyte-formed 3D epidermis leads to the formation of suprabasal clefts and irregular hyperkeratosis, resulting in impaired skin barrier function. They created an experimental keratinocyte-virus-epidermis (KVE) model using this barrier-dysfunctional 3D model. By infecting the keratin layer with HSV1, they examined whether boosting innate immunity could reduce HSV replication in this three-dimensional model. In addition, the researchers evaluated the efficacy of topical reagents that could help reduce HSV1 infection and mitigate for barrier dysfunction. (158)

8.3.17. SMAD Family Member 3 (SMAD3)

At 8 hpi, the gene was discovered in cells that were infected with influenza B and H3N2. SMAD3 is a gene that codes for a protein that is involved in the process of chemical signals being sent from the cell surface to the nucleus. This particular signalling route, which is referred to as the transforming growth factor-beta (TGF-)

pathway, makes it possible for outside influences to have an effect on a wide variety of cellular functions, one of which is protein synthesis. Through the TGF- signalling pathway, the SMAD3 protein can exert its influence on a variety of cellular activities, including cell growth and division (also known as proliferation), cell migration, and controlled cell death (also known as apoptosis). While pathogens can finely control SMAD signalling to aggravate disease pathology, the host can also modulate it in response to infection.

Because SMAD proteins are involved in the signalling process initiated by all ligands belonging to the TGF- superfamily, studying them presents an exciting opportunity. If we could understand how pathogens interfere with the function of SMAD, we might be able to shed light on new pathways involved in the advancement of cancer, developmental biology, and other cellular contexts in which these transcription factors play critical roles. Furthermore, because there is such a wide variety of pathogens that target SMAD signalling, this field has untapped potential for the creation of broad-spectrum antimicrobial treatments. (159)

8.3.18. Small Nuclear Ribonucleoprotein U5 Subunit 200 (SNRNP200)

It was discovered in cells infected by H3N2 8 hpi. It has been demonstrated through research that the RNA helicase SNRNP200, which is located in the spliceosome, has the ability to improve viral RNA detection and trigger the antiviral response via IRF3. Upon infection, SNRNP200 binds to viral RNA and then translocates to cytoplasmic structures that include TBK1, which makes it easier for IRF3 to get activated and for IFNB1 to be produced. This study discloses a previously unknown immunoregulatory function for the spliceosomal SNRNP200 helicase. Because it acts as both an RNA sensor and an adapter for TBK1, it helps activate the IRF3-mediated innate antiviral response. (160)

8.3.19. Catenin Alpha 1(CTNNA1)

At 8 hpi, the gene was discovered in cells infected with influenza B; for H3N2, it was detected at zero hours after infection by H3N2. Catenin Alpha 1, a member of the catenin family of proteins, is encoded by the CTNNA1 gene. Catenin Alpha 1 is an important component in cell adhesion because it links cadherins on the plasma membrane of the cell to actin filaments within the cell. The expression of genes occurs

in virtually all normal human tissues. Recent research has shown that deleting CTNNA1 expression enhances the activation of tumour necrosis factor alpha converting enzyme (TACE). This leads to an increase in the activity of transforming growth factor alpha (TGF- α), which in turn promotes cell proliferation and wound healing. CTNNA1 is an enzyme that converts tumour necrosis factor alpha into TGF- α . In addition, beta-catenin, which is one of the substrates that autophagy aims for and is encoded by the gene CTNNA1, is one of the targets of autophagy. Within the scope of this discussion, autophagy acts as a negative regulator for the pathway consisting of Yes-associated protein 1 (YAP1) and transcriptional co-activator with PDZ-binding motif (WWTR1/TAZ). In contrast, interruption of autophagy stimulates cell proliferation by activating YAP1-WWTR1/TAZ through the destruction of β -catenin. This results in increased cellular division. (161)

8.3.20. U2 Small Nuclear RNA Auxiliary Factor 2 (U2AF2)

The gene was found to be differentially expressed in cells infected with Influenza B at 8 hpi, for H1N1 at 0 hpi as well as 8 hpi and for H3N2 at 8 hpi. U2AF2 encodes U2 Small Nuclear RNA Auxiliary Factor 2, a protein that participates in pre-mRNA splicing and 3'-end processing. It recruits PRPF19 and the PRP19C/Prp19 complex/NTC/Nineteen complex to the C-terminal domain (CTD) of RNA polymerase II, thereby facilitating pre-mRNA binding and possibly linking transcription to splicing. U2AF2 defects have been linked to numerous human diseases. Certain malignancies have been found to contain U2AF2 mutations, albeit with a lower frequency than U2AF1 mutations. Significant associations exist between de novo mutations of U2AF2 and developmental delay and malformation. Purine substitutions associated with retinitis pigmentosa and neurofibromatosis have been found to reduce U2AF2 binding to RP2 and NF1 Py tracts. In autoimmune diseases such as multiple sclerosis, U2AF2 also regulates the splicing of an IL7R exon that is dysregulated. In addition, autism spectrum disorder is associated with the disruption of the association between U2AF2 and PTEN. Studies on the structure and function of U2AF2 mutations associated with these diseases provide invaluable insight into the normal functions and mechanisms underlying disease progression of the protein. As a basis for comparison with disease-associated mutations, it is essential to comprehend the protein's regular sequence specificity and adaptability. (162)

8.3.21. NOP56 Ribonucleoprotein (NOP56)

The gene was found to be active in cells infected with H3N2 after 8 hpi. The gene known as NOP56 is responsible for the encoding of nucleolar protein 56. This protein is found almost exclusively in the nucleus of nerve cells, more especially in the region of the brain known as the cerebellum, which is in charge of the coordination of movement. This protein is an essential component of the ribonucleoprotein complex, which consists of other proteins and RNA molecules. This complex is necessary for the formation of ribosomes, which are the cellular structures responsible for protein synthesis. NOP56, also known as NOL5A/SCA36, has been identified as a component of short nucleolar RNAs (snoRNAs), and its species-specific conservation is found to be exceptionally high. It participates actively in the formation of ribosomes as well as the processing of pre-RNA. As one of the essential components of box C/D small nucleolar ribonucleoprotein particles (snoRNPs), it facilitates the 2'-O-methylation of multiple nucleotides in ribosomal RNAs, tRNAs, snRNAs, and some mRNAs. NOP56 facilitates the final development and activation of the catalytic activity box C/D snoRNP effect, playing an essential role in the process. Although it has been hypothesised that non-coding RNAs, also known as ncRNAs, may play a role in the packaging of virions, the precise roles of NOP56 in this context have not been fully investigated. (163,164)

8.3.22. Protein Kinase N2 (PKN2)

PKN2 was found to be differentially expressed in cells infected with H1N1 at 0 hpi and 8 hpi. Serine/threonine protein kinase and Rho/Rac effector protein Protein Kinase N2 is essential for some signal transduction reactions in the cell. Protein Kinase N2 is a protein that is encoded by the PKN2 gene, and this gene is in charge of encoding Protein Kinase N2. It participates in the control of a variety of cellular processes, including cell cycle progression, the formation of the actin cytoskeleton, cell motility, cell adhesion, cancer cell invasion, and signalling for transcriptional activation.

Protein Kinase R, often known as PKR, is an additional important participant in the antiviral response. It accomplishes this by blocking the translation of mRNA, initiating apoptosis, and enhancing the reaction that is caused by interferon (IFN). PKR acts as

an antagonist against a wide variety of viruses, preventing the reproduction of RNA and DNA viruses alike. As a result of the powerful antiviral activity that PKR possesses, many viruses have developed mechanisms to defend themselves against its effects. They are able to accomplish this by utilising their own viral products or by hijacking the functions of cellular proteins in order to interfere with various stages of the PKR activation cascade. (165)

8.3.23. Ubiquitin Specific Peptidase 39 (USP39)

The gene was found to be differentially expressed in cells at 8 hpi with Influenza B and H3N2. The deubiquitinase enzyme USP39 has been linked to the immune system's ability to combat infections. via stabilising STAT1 in the type I interferon (IFN) signalling pathway, its expression affects antiviral activity via boosting innate antiviral immunity. USP39's deubiquitinase activity is essential for its capacity to control type I IFN signalling. (166)

8.3.24. DEAH-Box Helicase 16 (DHX16)

The gene was found to be differentially expressed in cells at 8 hpi with Influenza B. Aso, 0 hpi and 8 hpi for H1N1.. DHX16 is an RNA helicase that performs the function of a pattern recognition receptor. It does this by identifying specific viral RNA in order to activate RIG-I-dependent innate antiviral immune responses. It is able to recognise splicing signals within influenza A virus RNA in a unique manner, which ultimately results in efficient RIG-I-mediated antiviral reactions that are regulated by TRIM6 and unanchored polyubiquitin. In addition to this, DHX16 has the ability to detect illnesses brought on by other viruses, such as the SARS-CoV-2 virus. (167)

8.3.25. Calnexin (CANX)

The gene was found to be differentially expressed in cells at 0 hpi with H1N1. A protein called calnexin is the product of a gene called CANX, which is responsible for coding proteins. It has been associated with a number of disorders, such as the influenza virus and nephrogenic diabetes insipidus. In addition to this, it participates in particular cellular processes, such as the Early SARS-CoV-2 Infection Events and the Translation of Structural Proteins, which are both examples. (168,169)

8.3.26. Dynein Cytoplasmic 1 Light Intermediate Chain 1 (DYNC1LI1)

The gene was found to be differentially expressed in cells at 8 hpi with Influenza B. A protein that serves as a component of cytoplasmic dynein is encoded by the DYNC1LI1 gene, which is responsible for encoding the cytoplasmic dynein 1 light intermediate chain 1. The cytoplasmic dynein is a huge molecular motor that is composed of many subunits. It is responsible for carrying cellular components towards the minus ends of microtubules and for controlling the distribution of vesicular organelles. In addition to this, it is involved in a number of different processes that take place during mitosis. The development of protein complexes can be aided by DYNLL1 thanks to the contact that it has with a variety of other cellular proteins apart from the dynein complex. Because of this interaction, cellular proteins may be recruited to the Reverse Transcription Complex (RTC) of HIV-1, which may contribute to the reorganisation and/or stabilisation of the RTC. This, in turn, is expected to assist in the correct uncoating of HIV-1, which will ultimately lead to more efficient reverse transcription. (170)

8.3.27. Karyopherin Subunit Alpha 3 (KPNA3)

The gene was found to be differentially expressed in cells at 8 hpi with Influenza B. KPNA3, also known as Karyopherin Subunit Alpha 3, is a gene that is needed for nucleocytoplasmic trafficking. It is responsible for encoding a subunit of a nuclear transport receptor (NTR), which is necessary for the delivery of nuclear cargo. Nucleocytoplasmic transporters are essential components of the nuclear-cytoplasmic transport system. They play an important role in the facilitation of signal transduction, the regulation of the cell's internal and external environment, as well as the upkeep of cellular homeostasis. The process that facilitates the movement of macromolecules into and out of the nucleus depends heavily on karyopherins. In KPNA3 knockdown cells, which express low levels of KPNA3, the Porcine Circovirus Type 2 exhibits considerable viral replication, according to recent research by Lili Zhang and her coworkers. Conversely, viral replication is decreased in cells that overexpress KPNA3, which express high quantities of KPNA3. This shows that the levels of KPNA3 and viral replication are inversely correlated. Certain cellular transcription factors, like IRF3 and NF- κ B, are transported into the nucleus when a cell becomes infected with a virus, where they activate the transcription of genes that are in charge of combating the infection. (171)

8.3.28. 5'-3' Exoribonuclease 2 (XRN2)

The gene was found to be differentially expressed in cells at 8 hpi with H3N2. The 5'-3' exoribonuclease known as XRN2 plays an important role in the infection process caused by RNA viruses. According to research done at Stanford University by Cecilia D. Sedano, XRN2 destabilises the viral RNA genome to control how much RNA the Hepatitis C virus (HCV) produces. The fact that the levels of HCV RNA are recovered after XRN2 depletion in the presence of miR-122 suggests that miR-122 is no longer necessary to maintain the stability of viral RNA in the absence of XRN2. As a result, miR-122 is used by the HCV virus to build a protective oligomeric complex at the 5' end of the viral genome in order to counteract the effects that XRN2 has. XRN2 works as an efficient antiviral effector. This research shed light on an XRN2 activity that was previously unknown and highlighted its broad significance as a unique antiviral component in RNA virus infections. Its broad importance as a novel antiviral component in RNA virus infections was further underscored by the study. (172)

8.3.29. Treacle Ribosome Biogenesis Factor 1 (TCOF1)

The gene was found to be differentially expressed in cells at 8 hpi with H3N2. Nucleolar protein TCOF1, also known as Treacle, takes part in the transcription of ribosomal DNA genes by interacting with upstream binding factor (UBF). This process is known as ribosomal DNA gene transcription. There is a connection between mutations in this gene and the condition known as Treacher Collins syndrome, which is characterised by abnormal development of the skull and face. Treacle is necessary for ribosome biogenesis because it helps facilitate the recruitment of Pol I, UBF, and Nopp140 to the rDNA promoter. This is an essential step in the process. It emerges as a crucial regulator of vital cellular processes such as ribosome synthesis, mitosis, proliferation, and responses to DNA damage and apoptosis. These functions are all essential to the cell. Despite this, there are still a number of questions that remain unresolved. To begin, a significant amount of information is still lacking on the precise processes that determine the spatiotemporal regulation of TCOF1 expression. The research conducted on the transcriptional TCOF1 regulators has produced equivocal results thus far. In light of the fact that the formation of ribosomes can be regulated by non-coding RNAs, it is plausible that TCOF1 could be sensitive to control in this manner. Second, the particular processes through which TCOF1 mutations lead to the

development of Treacher Collins syndrome have not yet been fully understood, despite the fact that research has been ongoing in this area for quite some time. (173)

8.3.30. Tumor Protein P53 Binding Protein 1 (TP53BP1)

The gene was found to be differentially expressed in cells at 8 hpi with H1N1. The protein that plays a crucial role in repairing double-strand DNA breaks (DSBs) in response to DNA damage is encoded by the TP53BP1 gene, also known as Tumour Protein P53 Binding Protein 1. The homologous recombination (HR) repair protein BRCA1 is specifically inhibited while the non-homologous end joining (NHEJ) pathway is simultaneously promoted to repair double strand breaks (DSBs). Viruses can only live as long as they are inside their host cells, thus they have devised a variety of methods to exploit the machinery of their hosts' cells for their own benefit. p53 is a prominent target for viral interactions (Table 1) because it is a key cellular route that is governed by vital cellular pathways and because it is an important cellular transcription factor. It is possible for many viruses to target p53 in a variety of ways. (174)

Some viruses require functional p53 in order to proliferate, while other viruses destroy p53's function in order to do so. It is interesting to note that certain viruses regulate p53 in a stage-specific dual manner, meaning that they activate it during some stages of the viral cycle and inhibit it during other stages. As a result of this, preliminary research has indicated that p53 may be a possible therapeutic target for viral infections. As an illustration of a potential antiviral treatment, consider the activation of p53 and the induction of apoptosis that is p53-dependent. Recent developments in the treatment of head and neck squamous cell carcinoma (HNSCC) caused by HPV have involved the utilisation of 5-Azacytidine (5-aza). In host cells, 5-aza caused DNA to become less methylated, which resulted in a decrease in the production of HPV genes and a stabilisation of the p53 protein, both of which led to the induction of p53-dependent death. (175) In a similar vein, certain cancer chemotherapeutic drugs, such as 5-fluorouracil (5-FU), have been investigated as possible therapies for HIV-1 latently infected T-cells. 5-FU stabilises p53, which results in enhanced levels of apoptosis that is p53-dependent. (176)

8.3.31. Interferon Gamma Inducible Protein 16 (IFI16)

The gene was found to be differentially expressed in cells at 0 HPI and 8 hpi with H1N1. IFI16 is a nuclear DNA sensor that plays a critical part in the process of

launching the host's antiviral immunity as well as regulating the development of tumours. It does this by detecting various kinds of DNA, such as damaged nuclear DNA, double-stranded DNA (dsDNA), and single-stranded DNA (ssDNA), and then activating innate antiviral immunity and inflammasome responses. This also has the effect of reducing the development of tumours. In addition to this, it engages in cooperative interactions with a number of tumour suppressors, such as p53 and BRCA1. Pathways that cause programmed cell death are activated during an infection with the Influenza A virus (IAV). Viral infections are one type of stressor that can cause these pathways to become active. (177)

The function of the cytosolic DNA sensor IFI16 in the activation of programmed cell death pathways in IAV-infected cells was highlighted by a study led by Shalabh Mishra and his team. In order to better understand how these pathways are regulated in response to IAV infection, this was done. They came to the conclusion that IFI16 is an RNA sensor for the influenza A virus after discovering that it interacts with genomic RNA. When IFI16 is activated, IAV-infected cells are more likely to go through apoptosis and pyroptosis, as well as other types of programmed cell death. This is because IFI16 causes the production of type I and III interferons as well as pro-inflammatory cytokines, which are mediated by the STING-TBK1 and Pro-caspase-1 signalling axis. On the other hand, when IFI16 is inhibited, there is a decrease in inflammatory responses, and cell death is averted after IAV infection. These findings highlight the critical function that IFI16 plays in sensing and responding to IAV infection, as well as its significance in activating programmed cell death pathways. Additionally, these data highlight the significance of IFI16's role in activating these pathways. (178)

8.3.32. Par-3 Family Cell Polarity Regulator (PARD3)

The gene was found to be differentially expressed in cells at 8 hpi with Influenza B. PARD3 is a gene that is responsible for the transcription of a protein that is involved in processes such as asymmetrical cell division and cell polarisation. PARD3 is also known as Par-3 Family Cell Polarity Regulator. It targets the phosphatase PTEN to cell junctions, where it plays an essential part in the creation of tight junctions in epithelial cells and is implicated in the establishment of cell junctions. PARD3, a member of the PDZ family of proteins, was shown to be altered for the first time during an infection

with porcine epidemic diarrhoea virus (PEDV) according to a study that was led by Huimin Huang and carried out by his team. The study made use of quantitative membrane proteomics. PARD3, when infected with PEDV, proceeds via a pathway that is dependent on the proteasome for its destruction. The down-regulation of PARD3 can cause the tight junctions between cells to become disrupted, which can have an effect on both the apical and basolateral sides of cells. This ultimately makes PEDV infection more likely to occur. In order to guarantee their own reproduction and spread, viruses frequently interfere with the normal functioning of cells and the barriers that separate tissues by stealing proteins involved with tight junctions. According to the findings of the study, PARD3 is a new antiviral protein that protects against PEDV infection. These findings contribute to a better understanding of the underlying mechanisms that are involved in PEDV infection and provide vital insights into the role that membrane proteins play in PEDV infection. (179)

8.3.33. DEAD-Box Helicase 42 - DDX42

The gene was found to be differentially expressed in cells at 0 HPI and 8 hpi with H1N1. Natural inhibitors of HIV and other dangerous viruses, such as SARS-CoV-2 and Chikungunya virus, have been discovered, and one of them is called DDX42. DDX42 is a DEAD-box RNA helicase. Boris Bonaventure and the members of his research team have demonstrated that DDX42 takes advantage of the antiviral qualities of type 1 interferon to produce a cellular environment that is unfavourable for the multiplication of viruses. Their research reveals DDX42 to be an innovative and naturally occurring antiviral inhibitor. Investigating the part that this RNA helicase plays in regulating the replication of different positive-strand RNA viruses should yield important takeaways for researchers interested in the development of antiviral tactics. (180)

8.3.34. DEAD-Box Helicase 46 (DDX46)

The gene was found to be differentially expressed in cells at 8 hpi with H1N1. In the process of pre-mRNA splicing, the RNA helicase known as DDX46, which is a member of the DEAD-box family, is an extremely important factor. It is necessary for the splicing process to occur either before or during the creation of the A complex. DEAD-box helicases, such as DDX46, are essential components for recognising and processing RNA, and they play a pivotal role in the initiation of innate immune

responses to viruses. It has been established that RNA modification plays an important part in a variety of biological processes; however, its precise function in antiviral innate immunity is still unknown. Following viral infection, the synthesis of type I interferons is inhibited thanks to DDX46, a nuclear member of the DDX helicase family, according to the findings of a study that was headed by Qingliang Zheng and her team. DDX46 is able to interact with the transcripts of important antiviral signalling molecules such as Mavs, Traf3, and Traf6. These molecules are all implicated in antiviral responses. In addition, the DEAD helicase domain of DDX46 is important for the recruitment of ALKBH5, an enzyme that is in charge of removing the RNA modification known as N6-methyladenosine (m6A). This demethylation of m6A-modified antiviral transcripts causes them to be retained in the nucleus, which prevents their translation and, as a result, inhibits the generation of interferon. Therefore, DDX46 inhibits antiviral innate responses by sequestering certain antiviral transcripts in the nucleus. This is accomplished by deleting the m6A alteration on these transcripts, which is generally necessary for their export from the nucleus and translation. (181,182)

8.3.35. Junction Plakoglobin (JUP)

The gene was found to be differentially expressed in cells at 0 hpi with H3N2. JUP is a gene that is responsible for encoding a major cytoplasmic protein. This protein is an essential component that is present in the submembranous plaques of both desmosomes and intermediate junctions. Junction Plakoglobin, another name for JUP, is another name for this protein. The presence of a distinctive repeating amino acid motif known as the armadillo repeat identifies it as a member of the catenin family. It is also responsible for the formation of particular complexes with cadherins and desmosomal cadherins. There is evidence that Naxos illness is caused by mutations in this gene. It has been demonstrated by Ke Chen and colleagues that JUP is involved in the regulation of hypoxia-inducible factor 1 (HIF2) signalling during the evolution of clear cell renal cell carcinoma (ccRCC). They believe that JUP is a good candidate for future therapeutic approaches because of the role it plays. The discovery that the expression of JUP impacts insulin signalling and glucose absorption in adipocytes was made by Florentina Negoita and the other members of her research team in a study that they carried out. In addition to this, they found that salt-inducible kinase 2, also known as SIK2, is involved in the process of regulating the production of JUP in vitro. (183,184)

8.3.36. WD Repeat Domain 75 (WDR75)

The gene was found to be differentially expressed in cells at 8 hpi with H3N2. The WDR75 gene produces the human protein WDR75. It is a component of the WDR75 protein and a member of the WD40 superfamily. There are numerous varieties of eukaryotic cells that share this WD40 domain. It is well known that it plays a role in the control of crucial cellular processes such pre-mRNA processing and cytoskeleton assembly. WDR domains are crucial parts of multiprotein complexes involved in a wide range of signalling pathways, including immune-related functions, the detection and repair of DNA damage, ubiquitin signalling and protein degradation, cell cycle regulation, epigenetic control of gene expression, and chromatin organisation. (185,186)

8.3.37. Midasin AAA ATPase 1 (MDN1)

The gene was found to be differentially expressed in cells at 8 hpi with H3N2. MDN1, also known as Midasin AAA ATPase 1, is a gene that is responsible for encoding a protein that is involved in the process of ribosomal large subunit assembly. This protein plays a function in the process. Mdn1 is a member of the AAA protein family, also known as the ATPase Associated with different Activities family. This family of proteins utilises the energy that is obtained from the hydrolysis of ATP in order to carry out mechanical functions. In contrast to the majority of AAA proteins, which organise themselves into hexameric rings made up of identical AAA domains, Mdn1 is composed of six unique AAA domains that are grouped in a single polypeptide. This structure is analogous to the one that is seen in dynein, a microtubule-based motor protein. (187)

8.3.38. Lamin A/C (LMNA)

The gene was found to be differentially expressed in cells at 0 hpi with H3N2. LMNA is a gene that is responsible for encoding nuclear lamina proteins, specifically lamin A and lamin C. It is also referred to as Lamin A/C, which is another name for this gene. These proteins serve as significant structural and mechanotransductive elements of the nuclear lamina. They are essential for maintaining nuclear morphology, stabilising chromatin architecture, controlling cell cycle progression, regulating spatial-temporal gene expression, and transmitting mechanical signals inside of cells. (188,189)

8.3.39. Minichromosome Maintenance Complex (MCM):

The replication and controlled transcription of the influenza virus genome do not solely rely on the viral components found in virions. This has led to the hypothesis that certain factors within infected cells are essential for regulating these processes. Indeed, studies have shown that the viral ribonucleoprotein (vRNP) interacts with various cellular proteins.

It has been demonstrated by Kawaguchi A and Nagata K that IREF-1/MCM plays an essential part in boosting the replication of virus genomes by increasing the stability of replicating RNA polymerases. When IREF-1/MCM is not present, replicating RNA polymerases can only make very short RNA chains that are only partially complete. The contact that IREF-1/MCM and vRNP make with the PA protein is what facilitates their interaction with one another. It has been strongly indicated, on the basis of biochemical studies, that MCM contributes to the stabilisation of replicating polymerase complexes by increasing the contact between the viral polymerase and nascent cRNA. This interaction takes place in the process of replicating polymerase. In addition, the researchers discovered that virus genome replication decreased in cells in which MCM had been knocked down. This finding suggests that IREF-1/MCM works as a host factor that regulates influenza virus genome replication. (190)

8.3.40. STRING Analysis:

The network analysis using STRING database not only unveiled known host responses to influenza virus infection but also revealed novel interactions between host cellular proteins and influenza virus proteins. Previous studies have identified host cellular responses in infected cells, such as protein sumoylation, MAPK signalling, and c-Jun N-terminal kinase-mediated signalling. (66) Additionally, less characterized protein complexes related to cell cycle, epigenetic control of gene expression and chromatin organisation, although their specific functions during influenza virus infection remain unclear. The quantitative phosphoproteome analysis uncovered cellular proteins and biological processes involved during influenza virus infection, focusing on changes at 0 hours and 8 hours post-infection

CHAPTER 6:

CONCLUSION &

FUTURE PROSPECTS

9. SUMMARY AND CONCLUSION

The present study was undertaken to assess the phosphoproteomic profile of A549 cells after infection with different strains of Influenza viruses at 0 and 8 hpi.

1. pH1N1, H3N2 and Influenza B viruses were obtained from Department of Virology, Haffkine Institute.
2. The human lung cell line (A549) was purchased from the National Centre for Cell Science (NCCS), Pune.
3. MDCK cells were grown in minimum essential medium (MEM; Gibco) supplemented with 10 % fetal bovine serum (FBS; Gibco) and 1 % antibiotics (100 U/mL penicillin G, and 100 µg/mL streptomycin) (Gibco). A549 cells were grown in F-12K medium (Gibco) containing 10 % FBS and 1% antibiotics. Cells were incubated at 37°C with 5% CO₂.
4. The virus was propagated in MDCK cells with virus growth medium consisting of incomplete growth medium containing 2µg/ml of TPCK-treated trypsin from bovine pancreas (VGM).
5. Aliquots of stock virus were stored at –80°C.
6. Infection was done on A549 cells were seeded at 2×10^4 cells/well in a 6 well plate and cultivated in F-12K growth medium until near confluence. A549 cells were infected with the virus (HA titer – 1:216). Mock infection was carried out as a control using only F-12K medium. Three replicates of virus-infected and mock-infected cultures were prepared.
7. After 4 and 24 hpi, mock and virus-infected cells were subjected for RNA extraction using Genes 2 me SpinRNA Viral RNA Extraction Kit.
8. Gene expression analysis was done using SYBR green Chemistry (Takara).
9. The gene expression of each cytokine gene was expressed as fold change in the infected cells with respect to their respective time point uninfected controls and the fold change between the infected cells at 24 hpi with respect to 4 hpi. The triplicate fold change values were subjected to t-test. Graphs were plotted out and assessed using Graph Pad Prism Software (Graph Pad Inc. v. 9)
10. It was found that influenza type A is more common and contagious than influenza type B, and that changes in the HA protein of influenza type A led to variances in the expression of genes. It is possible that this is why the cytokine response to a pH1N1 infection was so much stronger than those to H3N2 and influenza B infections.
11. When compared to the cytokine response that occurred four hours after infection, the early cytokine response that occurred 24 hrs after infection showed a considerable increase in up-

- regulation. This is probably a protective response. However, this could lead to severe effects if there is an imbalance in the production of cytokines.
12. The expression of genes belonging to the IFITM family, which are known to be involved in influenza infection, was also investigated in this work.
 13. Additionally, post-translational alterations of host cells following infection were investigated, with the goal of gaining insight into interactions between viruses and their hosts as well as identifying possible therapeutic targets. The development of effective techniques to treat influenza and other viral illnesses requires a solid understanding of the processes involved.
 14. After 0 and 8 hpi, mock and virus-infected cells were subjected for cell lysis by incubating with 2% SDS lysis buffer and subjected to repeated sonication on ice (three times with 10-sec pulse and 30% amplitude each) for protein extraction.
 15. Protein concentration of A549 cells lysed with 2% SDS lysis buffer was quantified using bicinchoninic acid (BCA) assay.
 16. The protein samples were reduced and alkylated using IAA and DTT.
 17. The Peptide profile of the cells at 0 and 8 hpi, before and after digestion using mass spectrometry grade trypsin was checked using SDS-PAGE.
 18. Phosphopeptide enrichment was done using SIMAC method [FeNTA method followed by TiO₂ method].
 19. Enriched phosphopeptides were then subjected to iTRAQ labelling.
 20. iTRAQ-labeled phosphopeptides were subjected to LC-MS/MS analysis.
 21. Thermo Scientific Orbitrap Fusion Tribrid mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) connected to Easy-nLC-1200 nanoflow liquid chromatography system (Thermo Scientific) was used for data acquisition.
 22. MS/MS scans were carried out at a range of 110-2000 m/z using an Orbitrap mass analyzer at a resolution of 50,000 at 200 m/z. The maximum injection time was 200 ms.
 23. The proteomic database search was performed against the Homo sapiens proteome database (RefSeq version 108). Protein sequences were theoretically digested using protease trypsin with a maximum of 2 missed cleavages and searched against the LC-MS/MS generated peptide precursor and fragment ions (b and y ions) with 10 ppm and 0.05 Da peptide and fragment mass tolerance respectively.
 24. Fold change values were computed, and heat maps were generated using the Multiple Array viewer.
 25. Gene Ontology analysis was performed using the DAVID 8.6 functional annotation tool to classify and categorize the proteins.

26. Pathway analysis was conducted using the KEGG, to further understand the pathways associated with the identified proteins.
27. The proteins were classified based on their functions by referring to data from PANTHER database.
28. The identified genes, including DDX46, DDX42, U2AF2, SF3A1, HNRNPC, RBM25, ACIN1, HNRNPA1, HNRNPU, SRSF1, HNRNPK, DHX16, TP53BP1, HSP90AA1, IFI16, PKN2, TRAF2, ANTXR1, LIG1, RAD23B, HSP90AB1, HSP90AA1, HNRNPK, and EGFR, play crucial roles in viral infection.
29. These genes have a variety of roles, including RNA processing, splicing, immunological signalling, protein stability, and intracellular trafficking, which all have an impact on viral replication as well as the host's response and the interactions between hosts and viruses.
30. There is a possibility that certain genes are engaged in the processing of viral RNA, while others take part in the antiviral response of the host, and yet others have an effect on the stability of viral proteins and cellular signalling pathways.
31. These findings highlight the significance of these genes in determining the outcomes of viral infections and suggest their potential as targets for future antiviral therapy efforts. Our understanding will improve as a result of additional research into the specific mechanisms by which these genes contribute to viral infection, and this will open the way for the development of effective therapies against viral illnesses.

10. FUTURE PROSPECTS

The present study can be continued with future objectives as follows:

1. *In vivo* studies on suitable animal models may be pursued to understand the interplay of differential gene and protein expression including the PTM profiles of the host.
2. Similar studies on nasal and other respiratory samples from human volunteers suffering from influenza may be carried out to understand the role of these host factors in the human host.
3. Based on the differential gene expression and protein expression including PTM profiles, potential prognostic and host directed therapeutic markers may be identified and developed.
4. These strategies will help us to be better prepared with host specific interventions in case of future epidemics of the influenza viruses
5. Understanding the role of such molecular events will also help in improving virus yields for vaccine manufacturing.

The majority of studies on phosphoproteomics are centred around cancer biology. Also, a relatively lesser number of studies on phosphoproteomics in viral infections are focused on profiling studies. Under such circumstances it is difficult to correlate the results of one study with another for commenting on the potential mechanism/s of action or role of certain differentially expressed proteins in the biology of the viral infection. It is imperative that more studies with targeted proteomic approaches are undertaken in virus biology to be able to utilise the data generated in such proteomic studies for biomarker discovery.

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