Petri Jalovaara

The Role of Influenza A Virus NS1 Protein C-terminus in Counteracting Antiviral Responses

Helsinki Metropolia University of Applied Sciences
Bachelor of Laboratory Sciences
Laboratory Sciences
Bachelor's Thesis
Date 26 February 2014

Metropolia

Abstract

Author	Petri Jalovaara
Title	The Role of Influenza A Virus NS1 Protein C-terminus in Counteracting Antiviral Responses
Number of Pages	40 pages
Date	26 February 2014
Degree	Bachelor of Laboratory Sciences
Degree Programme	Laboratory Sciences
Instructors	Maria Anastasina, MSc, PhD Student Tiina Soininen, PhL, Senior Lecturer

Influenza A virus (IAV) is a significant human pathogen causing annual epidemics and occasional pandemics. The virus evolves rapidly avoiding vaccine-induced immunity and virus-targeted therapeutics. Development of novel options for the control of influenza requires detailed understanding of virus-host interactions.

The viral non-structural protein NS1 is a key regulator of IAV-host cell interactions. In this thesis we studied the role of NS1 C-terminus in modulation of cellular responses to IAV infection.

In our study gene expression and phosphokinase profiling were analyzed in human macrophages infected with a wild type IAV or with three mutant IAVs expressing differently C-terminally truncated NS1 proteins.

The current study revealed that one of the truncations in the NS1 C-terminus resulted in an improved ability of the virus to inhibit transcriptional activation of antiviral genes in the infected cells. Moreover, it resulted in the activation of five cellular phosphoproteins suggesting a possible involvement of the NS1 C-terminus in regulation of signal transduction pathways in the infected cells. Based on these observations, we suggest that NS1 amino acids 202-210 possess some features that may contribute to the ability of IAV to counteract host viral responses. This amino acid stretch should be studied further.

Understanding host responses and their underlying mechanisms could facilitate discovery of better options to control IAV, to mitigate annual epidemics and to prevent future influenza pandemics.

Keywords	influenza A virus, NS1, C-terminus, antiviral responses

Metropolia

Tiivistelmä

Tekijä Otsikko Sivumäärä Aika	Petri Jalovaara Influenssa A -viruksen NS1-proteiinin C-terminuksen merkitys virusvasteen estämisessä 40 26.02.2014
Tutkinto	Laboratorioanalyytikko (AMK)
Koulutusohjelma	Laboratorioala
Ohjaajat	Maria Anastasina, FM, tohtoriopiskelija Tiina Soininen, FL

Influenssa A -virus on merkittävä ihmispatogeeni, joka aiheuttaa vuosittain epidemioita ja toisinaan pandemioita. Virus muuntautuu nopeasti harhauttaen rokotteella hankitun immuniteetin ja virusinfektion hoitoon kehitetyt menetelmät. Uusien influenssan torjuntakeinojen kehittäminen edellyttää viruksen ja sen isännän välisten vuorovaikutusten syvällistä ymmärtämistä.

Viruksen ei-rakenneproteiini NS1 on keskeinen influenssa A -viruksen ja isäntäsolun vuorovaikutuksen säätelijä. Tässä opinnäytetyössä tutkittiin NS1:n C-terminuksen merkitystä influenssa A -virusinfektion aikaansaaman soluvasteen muuntamisessa.

Tutkimuksessa geenien ilmentymistä ja fosfokinaaseja analysoitiin ihmisen makrofageissa. Makrofagit infektoitiin luonnonkantaisella influenssa A -viruksella tai jollakin kolmesta mutatoidusta influenssa A -viruksesta, joiden NS1-proteiinin C-terminusta oli lyhennetty eri tavoin.

Tutkimus osoitti, että yksi NS1-proteiinin lyhennetty C-terminus edesauttoi viruksen kykyä estää infektoitujen solujen virusvastetta ilmentävien geenien transkriptioaktiivisuus. Se aktivoi myös solun viittä fosfoproteiinia, mikä viittaa NS1:n C-terminuksen mahdolliseen osallisuuteen infektoituneiden solujen signaalitransduktioreittien säätelyssä. Näihin havaintoihin perustuen on syytä olettaa, että NS1:n aminohapoilla 202 - 210 on ominaisuuksia, jotka saattavat parantaa influenssa A -viruksen kykyä torjua isännän virusvastetta. Kyseessä olevaa aminohappojaksoa olisi hyvä tutkia jatkossa lisää.

Isännän vasteiden ja niihin sisältyvien mekanismien ymmärtäminen saattaa auttaa löytämään parempia keinoja torjua influenssa A -viruksia, lieventää kausiepidemioita ja estää tulevia influenssapandemioita.

Avainsanat	influenssa A -virus, NS1, C-terminus, virusvasteet

Table of Contents

List of figures

List of images

List of tables

Abbreviations

BSA	Bovine serum albumin
FIMM	Institute for Molecular Medicine Finland
FBS	Fetal bovine serum
HA	Hemagglutinin
IAV	Influenza A virus
IFN	Interferon
ISGs	FN-stimulated genes
LSM	Lymphocyte separation medium
M1, M2	Matrix protein 1, 2
MDCK	Madin-Darby Canine Kidney
NA	Neuraminidase
NEP	Nuclear export protein
NES	Nuclear export sequence
NoLS	Nucleolar localization sequence
NP	Nucleocapsid protein
NS	Non-structural protein
NS1	Non-structural protein 1
NS2	Non-structural protein 2
PA	Polymerase activity protein
PABPII	Poly (A) binding protein II
PB1	Polymerase binding protein 1
PB2	Polymerase binding protein 2
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PI3K	Phosphaditylinositol-3-kinase
PKR	Protein kinase R
RdRp	RNA-dependent RNA polymerase
RIG-I	Retinoic-acid inducible gene I
RT-PCR	Reverse transcription polymerase chain reaction
Vero	Kidney Epithelian Cells
VGM	Virus-growth medium
vRNA	Viral RNA
vRNP	Viral ribonucleoprotein
WSN	A/Wilson-Smith/1933 H1N1

1 Introduction

Influenza A virus (IAV) is a respiratory virus and an important globally circulating human pathogen that causes annual epidemics (up to 5 million cases of severe illness and 250 000 - 500 000 deaths) and occasional pandemics. It represents a constant health threat and causes significant economic burden. [1, 2.]

Pandemic influenza viruses cause significant mortality in humans. According to research the following three essential prerequisites are needed for a pandemic to occur: transmission of a novel viral subtype to humans, viral replication causing disease in humans and efficient human-to-human transmission of the virus. [3.] In the course of the last 400 years at least 31 pandemics have been registered and during the last one hundred years, 4 influenza viruses have caused major pandemics (Figure 1): the 1918 H1N1 virus (Spanish flu), the 1957 H2N2 virus (Asian influenza), the 1968 H3N2 virus (Hong Kong influenza) and the 2009 H1N1 virus (Swine flu). [4, 5.]

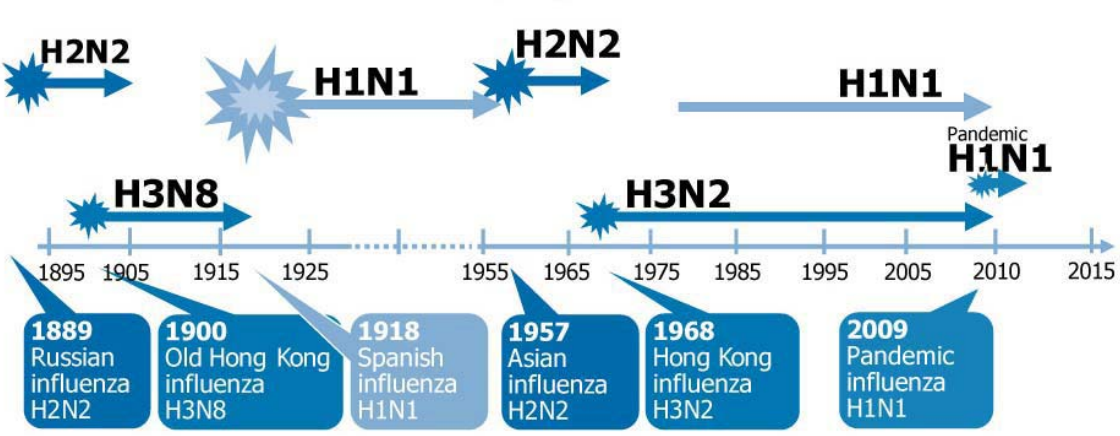


Figure 1. Recorded human influenza virus pandemics since 1885 (early subtypes inferred) [5]

The Spanish flu raged in 1918-1919 causing death of at least 50 million people mostly due to secondary bacterial pneumonias. It was most likely caused by the transmission of an avian influenza virus to humans. [3.] The global mortality caused by Asian influenza in 1957 was estimated to be 1-2 million deaths [6.] and the Hong Kong influenza resulted in 1-4 million deaths [7]. According to the World Health

Organization (WHO) 48 countries have reported severe illness and death cases of the most recent IAV pandemic (swine flu, H1N1/2009). This pandemic caused over 200 000 deaths. [8, 9.]

Recently in 2013, there have been more and more cases of influenza A (H7N9) virus registered in humans in China. This virus is one subtype among the larger group of H7 viruses, which normally circulate among birds. Even though there is so far no indication that this virus can be transmitted between people the disease is of great concern since most patients have been severely ill. [10.] Currently no vaccine is available for this influenza virus subtype [11].

These facts alone give us a compelling reason to study influenza A virus (IAV) further.

I had a little bird
And it´s name was Enza,
I opened the window,
And in-flew-Enza.

A skipping rope rhyme from 1919 [1]

2 Review of literature

2.1 Influenza A virus

Influenza A viruses belong to the family *Orthomyxoviridae* (name derives from their ability to bind sialic acid in mucoproteins). They are enveloped and usually rounded as can be seen in Figure 2 but can also be filamentous. The virions are 80-120 nm in diameter. The influenza A viruses are classified into subtypes depending upon their surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA). Up to this date 18 different HA (H1 to H18) and 11 NA subtypes (N1 to N11) have been identified. [12, 13, 14, 15.] Any HA or NA antigenic subtype pose a potential threat to public health. In humans antigenic subtypes H1, H2, H3 and N1, N2, N8, N9 have so far been detected. [10,16.]

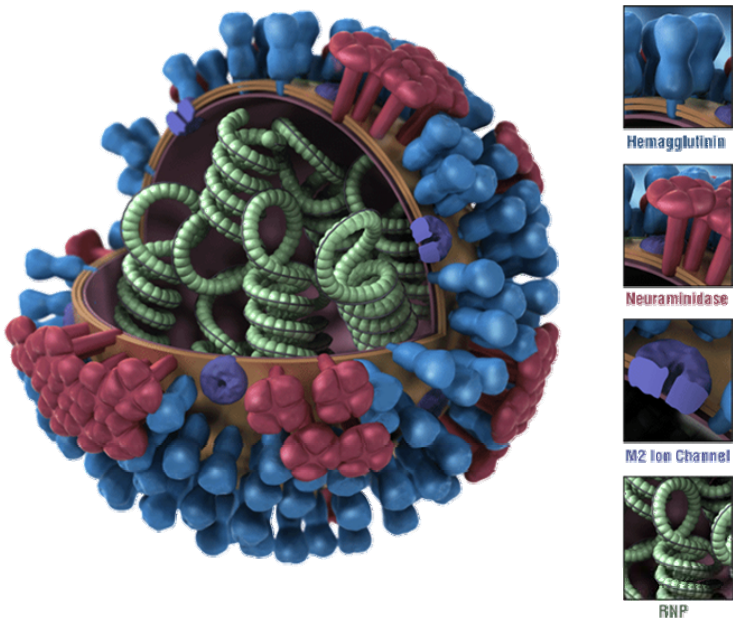


Figure 2. Influenza A virion [17]

IAV has a segmented ssRNA(-) linear genome encapsidated by nucleoprotein (NP) (Figure 2). The IAV genome contains 8 segments (Figure 3) which encode 12-14 proteins depending on strains. Segments size ranges from 890 to 2 341 nt, and the total size of the genome is 13,5 Kb. [12.]

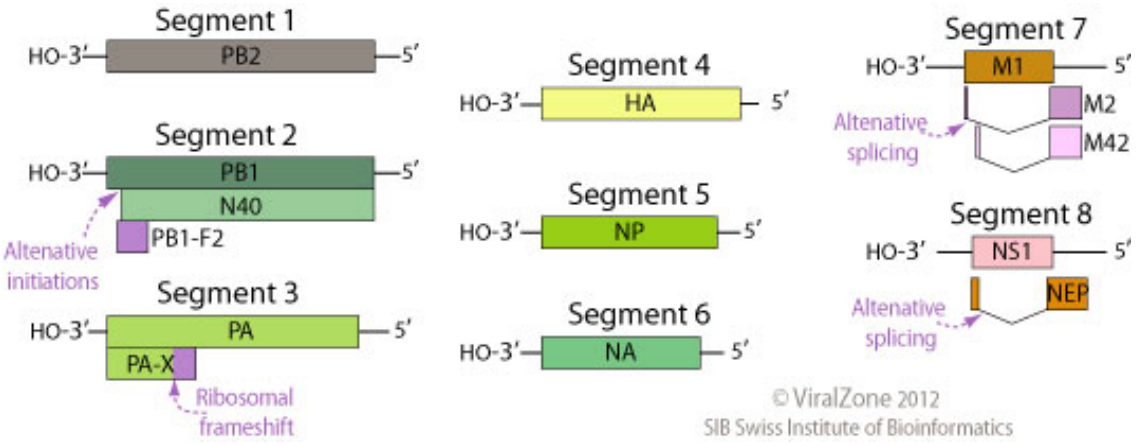


Figure 3. The genome of influenza A virus [12]

The IAV genome encodes for viral proteins as shown in Table 1: polymerase basic protein 2 (PB2), polymerase basic proteins 1-F1 (PB1-F1) and 1-F2 (PB1-F2), polymerase acidic proteins (PA and PA-X), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix 1 (M1), matrix 2 (M2), non-structural protein 1 (NS1) and non-structural protein 2 (NS2). [18.] Such novel influenza proteins as PA-N155 and PA-N182 have been identified in recent studies. According to them these proteins participate in replication processes. [19.]

Table 1. Influenza A virus gene segments and encoded proteins [20]

Influenza A virus gene segments and encoded proteins			
RNA segment	**Nucleotides**	**Protein**	**Amino acids**
1	2341	PB2	759
2	2341	PB1 PB1-F2	757 87-90
3	2230	PA PA-X	716 252
4	1778	HA	566
5	1565	NA	498
6	1413	NP	454
7	1027	M1 M2	252 97
8	875	NS1 NS2 (NEP)	230 121

Viral ribonucleoproteins (vRNP) are comprised of 6 proteins - the NP which forms viral capsids and regulates RNA synthesis and the five polymerase subunits i.e. proteins PB2 (regulation of transcription), PB1 (polymerase activity, cleavage of pre-mRNAs), PB1-F2 (enhancement of immune cell apoptosis), PA (regulation of transcription and replication) and PA-X (modulation of host antiviral response). They transcribe one mRNA from each genomic RNA segment forming ribonucleoprotein (RNP) complexes. [20.]

Hemagglutinin (HA) is a surface antigen responsible for binding the virus to the cell that is being infected (sialic acid-receptor binding) and subsequently for the penetration of viral RNA genome into the cell. The HA protein is a major antigenic determinant and induces a strong immune response. Approximately 25 % of viral proteins consist of HA. [20.]

The neuraminidase (NA) represents about 5% of viral proteins and it is the second major surface antigen playing a decisive role in late stage of infection. The NA protein removes sialic acid from sialyloligosaccharides and releases newly assembled virions from the cell surface. It also prevents the self-aggregation of virus particles. [20.]

The M1 matrix protein is the major structural component inside the virion and forms a shell around the vRNPs. The M1 protein is also considered to play a significant role during the replication and assembly of the virus and initiate the budding of the virus. [20.] The matrix protein M2 forms a proton-specific transmembrane (TM) ion channel, and it becomes active at an acidic pH. This protein plays an important role in both early and late stages of virus infection. In the beginning of infection the M2 ion channel functions between the steps of virus penetration and uncoating. [20.]

Non-structural proteins (NS1 and NS2) are encoded by the eighth gene segment of IAV genome. The NS1 is regarded to be responsible for optimal replication of the virus in the host cell. Importantly, the NS1 protein is assumed to play a crucial role in suppressing the host cell antiviral responses and inhibiting apoptosis by multiple mechanisms. The NS1 can also inhibit the nuclear export of host mRNA and the splicing of pre-mRNA. The NS2 is nowadays referred to as a nuclear export protein (NEP). It mediates the export of progeny vRNPs by acting as an adapter between the nuclear export machinery and the M1-vRNP complex. [21.]

An overview of the non-structural protein 1 (NS1) and the non-structural protein 2 (NS2) is presented in Figure 4. A more specific representation of the NS1 protein will follow later in this study.

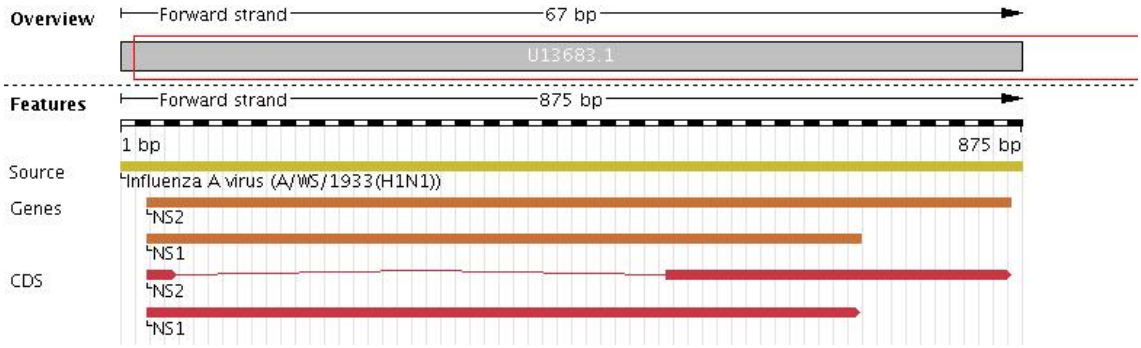
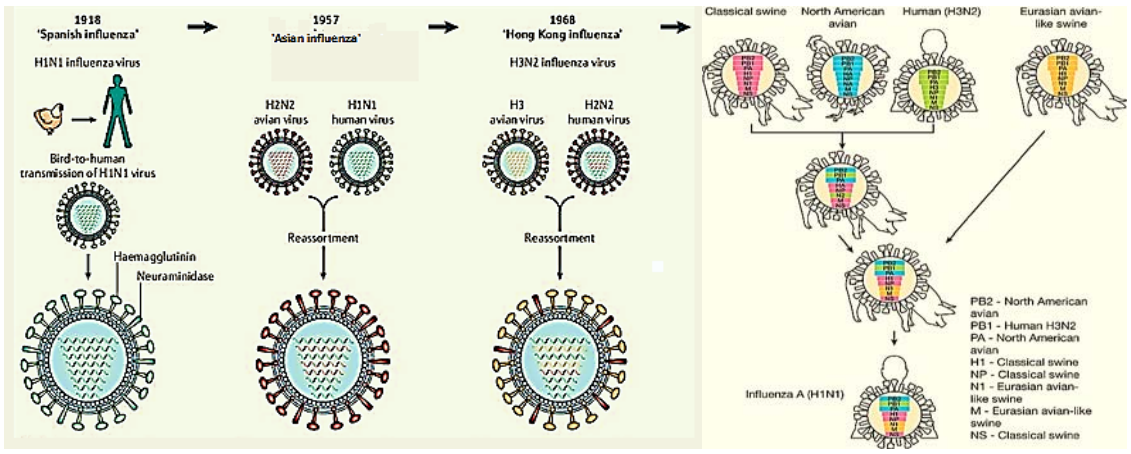


Figure 4. Influenza A virus (A/WS/1933(H1N1)) sequences of non-structural protein 1 (NS1) and non-structural protein 2 (NS2) [22]

2.1.1 Antigenic variation

All the pandemics mentioned earlier in this study began as a result of the introduction and successful adaptation of a novel hemagglutinin subtype to humans from an animal source, meaning that an antigenic shift took place. In 1918 all eight genetic segments are thought to have originated from avian influenza virus (Figure 5). In 1957, three new genetic segments from avian influenza virus were introduced (HA, NA, PB1). This subtype H2N2 virus had five segments from the 1918 virus. In 1968, two new genetic segments from H3 avian influenza virus were introduced (HA, PB1). Subtype H3N2 virus had the remaining segments from the H2N2 virus.



1918 Spanish flu A/H1N1 virus | 1957 Asian flu A/H2N2 virus | 1968 Hong Kong flu A/H3N2 virus | 2009 Swine flu A/H1N1 virus

Figure 5. Reassortment of the influenza A virus genome [24, 25]

The strain of 2009 pandemic influenza virus is a reassortant of avian, human, and swine influenza viruses (subtype H1N1). [23.] This process is illustrated in the figure on the previous page (Figure 5), which shows how a cell that is co-infected with different influenza viruses. The infected cell produces both parental viruses as well as reassortant ones which inherit some RNA segments from one strain and the remaining ones from the other strain.

Usually the RNA virus diversity is achieved by mutation. Influenza virus with a segmented genome, however, generates diversity through reassortment. In case of a cell being infected with two different influenza viruses the RNAs of both viruses are copied in the nucleus. When new virus particles are assembled at the plasma membrane, each of the 8 RNA segments may originate from either infecting virus. The progeny that inherits RNAs from both parents is called a reassortant. [23.]

The epidemiological behaviour of influenza virus in human population relates to the two types of antigenic variation of its envelope glycoproteins (HA and NA), i.e. antigenic drift and antigenic shift. Antigenic drift involves a minor change (point mutations) in the surface H-antigens resulting from an immune selective pressure. The antigenic shift means a complete change in H-antigen resulting from a replacement of the genomic RNA segment encoding surface antigens and leading to appearance of a novel potentially pandemic IAV. The new virus would be antigenically different from earlier viruses and would not have been able to evolve from them by mutation. Drifted viruses are less virulent than shifted ones. [26, 27.] These antigenic variations are shown in Figure 6.

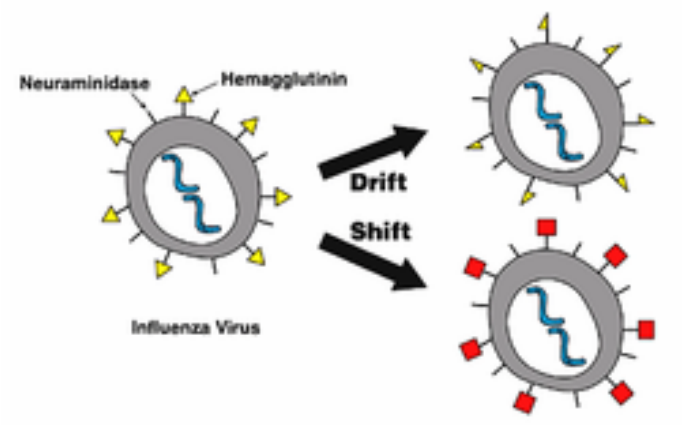


Figure 6. The two types of antigenic variation [27]

2.1.2 Life cycle of influenza A virus

There are three essential phases of replication for IAV as well as for all types of viruses – namely the initiation of infection, the replication and expression of the virus genome and the release of mature virions from the infected cell [28]. Unlike most RNA viruses, Orthomyxoviruses replicate in the cell nucleus. The influenza viral life cycle is presented in Figure 7.

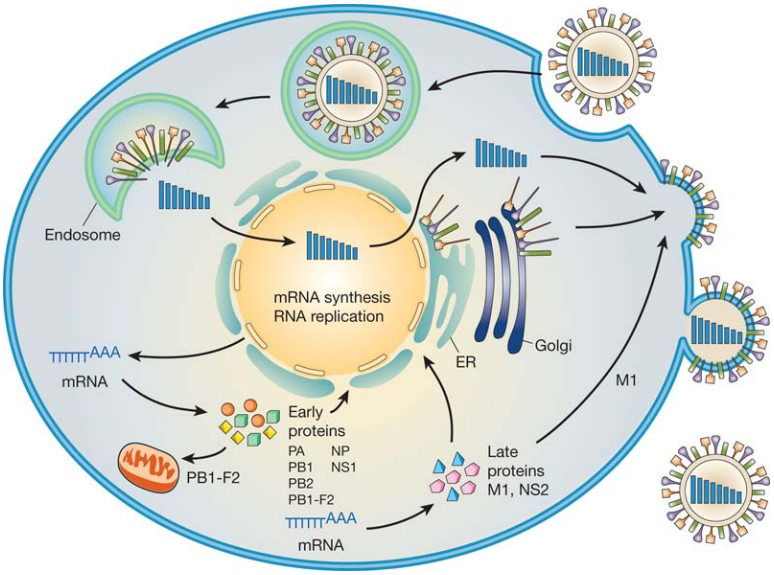


Figure 7. Schematic diagram of the influenza viral life cycle [25.]

It is typical for viruses that they are able to infect only a limited number of hosts (known as host range). The most common explanation for this is the "lock and key" mechanism meaning that a certain proteins on the virus particle must fit certain receptor sites on the particular host's cell surface. [29.]

The AIV binds to sialic acid-containing receptors on the surface of the host cell through HA protein and after endocytosis, fusion of virus membrane with the vesicle membrane and uncoating the viral genome (viral ribonucleoproteins; vRNPs) is released into the cytoplasm. Then, the vRNPs are transported to the nucleus for transcription. Viral RNA-dependent RNA polymerase (RdRp) transcribes all the genomic genes to produce mRNAs (Figure 8) and express viral proteins. Viral genomes are transcribed using fragments of the cellular mRNA as primers for mRNA synthesis initiation. The IAV is

able to efficiently shut off the host cell protein synthesis and selectively translate viral RNA. The virus "de-caps" host mRNA, making them susceptible to degradation.

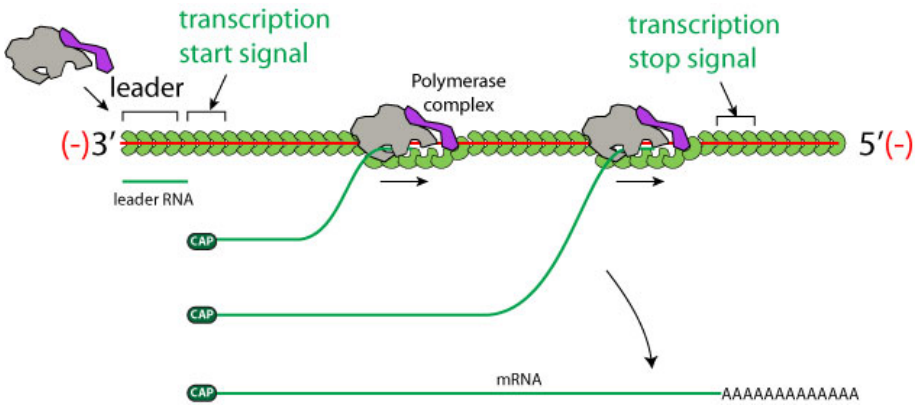


Figure 8. Negative stranded RNA virus transcription [12]

Six out of eight positive-sense viral messenger RNAs (mRNAs) are immediately exported out of the nucleus into the cytoplasm for protein synthesis (translation) by cellular ribosomes. Two mRNAs encoding non-structural and matrix proteins are further processed. The IAV exploits cellular translation for efficient production of its own protein components and enzymes. [29.]

The encapsidated vRNPs are then transported into the nucleus where viral genome replicates using the host's cellular machinery. The non-structural protein NS1 of IAV sets up multiple cellular pathways to facilitate virus replication. Viral RdRp performs genomic (-) RNA replication through antigenomic (+) RNA intermediate as presented in Figure 9. [25, 28, 30.]

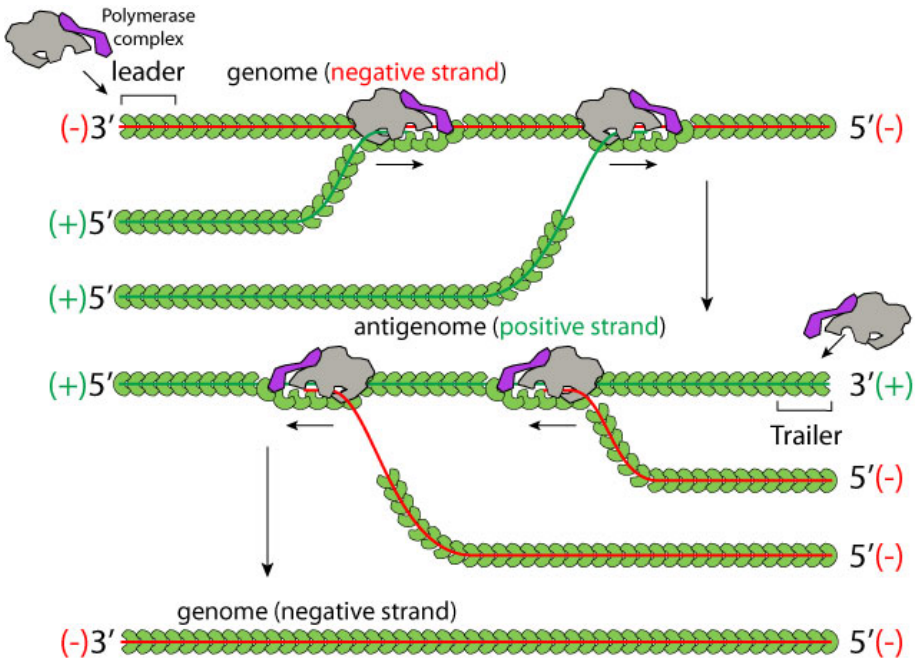


Figure 9. Negative stranded RNA virus replication [12]

Early viral proteins required for viral RNA replication and vRNP assembly (which also occur in the nucleus) are imported back into the nucleus. Late in the infection cycle, the newly synthesized vRNPs form and leave the nucleus with the assistance of M1 and NS2 (NEP) proteins. Finally, progeny virions assemble and bud at the plasma membrane. [25, 28, 30.]

2.2 Non-structural protein NS1

The viral non-structural protein NS1 is a phosphoprotein with a relatively low molecular mass (approx. 26 kDa). The NS1 is encoded by all strains of influenza A virus. It is a key regulator of virus-host interactions. It interacts with nucleic acids and multiple host proteins to limit cellular antiviral responses and thus secure effective replication of the virus. The length of NS1 proteins of human and avian IAV strains varies from 215 to 237 amino acids. The strains can differ in their primary sequences by more than 30%. This indicates specific adaptations to the respective host environment. [13, 31.] In this chapter the structure of the NS1 protein of influenza A virus is represented and the functions of this protein in virus-host interaction processes are reviewed.

2.2.1 Role of NS1 in molecular virus-host interactions

The viral NS1 has a crucial role in counteracting host cell responses in multiple ways in order to secure the production of viral particles. The NS1 protein has two major functional domains – N-terminus domain (amino acids 1 to 73) and C-terminus domain (amino acids 74 to 230) - with the help of which it may impact virulence by binding to cellular signalling proteins and interfering with the effects of several antiviral host factors. The NS1 C-terminus regulates host immune responses aiming at weakening innate and adaptive immunity by inhibiting host signal transduction and antiviral gene expression. [32.]

Innate immune responses are stimulated after the recognition of a pathogen by a pathogen-recognition receptor. The host innate interferon (IFN) response is a potent antiviral mechanism by which the host is able to limit virus replication and spread. Both RNA and DNA viruses induce IFNs by producing viral double-stranded RNA (dsRNA). The dsRNA-binding domain of NS1 interferes with host pathways during influenza infections. The infections lead to the activation of RIG-I (retinoic acid-inducible gene I). Type I interferons (such as IFN-α or IFN-β) are soluble cytokines that cells synthesize and secrete in response to virus infection and they are induced through the RIG-I pathway. The NS1 protein inhibits RIG-I-mediated innate immune responses by targeting RIG-I and blocking its signalling. In addition, NS1 targets TRIM25 (tripartite motif-containing protein 25) and interferes with caspase-1 (apoptosis-related cysteine peptidase) activation. The levels of IFNα/β mRNA are also reduced by NS1 interfering with mRNA splicing, the polyadenylation and nuclear export of cellular pre-mRNAs. [13, 25, 33, 34.]

The C-terminus domain interacts with cleavage and polyadenylation specificity factor (CPSF) and poly(A)-binding protein II (PABII) which are responsible for efficient 3’ end cleavage and polyadenylation of cellular pre-mRNAs in the nucleus. This NS1 function blocks cellular mRNA processing in IAV-infected cells yet not harming the synthesis of viral mRNA since its polyadenylation is run by a different mechanism.

NS1 suppresses the activity and function of the dsRNA-dependent PKR (cellular protein kinase R) which is encoded by IFN-stimulated genes (ISGs) by preventing it from phosphorylating eukaryotic translation elongation factor eIF-2α and allowing viral

protein synthesis to continue. NS1 binds to double-stranded RNA (dsRNA) to prevent the dsRNA-dependent activation of interferon–induced 2'-5'-oligoadenylate synthetase (OAS), and the subsequent activation of Ribonuclease L (RNasel) which is responsible for degradation of viral RNA. These cytoplasmic antiviral proteins – OAS, RNase L and PKR - are central regulators of viral transcription and translation processes, other innate defences and the host apoptosis. [13, 25, 32, 34.]

The Akt (also known as protein kinase B, PKB) and PI3K (phosphoinositide 3-kinase) play a significant role in several host cell processes, including the suppression of host apoptotic response, cell growth, transcription, proliferation and cytokine production. NS1 stimulates the PI3K/Akt pathway which is critical for efficient IAV replication, presumably by preventing premature apoptosis. The activation of this pathway occurs in the first 8 h of infection and is caused by expression of the viral NS1 protein. [13, 34.]

It has appeared that the C-terminus four amino acids of avian influenza virus NS1 proteins comprise the consensus sequence of a PDZ domain ligand (PL). PDZ domains are protein–protein recognition modules participating in organizing diverse cell-signalling assemblies. These domains have an effect on virulence, most probably through interaction with the cellular PDZ domain proteins SCRIB (scribble homolog), Dlg1 (disks large homolog 1), and membrane-associated guanylate kinase MAGI-1, -2, and -3, which regulate apoptosis. [13, 34.]

The nucleo-cytoplasmic trafficking of RNA and proteins is critical for correct cellular functions and survival. A well-known nuclear function of the IAV NS1 protein is the inhibition of the splicing and nucleo-cytoplasmic export of cellular mRNAs. In the later stage of the infection, the NS1 is present in the cytoplasm and binds to the 5'-untranslated region (5' UTR) of some viral mRNAs in order to stimulate their translation. [31, 35.]

2.2.2 Structure of NS1

Schematic representation of a 230 amino acid long NS1 protein is presented in yellow in Figure 10. The N-terminus forms a functional RNA-binding domain, while the C-terminus (effector domain) binds to distinct cellular proteins and affects their function thus predominantly mediating interactions with host-cell proteins. The last 20 amino acids of the C-terminus may be natively unstructured. NS1 contains two nuclear localization sequences (NLS1 and NLS2), and a nuclear export sequence (NES). A nucleolar localization sequence (NoLS) has been found in some strains and it is related to NLS2. NS1 interacts with nucleic acids and multiple cellular factors during infection. A number of binding sites for NS1 partners have already been identified in NS1. [13.]

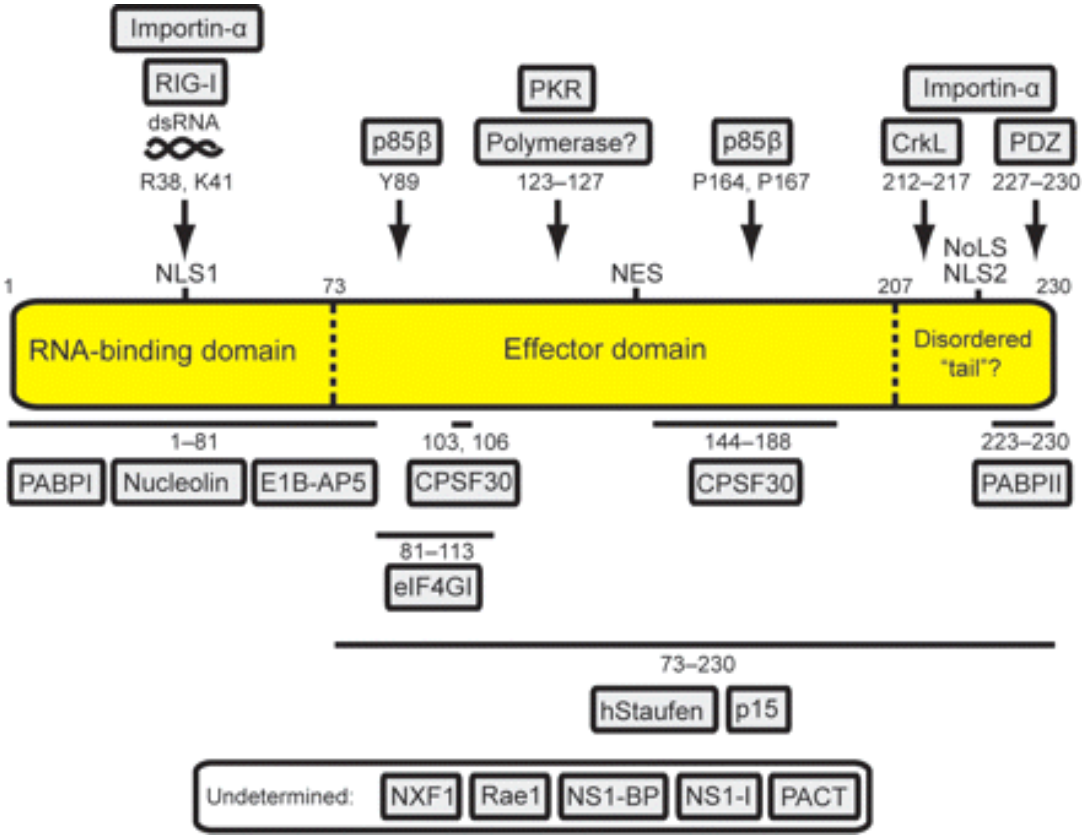


Figure 10. The structure of NS1 protein [13]

Residues Arg-38 [R38] and Lys-41 [K41] which are involved in RNA-binding are implicated in the inhibition of OAS/RNase L, Jun N-terminal kinase, and RIG-I. The NS1 protein contains also binding sites for poly(A)-binding protein I (PABPI), p85β, importin-α, nucleolin, NS1-BP, eIF4GI, hStaufen, NS1-I, PKR, PACT, CPSF30, poly(A)-

binding protein II (PABPII), Crk/CrkL, PDZ domain containing proteins, the viral polymerase, and components of the cellular mRNA nuclear export machinery (E1B-AP5, p15, NXF1, and Rae1). [13.]

The amino acids of wild type influenza A virus (strain A/Wilson-Smith/1933 H1N1) NS1 protein (total 230 a. a.) are presented in Figure 11 below.

```
        10         20         30         40         50         60
MDPNTVSSFQ VDCFLWHVRK RVADQELGDA PFLDRLRRDQ KSLRGRGSTL GLDIETATRA

        70         80         90        100        110        120
GKQIVERILK EESDEALKMT MASVPASRYL TDMTLEEMSR HWFMLMPKQK VAGPLCIRMD

       130        140        150        160        170        180
QAIMDKNIIL KANFSVILDR LETLILLRAF TEEGTIVGEI SPLPSLPGHT DEDVKNAVGV

       190        200        210        220        230
LIGGLEWNNN TVRVSETLQR FAWRSSNENG RPPLTPKQKR KMAGTIRSEV
```

Figure 11. Non-structural protein 1, influenza A virus, amino acids [36]

The development of novel options for the control of influenza requires identification of virulence determinants and detailed understanding of virus-host interactions. Naturally occurring viruses may differ from each other by a number of determinants, including the length of the NS1 protein. In previous studies clinical isolates of IAV containing C-terminus truncations of NS1 were found. The truncated region contains motifs which may be important for modulation of antiviral responses and infection progression.

3 Aim of the study

Exploring the mechanism of regulation of host responses is crucial for understanding the pathogenesis of IAV. The principal question which is addressed in this thesis is the role that the NS1 protein C-terminus presumably has in regulating virus-host interactions and counteracting cellular antiviral responses at transcriptional and post-transcriptional level. To understand how this is operated by the above mentioned viral protein terminus a research project whose material and methods will be described in the following chapter was begun.

4 Materials and methods

For this study mammalian cell lines and human primary cells were cultured. A wild-type influenza virus and mutant viruses made of it were also generated and propagated. The viruses were then sequenced and characterized. Finally gene expression and phosphokinase profiling were performed. These stages are shown in Figure 12 and described more in detail later in this chapter.

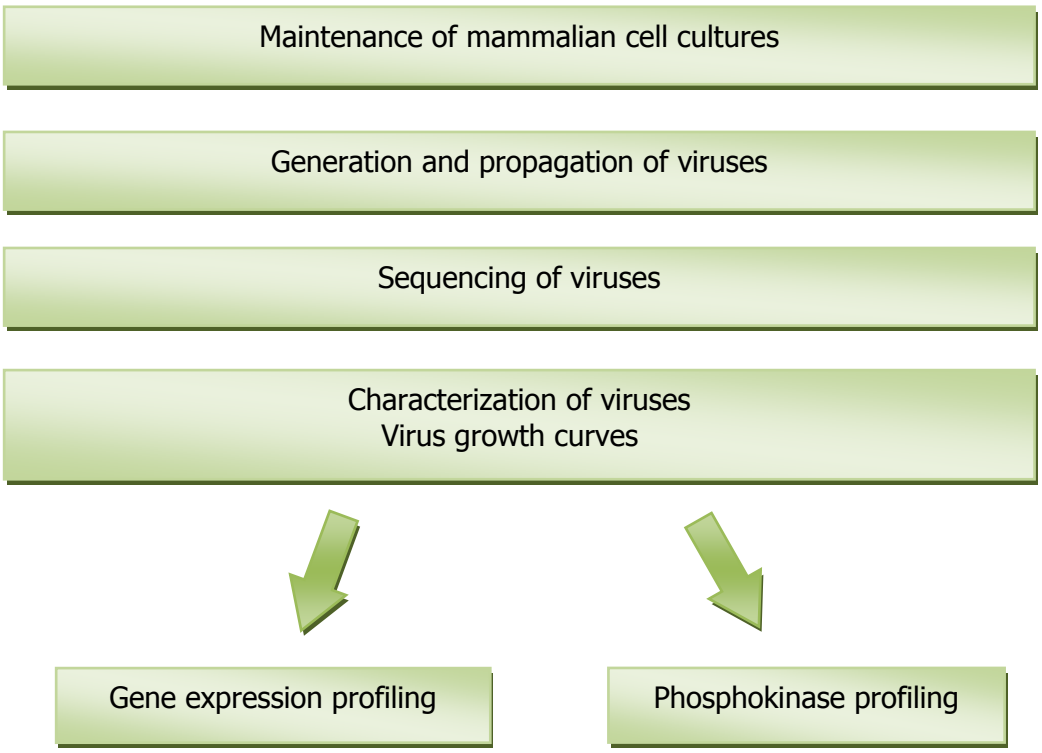


Figure 12. The stages of the study

4.1 Cell Culture

For this study the Vero and Madin-Darby canine kidney (MDCK) immortalized mammalian cell lines and primary human macrophages were used. The MDCK cells are kidney epithelial cells that were isolated from a dog in 1958 by S.H. Madin and N.B. Darby and have been widely used for influenza A virus studies for decades [37]. Vero cells are kidney epithelial cells isolated from African green monkey in the 1960s and are also widely used in virology with the advantage that these cells have abrogated

IFN alpha and IFN beta signalling allowing propagation of attenuated mutant viruses [38, 39]. To avoid possible effects of cell immortalization on IAV infection primary cells were used for critical experiments. Pneumocytes and alveolar macrophages express similar responses to IAV infection, hence human macrophages were chosen as primary cells to be used in this study.

4.1.1 Vero and MDCK

Vero and MDCK cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplied with 200 mM 2-Amino-4-carbamoylbutanoic acid (L-Glutamine, Lonza), 10% heat-inactivated fetal bovine serum (FBS, Gibco) and 50 U/ml penicillin-streptomycin mix (PenStrep, Lonza). Cells were cultured as a monolayer attached to the flask bottom in 37 °C in 5% CO_2.

Cells were regularly passaged for experimental needs and to prevent inhibitory over-growth. For this, the cultured medium was removed from a confluent flask and cells were washed twice with phosphate buffered saline (PBS, 137 mM NaCl, 2,7 mM KCl, 8 mM Na_2HPO_4, 1,46 mM KH_2PO_4, Sigma). To detach the cells, a monolayer was covered with Trypsin-EDTA solution (Lonza) pre-warmed to 37 °C. After a 5 minute incubation at 37 °C cells were detached from the flask bottom and growth medium was added to inactivate trypsin and to achieve desired dilution for passaging. A fraction of cell suspension was transferred in a new flask containing fresh medium and distributed evenly by tilting the flask several times.

4.1.2 Macrophages

During influenza viral replication in bronchial epithelial cells macrophages being one of the earliest targets to be infected are indispensable in alveolar host defense and controlling influenza virus in pulmonary organs. Macrophages were used in this study since it is presumed that pneumocytes and alveolar macrophages (lung macrophages) express similar genetic responses when IAV replicates in them. Scientifically, the macrophages may even be a better research object since they react in a "more professional" way. This is due to the fact that macrophages communicate with B- and

T-cells and the latters need information from macrophages to be able to induce an immune response.

Macrophages were differentiated from peripheral blood mononuclear cells (PBMCs) extracted from blood donated by a healthy individual. PBMCs were separated by density gradient centrifugation of blood in Lymphocyte Separation Medium (LSM, D= 1,077 g/ml, PAA Laboratories). Eight ml of heparinized blood was carefully pipetted on top of 7 ml of LSM of in 15 ml tubes and centrifuged at 1200 x g (without break) for 20 minutes. After centrifugation the PBMCs were concentrated in the interphase between the plasma and separation solution and were collected with a sterile pipette (Figure 13).

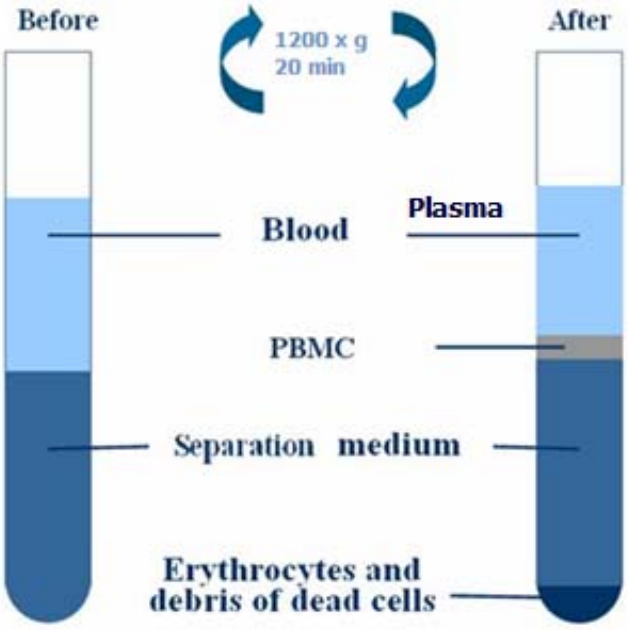


Figure 13. Extraction of PBMCs from blood

Following collection, the PBMCs were washed with PBS (PBS 3 x volume) and centrifuged at 400x g for 15 minutes. The supernatant was discarded and the cell pellets were suspended in PBS for a second wash and centrifugation at 200x g for 10 minutes. The supernatant was discarded again and the cell pellet was resuspended in 1 ml of PBS. To determine number of cells for further cultivation, cells were counted using a haemocytometer. The PBMCs were suspended in RPMI 1640 (Roswell Park Memorial Institute - 1640, Lonza) supplied with 1 M pH 7,3 N-(2-

Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES, PAA Laboratories), 200 mM L-Glutamine and 50 U/ml PenStrep and the desired amount of cells was seeded on a growth dish. Monocytes were let to attach to the dish bottom for a minimum of 1 hour after which cells were washed twice with PBS to remove unattached cells. The monocytes were differentiated into macrophages in serum-free medium (SFM, GIBCO®) supplied with 20mM L-Glutamine, 50U/ml of PenStrep and 10 ng/ml of human granulocyte macrophage-colony stimulating factor (GM-CSF, Sigma-Aldrich) for 7 days at 37°C and 5% CO_2. The growth medium was changed to a fresh one every second day. During the differentiation into macrophages the monocytes changed their size and shape.

4.2 Virus work

In this study a wild type A/Wilson-Smith/1933 H1N1 IAV strain (wtWSN further in the text) and its mutants A/WSN/33-202 (WSN-202 further in the text), A/WSN/33-210 (WSN-210 further in the text) and A/WSN/33-220 (WSN-220 further in the text) were used. The mutants were generated using reverse genetics system for A/WSN/33 IAV.

Since a gene mutation can only be made by having a gene inserted into a plasmid and there is not yet any plasmid of influenza A/H1N1/pdm09 (swine flu) available, the different mutations were made of A/WSN/33 (Figure 14, on the next page). A WSN-202 virus contains mutation W203Stop in NS1 protein (in the NS1 protein of WSN-202 a tryptophan in 203 was changed to a Stop codon resulting in a 28-amino-acid C-terminus truncation) and expresses NS1 with a C-terminus 28-amino-acid truncation. A WSN-210 virus contains mutation R211Stop in NS1 protein and expresses NS1 with a C-terminus 20-amino-acid truncation. A WSN-220 virus contains mutation K221Stop in NS1 protein and expresses NS1 with a C-terminus 10-amino-acid truncation.

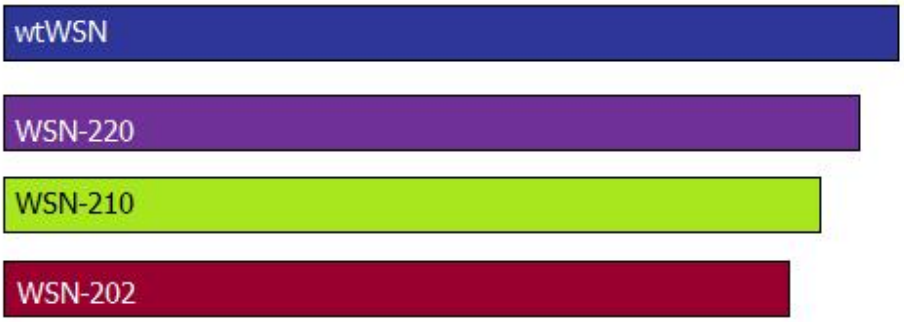


Figure 14. NS1 (wtWSN and its mutant viruses)

4.2.1 Virus infections

For infection of MDCKs and Vero cells were washed two times with PBS and the virus was added in virus-growth medium (VGM; DMEM supplied with 20 mM L-Glutamine, 50 U/ml PenStrep, 0.2% bovine serum albumin (BSA, Gibco) and 1 µg/µl N-p-Tosyl-L-phenylalanine chloromethyl ketone treated trypsin (TPCK-trypsin, Sigma-Aldrich)). For infection of macrophages, medium was discarded and the virus was added in fresh SFM supplied with 20mM L-Glutamine and 50 U/ml PenStrep. Infected cells were incubated in 37 °C and 5% CO_2.

4.2.2 Propagation of viruses

In this study wtWSN, WSN-202, WSN-210 and WSN-220 were propagated in Vero cells which are immunocompromised thus allowing replication of mutant and attenuated viruses. For virus propagation Vero cell monolayers in T175 flasks were washed twice with PBS. Next, 20 ml of VGM was added to the flasks. Following this, cells were infected with viruses at multiplicity of infection (MOI) 0,01. MOI was calculated as the ratio of infectious virus particles number to the number of cells to be infected. The infected cells were incubated until cytopathic effect (CPE; changes in cell morphology and cell death caused by infection and followed by detachment from the flask bottom) was observed. When CPE > 90% was achieved viruses were harvested as follows: VGM from infected cells was collected and at 1200x g for 10 min to pellet dead dells

and cell debris. After centrifugation the clarified supernatant was collected, aliquotted and stored at - 80 °C.

4.2.3 Virus sequencing

To ensure that propagated viruses did retain the generated mutations in the NS1 gene all virus stocks were sequenced prior to use. For sequencing total RNA was extracted from 500 µl of virus stocks using RNeasy Mini kit (Qiagen) according to manufacturer's protocol.

The extracted viral RNA was amplified for sequencing. The NS segment DNA was generated in a one-step RT-PCR using Invitrogen SuperScript™ One-Step RT-PCR system (Life Technologies), a common Uni12 oligo, 32, (5´-AGCAAAAGCAGG / 31,4 µg/OD) for first-strand cDNA synthesis and specific WSN NS1 oligos (forward: 5'-ATGGACCCAAACACTGTGTCAA / 30,7 µg/OD, reverse: 5'-TCAAACTTCTGACCTAATTG / 32,3 µg/OD) for amplification. In one-step RT-PCR all reaction components were mixed in one tube prior to starting the reactions.

Mixture (for one reaction):

Rnase-free water	1,9	µl
vRNA	10	µl
Primers	0,05	µl (each)
Buffer	12,5	µl
Enzyme mix	0,5	µl

This method offered simplicity and convenience and minimized the possibility for contamination. The reaction was set up as shown in Table 2 on the next page.

Table 2. One-step RT-PCR programme

One-step RT-PCR		Temperature	Time	
Reverse transcription		42°C	15 min	
		55°C	15 min	
		60°C	5 min	
Denaturation		94°C	2 min	
Primary amplification	Denaturation	94°C	30 sec	5 cycles
	Annealing	45°C	30 sec	
		45°C - 68°C	46 sec; 0,5°C/sec, slow ramp	
	Extension	68°C	3 min	
Secondary amplification	Denaturation	94°C	30 sec	30 cycles
	Annealing	57°C	30 sec	
	Extension	68°C	3 min	
		68°C	5 min	

To ensure that the amplification had been successful the DNA products were separated by electrophoresis in a 1 % agarose gel in tris-acetate buffer (1XTAE, 40mM Tris, 20mM acetic acid, and 1mM EDTA). For further visualization of DNA 10 mg/ml Ethidium Bromide (VWR International) was added to the gel. DNA was visualized in 260 nm UV light. The DNA concentration was measured using Nanodrop 1000 Spectrophotometer (Thermo Scientific). Prior to sequencing reaction the PCR mix was treated with exonuclease I (ExoI, Thermo Scientific) to degrade residual Uni12 oligonucleotides and thermosensitive alkaline phosphatase (FastAP, ThermoScientific) to prevent incorporation of unlabelled dNTPs during sequencing reaction. For this 10 units of ExoI and 1 unit of FastAP were added to 5 µl of PCR mix. The reaction was incubated at 37 °C for 30 minutes after which the enzymes were inactivated by incubation at 90 °C for 10 minutes. Sequencing was done by the Sanger method in a local sequencing facility using a NS1 specific primer 5'-TTCACCACTGCCCTCTCTTC.

4.2.4 Plaque assay

Determining virus titre, the quantity of infectious virus particles in 1 ml of virus stock, is critical for calculation of MOI in further experiments. In this work virus titres were determined by plaque assay for influenza. For this, 10-fold serial dilutions of virus stocks in VGM were prepared. To ensure accuracy of dilutions, tips were changed between every dilution.

MDCK cells grown in 6-well plates were used for plaque assay. Cells were washed with PBS and 200 µl of virus dilutions were added to the wells. For control, one well was left non-infected and one well was infected with a virus of known titre. The infected cells were incubated for 1 hour with tilting every 15 minutes to prevent cells from drying and ensure even virus spread. After a 1 hour incubation cells were overlaid with minimal essential medium (MEM, Gibco) supplied with 1,2% Avicel (FMC BioPolymer), 2mM L-Glutamine, 50 U/ml PenStrep, 0,2% BSA, and 1 µg/ml TPCK-trypsin to restrict the spread of new viruses to neighbouring cells (Image 1A).

After 2 days of incubation, cells were fixed by adding 4 ml of fresh 4% formaldehyde in PBS. After 3 hours of incubation at room temperature the fixative was taken away, the cells were washed twice with PBS and 1 ml of staining solution (20% ethanol (Altia Oy), 3.6% formaldehyde, 1% methanol (Sigma-Aldrich), 0.1% crystal violet (Sigma Aldrich)) was added to each well. After 5 min incubation with staining solution cells were washed with tap water. In plaque assay each infectious particle produces a circular zone of infection, which results in cell death (Image 1B).

A B

Image 1. A: Overlaid cells, B: Plaques

These infection zones are visualized as plaques – unstained circular zones in cell monolayer where the cells have been killed by viral infection. The plaques were calculated and a virus titre was determined as in formula 1:

[1] *Virus titre (pfu/ml) = [number of plaques] x [dilution coefficient] x 5*

4.2.5 Virus growth curves

To obtain virus growth curves titres of viruses produced during a multiple cycle infection of MDCK cells with wt A/WSN/33 and its mutants were calculated. For this, MDCK cells were infected with IAVs at a MOI = 0,001 for a multiple cycle infection and supernatants containing new progeny viruses were collected at 12, 24, 36, 48 and 60 hours post infection. The supernatants were clarified from dead cells and cell debris by centrifugation at 1200x g for 5 minutes and stored at -80 °C. The virus titres in supernatants were determined by plaque assays.

4.3 Gene expression profiling

In the field of molecular biology, gene expression profiling is the measurement of the activity of thousands of genes at once in order to create an overall picture of cellular function. These profiles show, for example, how the cells react to a particular treatment.

For gene expression profiling human macrophages were infected with wild type and mutant A/WSN/33 viruses at MOI = 2. After 8 hours post infection total RNA was extracted from the cells using RNeasy Mini Kit (Qiagen) according to the manufacturer's recommendations. The experiment was done in triplicates. Total RNA extracted from infected or non-infected macrophages was used to analyse gene expression using Illumina HT-12 v4 Expression BeadChip Kit according to the manufacturer's instructions. Raw microarray data were quantile-normalized using the bioconductor R package Bead Array [40] and Limma [41]. The normalized data were further processed using a variance and intensity filter. Genes differentially expressed between different samples were determined using the limma R/Bioconductor package.

The Benjamini-Hochberg multiple testing correction method was used to filter out the differentially expressed genes on the basis of a q-value threshold ($q<0.05$). The resulting data were sorted by logarithmic fold change value (log_2FC), genes with absolute $log_2FC>3$ were selected for further analysis.

4.4 Phosphokinase profiling

For phosphokinase profiling human macrophages were infected with wild type wtWSN, WSN-202, WSN-210, WSN-220 at MOI = 2. Eight hours post infection the medium was removed and cells were frozen at -80 °C. Phosphokinase profiling was carried out using Human Phospho-Kinase Array (Proteome Profiler^{tm} Antibody Arrays, R&D Systems) according to the manufacturer's instructions. Quantification of kinase production was done using ImageJ software.

5 Results

5.1 Characterization of viruses

First we characterized the mutant viruses to be used in the experiments through growth-curve analysis. To address any possible effects of introduced NS1 truncations on virus replication, we studied virus propagation in MDCK cells. For this we infected the cells with a wild type WSN and mutants expressing truncated NS1 protein - WSN-202, WSN-210 and WSN-220 with an MOI of 0,001. We then determined virus titres using plaque assays every 12, 24, 36, 48, 60 hours post infection. The data was reported as mean titres with standard deviations for three independent experiments as shown in Table 3 on the next page.

Table 3. The virus titration data

	12			24			36			48			60		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
wt WSN	1,5E+05	1,0E+05	3,0E+05	6,5E+08	7,5E+08	7,0E+08	1,0E+09	2,0E+09	2,0E+09	4,0E+08	2,5E+08	3,5E+08	1,5E+08	1,3E+08	1,0E+08
202	1,0E+04	5,0E+03	5,0E+03	2,0E+07	4,5E+07	1,5E+07	3,0E+08	5,0E+08	4,0E+08	2,0E+08	3,5E+08	2,5E+08	3,0E+08	2,0E+08	1,0E+08
210	1,5E+04	1,0E+04	2,5E+04	7,5E+07	1,5E+08	9,5E+07	5,0E+08	1,5E+09	1,9E+09	3,5E+08	5,0E+08	1,0E+09	4,0E+08	1,0E+08	3,5E+08
220	5,0E+04	4,0E+04	2,5E+04	5,0E+08	7,0E+08	5,5E+08	2,0E+09	1,5E+09	2,0E+09	4,5E+08	1,0E+09	7,5E+08	4,0E+08	2,5E+08	3,5E+08
	AVERAGE	STDEV		AVERAGE	STDEV		AVERAGE	STDEV		AVERAGE	STDEV		AVERAGE	STDEV	
wt WSN	1,8E+05	1,0E+05		7,0E+08	5E+07		1,7E+09	6E+08		3,3E+08	7,6E+07		1,3E+08	3E+07	
202	6,7E+03	2,9E+03		2,7E+07	2E+07		4,0E+08	1E+08		2,7E+08	7,6E+07		2,0E+08	1E+08	
210	1,7E+04	7,6E+03		1,1E+08	4E+07		1,3E+09	7E+08		6,2E+08	3,4E+08		2,8E+08	2E+08	
220	3,8E+04	1,3E+04		5,8E+08	1E+08		1,8E+09	3E+08		7,3E+08	2,8E+08		3,3E+08	8E+07	

At 24 hpi, when the wild-type virus had reached a titre of 7,0 x 10^8 pfu/ml, the titres of WSN-220, WSN-210 and WSN-202 were 1,2-fold, 6,3-fold and 25-fold lower, respectively.

At 36 hpi, titres of wtWSN, WSN-220 and WSN-210 were almost the same while WSN-202 lagged the first three ones by ~ 4-fold. At later stages of the observation period there were only minor differences between the viruses (Figure 15).

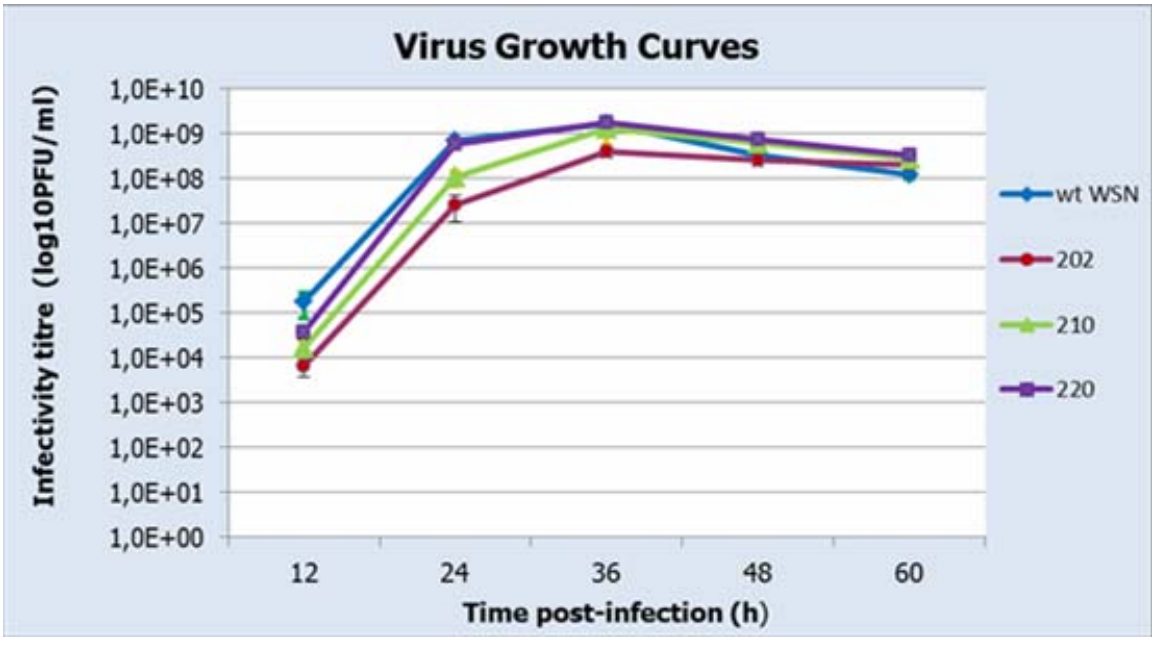


Figure 15. Virus growth curves

We observed that the growth kinetics of WSN-220 strongly resembled those of the wild type as compared to WSN-202 and WSN-210 which showed a slight delay in growth and resulted in lower titres in the first half of the observation period. However, in the latter half they reached the titres of wtWSN and WSN-220, suggesting that the mutant viruses reach approximately the same titres as wtWSN but with a delay. Still, these results reveal that the NS1 protein with a 28-amino-acid-truncated C-terminus may impair viral infectivity to some extent. The analysis of the virus growth kinetics showed that wtWSN and all the created mutants had similar growth kinetics. The virus growth characteristics were mirrored in the phenotype of plaques in the plaque assay on MDCK cells where the mutants yielded well detectable plaques with the morphology similar to wtWSN (Image 2).

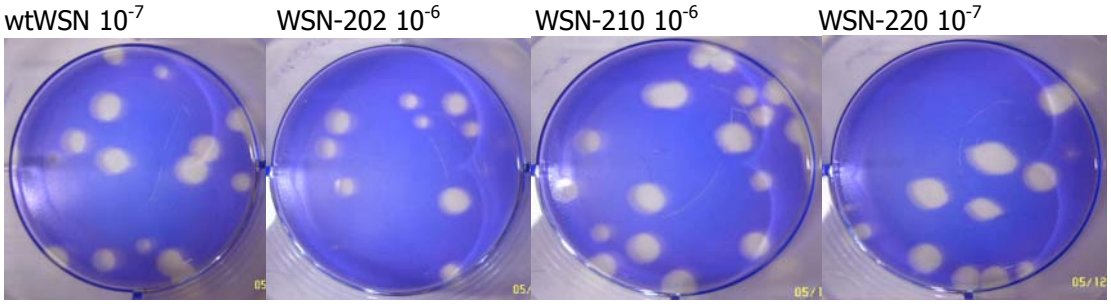


Image 2. Plaque assays 24 hpi.

5.2 Gene expression profiling

To observe whether or not the NS1 C-terminus affects cellular gene expression at transcriptional level we analysed expression of cellular genes in human macrophages infected with wtWSN, WSN-202, WSN-210 and WSN-220 viruses at 8 hours post infection. We found that the infection of macrophages with the wild type virus and the mutant viruses resulted in a strong (over 8-fold) overexpression of 74 cellular genes which are presented in a heat map (Figure 16).

We compared the effects of different viruses on gene expression and observed that a virus with a 10-amino-acid truncation in NS1 (WSN-220) affected cellular gene expression quite similarly to that of wtWSN. The viruses with larger truncations had differential effects on gene expression. The truncation of 28 amino acids in NS1 (WSN-202) resulted in stronger up-regulation of bigger number of genes which were

observed. Remarkably, the deletion of 20 amino acids (WSN-210) prevented activation of the majority of cellular genes. The number of up-regulated genes by each of the examined viruses is shown in Table 4.

Table 4. Summary of the number of up-regulated genes by each virus.

Virus	Up-regulated genes #, > 8-fold
wtWSN	51
WSN-202	74
WSN-210	5
WSN-220	52

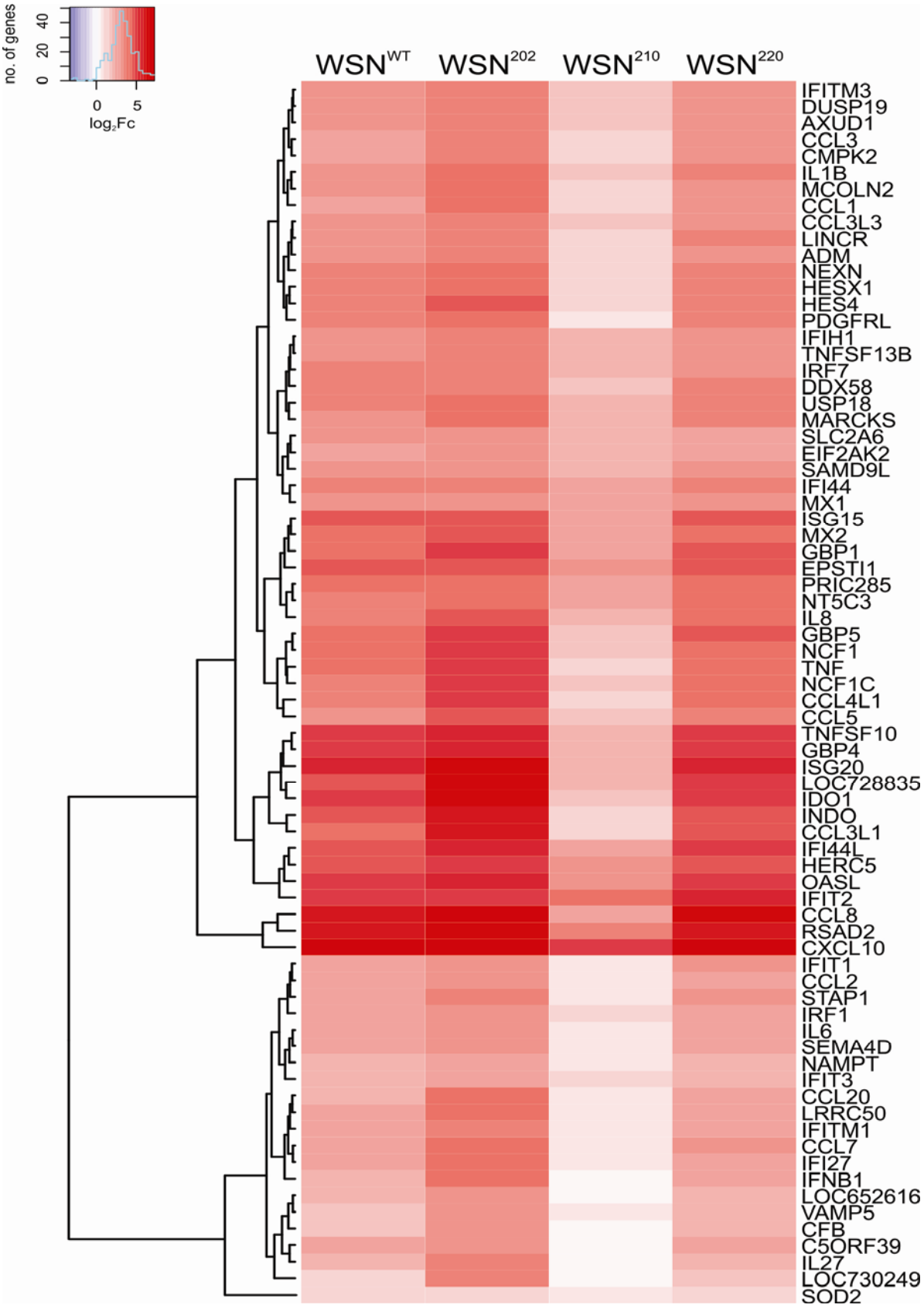


Figure 16. Heat map of log_2-transformed fold change of gene expression level between virus-infected cells and mock ($log_2FC>3$).

A heat map of gene expression values shows the effect of wild type and mutant virus infection on expression of cellular genes. The red colour indicates up-regulated expression.

We observed that most of the affected genes were related to cellular antiviral responses and were involved in, e.g. signalling in the immune system, chemokine signalling, cytokine-cytokine receptor interaction, cytokine signalling and interferon signalling.

Altogether, our results indicate that, compared to the wild type virus, a 10-amino-acid truncation in NS1 (WSN-220) does not have a noticeable effect on cellular gene expression. A truncation of 20 amino acids (WSN-210) allows the virus to suppress cellular gene expression more efficiently compared to wt. A 28-amino-acid truncation (WSN-202), in contrast, results in clearly reduced ability of the virus to inhibit cellular responses. On the basis of this finding it is plausible that some determinants in the C-terminus amino acid stretch 202-210 make the virus more efficient in counteracting host responses whereas stretches 210-220 and 220-230 bring this enhanced ability back to a normal wild type virus level.

5.3 Phosphokinase profiling

To reveal the possible mechanisms by which the C-terminus of NS1 could affect cellular antiviral responses, we studied activation of phosphokinases in human macrophages infected with wtWSN, WSN-202, WSN-210 and WSN-220 or non-infected.

Activation of phosphokinases by each virus was compared to uninfected cells. The value of each kinase in control (uninfected) cells was set to 1. Then the kinase activation levels in cells infected by wtWSN and the mutant viruses were normalized to mock-treated control cells. We observed that kinases HSP27, ERK1/2, JNK, Akt and c-JUN as shown in Figure 17 on the next page were most activated in human macrophages infected with examined viruses.

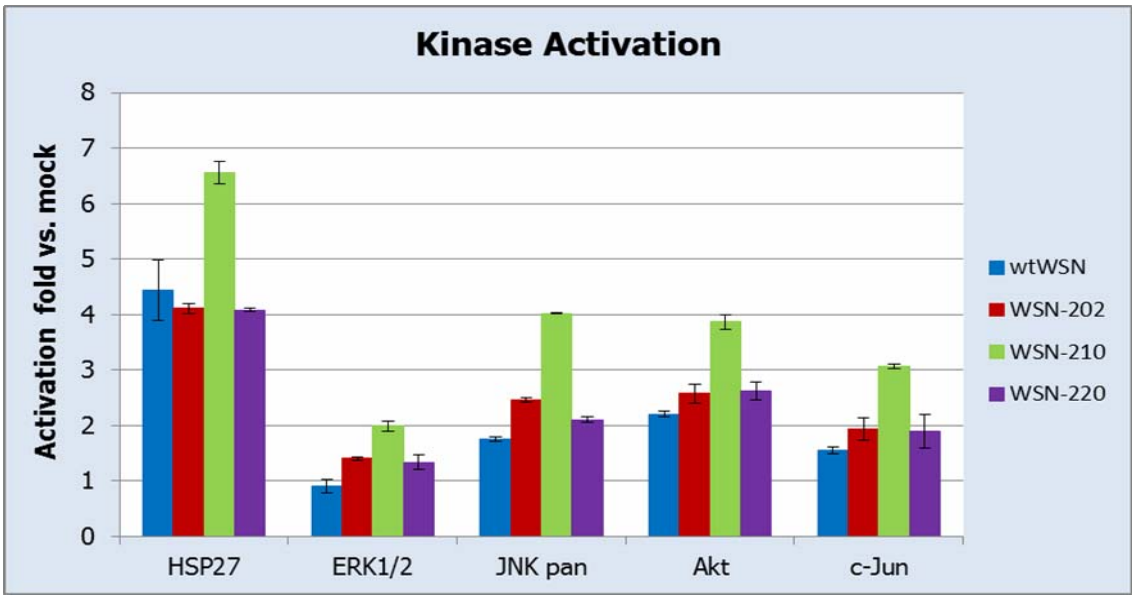


Figure 17. Kinase activation

Interestingly, whereas wtWSN, WSN-202 and WSN-220 did not have major differences in activation of kinases, WSN-210 resulted in increased phosphorylation of HSP27, ERK1/2, JNK, Akt and c-JUN. Since WSN-210 had also the strongest effect on transcription this result indicates that these effects may appear due to modulation of signaling pathways by a 210-amino-acid long NS1.

All the examined viruses activated phosphorylation of kinases - except wtWSN in relation to ERK1/2. Phosphokinase profiling results showed that WSN-210 induces strongest activation what comes to all five kinases examined (Figure 14). The mutants WSN-202 and WSN-220 induced a similar level of kinase phosphorylation compared to each other. All the mutant viruses lead to increased phosphorylation of kinases compared to wtWSN with the exception of heat shock protein HSP27.

HSP27 is a chaperone of the small heat shock protein group whose common functions are chaperone activity, thermotolerance, inhibition of apoptosis, regulation of cell development and cell differentiation [42]. The activation of HSP27 inhibiting cell apoptosis was significantly up-regulated by all the viruses. This extends viral replication which naturally assists virus propagation. Our data revealed that the WSN-210 with a 20-amino-acid truncation in NS1 induces the strongest activity of this kinase indicating that it might be able to replicate longer than the other studied viruses and thus it is more efficient in infecting new cells.

ERK1/2 (the extracellular signal-regulated kinase) and JNK (the Jun-N-terminal kinase) belong to the mitogen-activated protein kinase (MAPK) family which is activated by virus infection in mammals. MAPK is responsible for important signaling pathways regulating cell activation, differentiation, apoptosis and immune responses. ERK1/2 due to its involvement in the transportation of viral NS2 protein and nucleoprotein (NP) and JNK have a key role in virus replication and proinflammatory responses in various human cells. [43.]

ERK1/2 and JNK1/2 contribute strongly to the induction of IFN-β and subsequently to mRNA transcript levels of IFN-inducible antiviral proteins Mx which is responsible for a specific antiviral state against influenza virus infection and 2'-5'-oligoadenylate synthetase which is an antiviral enzyme counteracting viral attack by degrading viral RNA. [44.] The WSN-210 virus induces the strongest activity of these phosphokinases in macrophages indicating that it might encounter more difficulties in replicating in host cells than, for example, the wild type virus. However, this does not seem to affect the ability of WSN-210 to suppress the host cell antiviral gene expression and replicate successfully.

Three closely related serine/threonine-protein kinases (Akt1, Akt2 and Akt3) are called the Akt kinase. Akt regulates many processes which are mediated through serine and/or threonine phosphorylation of a range of downstream substrates. Akt also influences cell survival via the phosphorylation of MAP3K5 (apoptosis signal-related kinase). Phosphorylation of 'Ser-83' decreases MAP3K5 kinase activity stimulated by oxidative stress and thereby prevents apoptosis. [44.]

In several studies it was shown that the phosphatidylinositol-3-kinase (PI3K) and its downstream effector protein kinase Akt were induced upon IAV infection. The PI3K/Akt signaling is activated by various mechanisms and is required for various functions during viral infection. The early PI3K activity is required for efficient virus uptake and the later activation phase of PI3K prevents premature apoptosis. The virus-induced PI3K/Akt signaling pathway is an excellent example of a seemingly antiviral acting signaling pathway which is misused by the virus to ensure efficient replication and propagation. [45.] It appeared in our study that the Akt kinase was up-regulated in infected macrophages by 2- to 4-fold promoting efficient viral replication by supporting antiapoptotic signaling.

The c-Jun (transcription factor AP-1) kinase promotes activity of transcriptional activator NR5A1 (Nuclear receptor subfamily 5 group A member) [46]. Our results showed that this kinase activation was up-regulated 2- to 3-fold by all the viruses, WSN-210 again causing the highest activation level.

6 Discussion

Influenza A viruses have evolved various strategies to counteract multiple host antiviral defence mechanisms, often through the actions of the NS1 protein. In this study we wanted to find out the role that the NS1 C-terminus plays in the suppression of antiviral responses.

The current study revealed that the wtWSN and all its mutations WSN-202, WSN-210 and WSN-220 were able to suppress antiviral responses in host cells regardless of the length of NS1 C-terminus. Nevertheless, the virus with the shortest NS1 C-terminus seemed to bring out altogether more antiviral responses in infected cells than the other ones. The gene expression and kinase profiles of wtWSN and WSN-220 resembled each other much. The WSN-210 virus stood out from the rest clearly.

The experiments showed that WSN-210 had the strongest influence on kinase activity as well as the most effective inhibition ability in regard to antiviral gene expression. Interestingly, these findings suggest that a WSN virus induces less antiviral responses and/or is able to suppress them best having a 20-amino-acid truncation in its NS1 C-terminus in comparison with the other WSN mutations with a shorter or longer NS1 protein and wtWSN.

Based on this observation, we conclude that NS1 amino acids 211-230 may contain determinants affecting IAV capability to counteract host antiviral responses and, perhaps, pathogenicity compared to a 210-amino-acid long NS1. These may be sites targeted by host antiviral responses, for example. Again, it seems that a virus with too short a NS1 C-terminus (WSN-202) has also a weakened ability to respond to host defence mechanism. It might be worth studying further the amino acid stretch 202-210.

In summary, our data indicated that NS1 C-terminus may contribute to the suppression of cellular antiviral responses.

Ongoing molecular biology analyses deepen our understanding of virus-host interactions and support outbreak investigations of IAV. Understanding host responses and their underlying mechanisms may help us to find a way to take better control over IAV and to prevent future influenza pandemics.

Acknowledgments

This Bachelor's thesis was done at the Institute for Molecular Medicine Finland (FIMM). I would like to thank Dr. Denis Kainov for offering me the opportunity to work on this influenza project and to get acquainted with the world of virus research.

I am especially grateful to PhD student Maria Anastasina for professional guidance through the secrets of IAV, constructive feedback and endless patience during this study. I also thank my co-workers for all the support and advice. I express my gratitude to my instructor PhL Tiina Soininen for her support and valuable comments.

And last but not least, I extend my special thanks to my wife and my little daughter for understanding, supporting and encouraging me. I love you!

References

1 Brummer-Korvenkontio, M. 2007. Virusten ja prionien luonnonhistoria: Helsinki: Yliopistopaino.

2 Influenza: Overview. 2003. World Health Organization (WHO). http://www.who.int/mediacentre/factsheets/2003/fs211/en/. 28.01.2014.

3 Knobler, S. L., Mack, A., Mahmoud, A., Lemon, S. M. 2005. The Threat of Pandemic Influenza. Are We Ready? Washington (DC): National Academies Press.

4 Lazzari, S., Stohr, K. 2004. Avian influenza and influenza pandemics. Bull World Health Organization, 82(4), p. 242.

5 ECDC Risk Assessment. 2009. 2009 influenza A(H1N1) pandemic. 7th edition. European Centre for Disease Prevention and Control. Stockholm: ECDC.

6 Encyclopædia Britannica.2014. Asian flu of 1957. http://www.britannica.com./ EBchecked/topic/1663331/Asian-flu-of-1957. 05.01.2014

7 Encyclopædia Britannica. 2014. Hong Kong flu of 1968. http://www.britannica.com/ EBchecked/topic/1663333/Hong-Kong-flu-of-1968. 05.01.2014.

8 Pandemic (H1N1) 2009. 2013. World Health Organization (WHO). http://www.who.int/csr/disease/swineflu/en. 06.01.2014.

9 Simonsen, L., Spreeuwenberg, P., Lustig, R., Taylor, R. J., Fleming, D. M., et al. 2013. Global Mortality Estimates for the 2009 Influenza Pandemic from the GLaMOR Project: A Modeling Study. PLOS Medicine, 10(11), pp. 1-15.

10 Influenza at the Human-Animal Interface (HAI). 2013. World Health Organization (WHO). http://www.who.int/influenza/human_animal_interface. /influenza_h7n9/en/index.html. 15.12.2013.

11 Human infection with influenza A(H7N9) virus in China – update. 2013. World Health Organization (WHO). http://www.who.int/csr/don/2013_04_04/en/. 15.12.2013.

12 Le Mercier, P., Hulo, C., Masson, P. 2010. Influenzavirus A. Swiss Institute of Bioinformatics. http://viralzone.expasy.org/complete_by_species/6.html. 02.01.2014.

13 Hale, B. G., Randall, R. E., Ortín, J., Jackson, D. 2008. The multifunctional NS1 protein of influenza A viruses. Journal of General Virology, 89(10), pp. 2359-2376.

14 Tong, S., Zhu, X., Li, Y., Shi, M., et al. 2013. New world bats harbor diverse influenza A viruses. Public Library of Science Pathogens, 9(10):e1003657

15 Briedis, D.J. 2011. Influenza Viruses. Fundamentals of Molecular Virology. 2nd edition. Hoboken: Wiley, p. 210.

16 Shoham, D. 2011. The Modes of Evolutionary Emergence of Primal and Late Pandemic Influenza Virus Strains from Viral Reservoir in Animals: An Interdisciplinary Analysis. Cairo: Hindawi Publishing Corporation.

17 Images of the H1N1 Influenza Virus. 2010. Centers for Disease Control and Prevention. http://www.cdc.gov/h1n1flu/images.htm. 02.01.2014.

18 Samji, T. 2009. Influenza A: Understanding the Viral Life Cycle. Yale Journal of Biology and Medicine, 82(4), pp. 153–159.

19 Muramoto, Y., Noda, T., Kawakami, E., Akkina, R., Kawaoka, Y., 2013. Identification of novel influenza A virus proteins translated from PA mRNA. Journal of virology, 87(5), pp. 2455-2462.

20 Lamb, R.A., Krug, R.M. 1996. Fields Virology, Orthomyxoviridae: the viruses and their replication. Philadelphia: Lippincott-Raven.

21 Influenza NEP, NS2 Protein (Nuclear Export Protein, Nonstructural Protein 2). Sino Biological Inc. http://www.sinobiological.com/Influenza-NS2-Protein-Nuclear-Export-Protein-a-2517.html. 16.01.2014.

22 Sequence: U13683.1 2006. EMBL-EBI. http://www.ebi.ac.uk/ena/data/view/U13683. 20.01.2014.

23 Trifonov, V., Khiabanian, H., Rabadan, R. 2009. Geographic Dependence, Surveillance and Origins of the 2009 Influenza A (H1N1) Virus. New England Journal of Medicine, 361, pp. 115-119.

24 Influenza virus, Structure and Function. 2009. Yonsei University.Department of Biotech Engineering, https://web.csblab.or.kr/1106. 15.01.2014.

25 Neumann, G., Noda, T., Kawaoka, Yoshihiro. 2009. Emergence and pandemic potential of swine-origin H1N1 influenza virus. Gabriele Neumann, Takeshi Noda & Yoshihiro Kawaoka, Nature, 459, pp. 931-939.

26 Rachakonda, P. S. 2005, Studies on conformational stability of the ectodomain of influenza virus hamaglutinin. Mathematisch-Naturwissenschaftlichen Fakultät I der Humboldt-Universität zu Berlin. http://edoc.hu-berlin.de/dissertationen/rachakonda-p-sivaramakrishna-2005-02-15/HTML/chapter1.html. 16.01.2014.

27 Swine Flu: An Overview with updates. 2009. Yadav Pathology Laboratory. http://yadavpathlabbhiwani.blogspot.fi/2009_07_12_archive.html

28 Alan J. Cann, A. J. 2012. Principles of Molecular Virology, 5th edition, Waltham: Elsevier Ltd.

29 Bailey, R. 2014. Virus replication. http://biology.about.com/od/virology/ss/Virus-Replication.htm. 12.01.2014.

30 Müller, K. H., Kakkola, L., Nagaraj, A. S., Cheltsov, A. V. Maria Anastasina, M., Kainov, D. E. 2012. Emerging cellular targets for influenza antiviral agents. Trends in Pharmacological Sciences, 33(2), pp. 89-99.

31 Enami, M. 1997. Structure and function of influenza virus NS1 and NS2 proteins. Nihon Rinsho – Japanese journal of clinical medicine, 55(10), pp. 2605-9.

32 Satterly, N., Tsai, P. L., van Deursen, J., Nussenzveig D. R., Wang, Y., Faria, P. A., Levay, A., Levy, D. E., Fontoura , B. M. 2007. Influenza virus targets the mRNA export machinery and the nuclear pore complex. Proceedings of the National Academy of Sciences of the United States of America, 104(6), pp. 1853-8.

33 Skorvanova, L., Betakova, T. 2013. Cytokines and Influenza Virus. Journal of Infectious Diseases and Therapeutics, 1, pp. 25-32.

34 A comprehensive map of the influenza A virus replication cycle. 2013. BMC Systems Biology. http://www.biomedcentral.com/1752-0509/7/97. 20.01.2014.

35 Kuss, S. K., Mata, M. A., Zhang, L., Fontoura, B. M. A. 2013. Nuclear Imprisonment: Viral Strategies to Arrest Host mRNA Nuclear Export. Viruses, 5(7), pp. 1824-1849.

36 Non-structural protein 1, Influenza A virus (strain A/Wilson-Smith/1933 H1N1). 2013. http://www.uniprot.org/blast/?about=Q82506[1-230]. 18.12.2013.

37 Lugovtsev, V. Y., Melnyk, D., Weir, J. P. 2013. Heterogeneity of the MDCK Cell Line and Its Applicability for Influenza Virus Research. Public Library of Science ONE, 8(9): e75014.

38 Govorkova, E.A., Murti, G., Meignier, B., de Taisne, C., Webster, R. G. 1996. African green monkey kidney (Vero) cells provide an alternative host cell system for influenza A and B viruses. Journal of Virology, 70(80), pp. 5519–5524.

39 Randall, R. E., Goodbourn, S. 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. Journal of General Virology, 89, pp. 1–47

40 Dunning, M.J., Smith, M.L., Ritchie M. E., Tavaré, S. 2007. Beadarray: R classes and methods for Illumina bead-based data. Oxford Journals: Bioinformatics, 23(16), pp. 2183-4.

41 Smyth, G. K., Michaud, J., Scott, H. S. 2005. Use of within-array replicate spots for assessing differential expression in microarray experiments. Oxford Journals: Bioinformatics, 21(9), pp. 2067-75.

42 Rajaiya,J., Yousuf,M. A., Singh, G., Stanish, H., Chodosh, J. 2012. Heat Shock Protein 27 Mediated Signaling in Viral Infection. Biochemistry, 51(28), pp. 5695–5702.

43 Pleschka, S., Wolff, T., Ehrhardt, C., Hobom, G., Planz, O., Rapp, U. R., Ludwig, S. 2001. Influenza virus propagation is impaired byinhibition of the Raf/MEK/ERK signalling cascade. Nature Cell Biology, 3, pp. 301–305.

44 Gao, W., Sun, W., Qu, B., Cardona, C.J., Powell, K., et al. 2012. Distinct Regulation of Host Responses by ERK and JNK MAP Kinases in Swine Macrophages Infected with Pandemic (H1N1) 2009 Influenza Virus. Public Library of Science ONE, 7(1), e30328.

45 The role of the PI3K signaling pathway upon influenza virus infection in vitro and in vivo. 2013. http://zmbe.unimuenster.de/institutes/imv/ehrhardt/ehrhardtThema2.html. 05.01.2014

46 P05412 (JUN_HUMAN). Uniprot. http://www.uniprot.org/uniprot/P05412. 07.02.2014