Influenza A Virus-Host Interactions and Their Control by Viral Non-Structural Protein NS1

Maria Anastasina

Institute for Molecular Medicine Finland
and
Division of Microbiology
Department of Biosciences
Faculty of Biological and Environmental Sciences
and
Doctoral Program in Biomedicine
University of Helsinki

Academic Dissertation

To be presented for public examination with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki in the lecture hall 2402 of Biocenter 3, Viikinkaari 1 on Friday, 17th of April, 2015, at 12 o'clock noon

Helsinki 2015

Supervisors	Docent Denis Kainov, Ph. D. Institute for Molecular Medicine Finland, University of Helsinki, Finland
	Professor Sarah Butcher, Ph. D. Research Director Structural Biology and Biophysics Program, Institute of Biotechnology, University of Helsinki, Finland
Reviewers	Docent Thedi Ziegler, Ph. D. Research Center for Child Psychiatry Institute of Clinical Medicine University of Turku, Finland
	Docent Maija Vihinen-Ranta, Ph. D. Nanoscience Center, Department of Biological and Environmental Science, University of Jyväskylä, Finland
Opponent	Professor Stephan Ludwig, Ph. D. Institute of Molecular Virology Centre for Molecular Biology of Inflammation University of Münster, Germany
Custos	Professor Dennis Bamford, Ph. D. Department of Biosciences and Institute of Biotechnology University of Helsinki, Finland
Thesis Committee	Professor Kalle Saksela, Ph. D. Department of Virology, Haartman Institute University of Helsinki, Finland
	Professor Dennis Bamford, Ph. D. Department of Biosciences and Institute of Biotechnology University of Helsinki, Finland

ISBN 978-951-51-0960-6 (paperback)
ISBN 978-951-51-0961-3 (PDF)

Hansaprint Oy, Helsinki 2015

To my family

Contents

List of Original Publications

This thesis is based on the following publications and manuscripts which are referred to in the text by their roman numerals. In addition, unpublished data will be presented.

I **Anastasina M***, Le May N*, Butcher SJ, Egly JM, Kainov DE. Influenza A non-structural protein NS1 binds DNA to control host antiviral gene expression. *Manuscript.* (* = equal contribution).

II Kainov DE, Müller KH, Theisen LL, **Anastasina M**, Kaloinen M, Muller CP. Differential effects of NS1 proteins of human pandemic H1N1/2009, avian highly pathogenic H5N1, and low pathogenic H5N2 influenza A viruses on cellular pre-mRNA polyadenylation and mRNA translation. *Journal of Biological Chemistry.* 2011 Mar 4;286(9).

III **Anastasina M**, Schepens B, Saksela K, Saelens X, Kainov D. The length of C-terminus of influenza NS1 is essential for virus-host interplay. *Manuscript.*

IV **Anastasina M**, Terenin I, Butcher S, Kainov D. A technique to increase protein yield in a rabbit reticulocyte lysate translation system. *Biotechniques.* 2014. 56(1):36-9.

Candidate's independent contribution to this work:

I MA participated in design and performed experiments on DNA binding by NS1, generated recombinant viruses, did transcriptional and cytokine profiling of infected cells, did protein localization and ChIP experiments and interpreted the obtained data. NLM did *in vitro* transcription experiments and studied immune responses activation in transfected cells. MA, SB and DK wrote the manuscript

II MA did experiments addressing mRNA concentration effects on *in vitro* translation, effects of NS1 on mRNA stability and loss- and gain-of-function experiments with NS1 mutants. MA interpreted the obtained results and contributed to writing of the manuscript.

III MA generated recombinant viruses, designed and performed gene expression analysis, did cytokine and phosphoprotein profiling in infected cells and interpreted the data. MA and DK wrote the manuscript.

IV MA designed and performed experiments on cellular mRNA translation and polysome formation in rabbit lysate and interpreted the data. MA, SB, and DK wrote the manuscript.

Summary

Viruses infect all domains of life. They establish complex interactions with their host cells to subvert and hijack multiple cellular processes and warrant their own replication. Understanding virus-host interactions is critical to control spread of pathogenic viruses, develop vaccines and search for antivirals. Besides that, understanding virus-host interactions allows deciphering complex cellular processes and provides useful tools for biotechnology.

My research is dedicated to influenza A virus, an important pathogen that infects humans worldwide, represents a constant health care threat and elicits continuous efforts to control the human spread of the disease. Influenza A expresses a non-structural protein NS1 that is a key regulator of viral interactions with the host cell and an important virulence factor. Versatile functions of NS1 modulate multiple cellular functions to secure viral replication.

This work addresses several aspects of NS1-mediated modulation of core cellular processes. We discovered that NS1 binds to dsDNA and inhibits transcription of cellular genes, thus limiting antiviral responses. We found that NS1 secures general protein synthesis and mapped several residues within NS1 that are essential for this function. Further, we showed that the length of C-terminal "tail" of NS1 is essential for control of cellular antiviral responses and virus pathogenicity. The presented results increase the understanding of influenza A virus-host interactions and can be further utilized in the search for antivirals and vaccine development. In addition, this work provides a biotechnological application of influenza A NS1 protein for improvement of cell-free translation system.

Abbreviations

5'meG 5' methylated guanine

aa amino acid

AP-1 activator protein 1

CARD caspase recruitment domain

ChIP chromatin immunoprecipitation

CPSF cleavage and polyadenylation specific factor

cRNP complementary ribonucleoprotein

CTD C-terminal domain

CTL cytotoxic T lymphocyte

DC dendritic cell

ED effector domain

eIF2α translation initiation factor 2α

eIF4GI initiation factor 4GI

EMCV encephalomyocarditis virus

GCN2 general control non-derepressible kinase 2

HA hemagglutinin

HRI heme-regulated inhibitor

hStaufen human homolog of *Drosophila melanogaster* Staufen protein

IFITM3 interferon-inducible transmembrane protein 3

IFN interferon

IKK inhibitor of nuclear factor kappa-B kinase

IL interleukin

IRES internal ribosome entry site

IRF interferon regulatory factor

ISG interferon-stimulated gene

ISG15 ubiquitin-like protein ISG15

JAK Janus kinases

JNK c-Jun N-terminal kinase

K_d dissociation constant

M1 matrix protein

M2 M2 proton channel

MAPK mitogen-activated protein kinase

MAVS mitochondrial antiviral-signaling protein

MDA5 melanoma differentiation-associated protein 5

MxA myxovirus resistance gene product

NA neuraminidase

NEP nuclear export protein

NES nuclear export sequence

NFkB nuclear factor kappa-light-chain-enhancer of activated B cells

NLR NOD-like receptor

NLRP3 LRR- and pyrin domain-containing protein 3

NLS nuclear localization sequence

NP nucleoprotein

NS1 non-structural protein

OAS 2'-5'-oligoadenylate syntethase

PA polymerase acidic protein

PABP poly(A)-binding protein

PAMP pathogen-associated molecular pattern

PB1 polymerase basic protein 1

PB2 polymerase basic protein 2

PDZ postsynaptic density protein 95, *Drosophila* disc large tumor suppressor, and zonula occludens 1 protein

PERK PKR-like endoplasmic reticulum kinase

PI3K phosphoinositide-3-kinase

PKR protein kinase R

poly(A) polyadenine stretch

poly(I:C) polyinosinic:polycytidylic acid

PRR pattern recognition receptor

RBD RNA-binding domain

RdRp RNA-dependent RNA polymerase

RIG-I retinoic acid inducible gene I

RLR RIG-I-like receptor

RNAse L ribonuclease L

RRL rabbit reticulocyte lysate

SH3 Src-homology 3 domain

ssRNA single-stranded RNA

STAT signal transducer and activator of transcription

SUMO small ubiquitin-like modifier protein

TLR Toll-like receptor

UTR untranslated region

vRNA viral RNA

vRNP viral ribonucleoprotein

1 Review of the Literature

1.1 Introduction

Viruses are seemingly simple in comparison to organisms that they infect and consist of just a genome, structural proteins and sometimes a lipid bilayer envelope. Because of this simplicity they have very limited capacity to encode factors essential for their own replication. Viruses are obligate parasites of cells and have evolved multiple strategies to interact with the cell at each step of viral life cycle. For this, specific viral proteins interact with the numerous host factors and subvert cellular processes to fulfill the needs of virus replication.

The initial interaction event between the virus and a susceptible cell occurs when viral receptor-binding proteins recognize cellular proteins, carbohydrates or lipids exposed on the cell surface (Grove and Marsh, 2011). Viruses enter the cell via non-endocytic or endocytic routes, which often require interaction with cellular factors, such as clathrin-coated vesicle components (Dimitrov, 2004; Yamauchi and Helenius, 2013). Interaction with the cellular cytoskeleton is widely used by viruses to accelerate and direct their antero- and retrograde transport through the crowded intra-cellular environment (Ploubidou and Way, 2001). Uncoating and release of viral genomes is often triggered by interaction with specific cellular proteins (Suomalainen and Greber, 2013; Haywood, 2010). Furthermore, viruses that replicate their genomes in the nucleus translocate viral components there in an active way which requires binding to the proteins of nuclear pore complex (Kobiler et al., 2012).

Independently on whether they encode their own RNA polymerase or use the cellular enzyme, complex interactions with the cellular transcription machinery ensure effective synthesis of viral mRNA(s). For example, poliovirus and Rift Valley fever virus, which use their own enzymes for RNA synthesis both shut down host transcription, which is not essential for viral replication (Le May et al., 2004; Kundu et al., 2005). In contrast, the viruses that require host transcription machinery, for example

herpes simplex virus, set up regulatory interactions with cellular transcription factors to support their own DNA synthesis (Wysocka and Herr, 2003).

Furthermore, being restricted in their encoding capacity viruses lack their own functional translation machinery and fully rely on host protein synthesis (Walsh and Mohr, 2011). Even *Pandoravirus salinus* with the biggest known viral genomes of 2.77 mega base pairs encodes only few required translation factors remaining dependent on protein synthesis of its *Acantamoeba* host (Philippe et al., 2013). Viruses target the cellular translational machinery to secure preferential translation of viral transcripts or to shut off host translation when it is not required. For instance, vesicular stomatitis virus utilizes specific 3' structures on its mRNA and also interacts with the ribosome to secure preferential synthesis of viral proteins (Whitlow et al., 2006; Lee et al., 2013a). Viruses further utilize cellular processes for transport of their components or assembled virions and for escape from the cell (Bartenschlager et al., 2011; Lyles, 2013). Thus, human immunodeficiency virus type 1 usurps cellular endosomal sorting complexes required for transport pathway to facilitate its effective budding (Morita et al., 2011), whereas adenoviruses induce autophagy to enable cell lysis and viral exit (Jiang et al., 2011).

The cells of plants, many invertebrates and vertebrates respond to infection with robust induction of innate immune responses and no successful virus replication would be possible without control over these responses. For this, viruses have evolved a multitude of approaches to counteract their recognition by cellular detectors (Zinzula and Tramontano, 2013), subvert signal transduction, prevent activation of antiviral genes (Short, 2009), and, when necessary, limit apoptosis (Galluzzi et al., 2008).

The number of viral strategies to interact with the host cell is overwhelming. Although research helped to deduce the basic strategies of viral life cycle from the nature of viral genomes already in 1971 (Baltimore, 1971), the exact mechanisms of viral replication are so diverse and complicated that we are still striving to understand them. Studies of virus-host interactions are largely driven by attempts to improve the

surveillance of pathogenic viruses, search for antivirals and development of vaccines (Webby and Webster, 2003; Schwegmann and Brombacher, 2008). However, viruses have also proven many times to be valuable tools for understanding cell functions, from the initial discovery of DNA as genetic material (Hershey and Chase, 1952), up to recent advances in tackling complex processes such as endocytosis (Pelkmans and Helenius, 2003). Finally, understanding virus-host interactions brings novel applicable tools widely used in biotechnology, such as viral vectors for gene transfer (Vannucci et al., 2013) or baculovirus systems for heterologous protein production (van Oers, 2011). Although a great deal of mechanisms have been already discovered, the amount of information that we get now is growing exponentially and the majority of discoveries is perhaps still ahead.

1.2 Influenza A virus: an overview

This work is dedicated to influenza A virus, a member of the *Orthomyxoviridae* family. Influenza A viruses are commonly classified based on their surface antigens—hemagglutinin (HA) and neuraminidase (NA). HA subtypes 1–16 and all NA subtypes 1–9 are found in wild birds which, apparently, represent the main natural reservoir for influenza A virus (Stallknecht and Brown, 2007). In addition, H17N10 and H18N11 viruses were recently found in bats that seem to represent a sylvatic mammalian reservoir for influenza A viruses (Tong et al., 2012; Tong et al., 2013). Certain viral subtypes can also infect domesticated birds and multiple species of mammals, including humans. Whereas influenza A virus is usually asymptomatic in its natural hosts, it can cause mild to severe intestinal infections in poultry and asymptomatic to severe respiratory infections in mammals (Webster et al., 1992).

Although the first human influenza A virus was isolated in 1933 (Smith et al., 1933) and one of the first well-publicized influenza A pandemics occurred in 1918 (Taubenberger et al., 1997), numerous records indicate that humankind has been facing influenza epidemics and probably also pandemics for at least several centuries (Potter,

2001). There is molecular evidence that influenza A HA subtypes 1, 2, 3, 5, 7, 9 and 10 can infect humans, but currently only influenza A of H1 and H3 subtypes are circulating in humans and are causing annual epidemics.

Circulating strains of influenza A cause seasonal infections in humans. In most countries these infections result in annual epidemics which, according to World Health Organization (WHO), affect up to 10 % of the population worldwide and result in up to 500,000 deaths. In addition to these annual epidemics, global pandemics can occur when humans are infected with viruses capable of human-to-human spread to which they are immunologically naïve. Although influenza A pandemics are relatively rare events, humankind has faced three major pandemics in the twentieth century and already one in the twenty-first (Lagacé-Wiens et al., 2010; Fineberg, 2014). Whereas the mortality of seasonal influenza is modest, the mortality of pandemic influenza is unpredictable and can vary: for example, during the H1N1 pandemic in 2009 it was below 0.5 %, but during the H5N1 outbreak in 1997 it reached 60 % (Forrest and Webster, 2010; Noah and Noah, 2013). In addition to annual influenza-related deaths, the virus imposes an enormous economic burden on multiple sectors of societies (Szucs, 1999; Noah and Noah, 2013).

Influenza A virus genome is composed of eight single-stranded RNA (ssRNA) molecules of negative polarity (Palese, 1977). It is replicated with the viral RNA-dependent RNA polymerase (RdRp) which is error-prone and produces between 1.5 and 7.5×10^{-5} misincorporations per nucleotide. Because RdRp also lacks proofreading activity these misincorporations cannot be repaired and on average one mutation appears in the viral genome after each cycle of RNA replication (Parvin et al., 1986; Drake, 1993). The gradual evolution of influenza A viruses due to frequent mutations in viral proteins is referred to as antigenic drift. In addition, the viral genomic segments can reassort during the co-infection of the same cell with two or more influenza A viruses and give rise to progeny virions that contain segments derived from both "parental" viruses (McGeoch et al., 1976; Desselberger et al., 1978). If the "parental" viruses be-

long to different subtypes, the reassortant progeny virion(s) may harbor major changes that are referred to as antigenic shift. Antigenic drift, antigenic shift and inter-species transmission are the key drivers of viral evolution (Forrest and Webster, 2010). Antigenic drift limits efficacy of vaccines and antivirals and antigenic shift imposes constant risk for new pandemics. Thus, improvements of options to control influenza are needed. These efforts require careful virus surveillance, vaccine development and search for antivirals with novel mechanisms of action. This is impossible without a comprehensive understanding of influenza A virus-host interactions.

1.3 Influenza A virus organization and replication cycle

Influenza A virions are pleiomorphic, i.e. their shapes are not uniform and can be spherical, kidney- or rod-shaped with an average size of 100–150 nm (Fujiyoshi et al., 1994). The outer shell of the virion is composed of the host-derived lipid bilayer in which viral HA, NA and M2 proton channel (M2) are incorporated. This shell is underlined with the viral matrix protein (M1) (Harris et al., 2006). Each virion encompasses eight genomic RNA segments packed in viral ribonucleoproteins (vRNPs)—supercoiled ring-like structures in which paired 5' and 3' ends of the viral RNA are associated with heterotrimetic viral polymerase complex and the rest of the RNA is densely covered with viral nucleoprotein (NP) (Arranz et al., 2012). In the virion the vRNPs are associated with the M1 protein (Rees and Dimmock, 1982; Ye et al., 1999). Eight genes of all influenza A viruses encode 10 essential viral proteins: HA, NA, M1, M2, NP, polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA), non-structural protein (NS1), and nuclear export protein (NEP) (Lamb, 1983). In addition, some influenza A strains may encode accessory proteins PB1-F2, PB1-N40, PA-X, PA-155 and PA-182. Whereas HA, NA, M1, M2, NP, PB1, PB2, PA and NEP are structural components of viral particle, NS1, PB1-F2, PB1-N40, PA-X, PA-155 and PA-182 are considered to be non-structural and are involved in regulation of virus-host interactions (Chen et al., 2001; Hale et al., 2008c; Wise et al., 2009; Jagger

et al., 2012; Muramoto et al., 2013).

Influenza A replication cycle begins when the viral HA binds to the specific virus receptor on cell surface. The key, but possibly not the only, receptors for influenza A virus are sialic acids linked to cellular surface glycoproteins or glycolipids (Martín et al., 1998; Skehel and Wiley, 2000; Stray et al., 2000). HA molecules of avian influenza A viruses recognize α-2,3-linked sialic acids and HAs of human influenza A viruses recognize α-2,6-linked sialic acids (Connor et al., 1994; van Riel et al., 2010). HA recognition of specific sialic acids to a large extent determines viral host specificity. After receptor binding the viruses are endocytosed via clathrin-dependent or clathrin- and caveolin-independent routes and are transferred towards the perinuclear space in endosomes (Dourmashkin and Tyrrell, 1974; Matlin et al., 1981; Sieczkarski and Whittaker, 2002; Lakadamyali et al., 2003). Acidification of late endosomes in perinuclear space triggers two essential events that allow virus uncoating and delivery of vRNPs to the cytoplasm. First, low pH mediates the conformational change in the HA and enables fusion of viral and endosomal membranes (Carr and Kim, 1993). Second, acidification of the virus interior leads to dissociation of vRNPs from M1 which is a prerequisite for their import into the nucleus, a site of viral RNA transcription and replication (Bui et al., 1996; Stauffer et al., 2014).

Cytoplasmic transport of the vRNPs towards the nucleus is passive: it does not require cellular cytoskeleton components and appears to rely on diffusion (Martin and Helenius, 1991; Babcock et al., 2004). The nuclear import of vRNPs, in contrast, occurs in an active way (Kemler et al., 1994). For this, importin α isoforms 1 and 5 likely recognize the surface-exposed nuclear localization sequences (NLSs) on viral NPs and, together with the importin β, mediate traverse of vRNPs through the nuclear pore complex and its delivery to the nucleus (Martin and Helenius, 1991; O'Neill et al., 1995; Cros et al., 2005; Moeller et al., 2012; ying Chou et al., 2013).

In the nucleus the viral RNAs (vRNAs) are transcribed *in cis* by the viral polymerase associated with the vRNP (Moeller et al., 2012). Synthesis of viral mRNA is

initiated using 10–13 nucleotide long primers with 5' methylated guanine (5'meG) cap (Beaton and Krug, 1981; Plotch et al., 1981). These primers are stolen from cellular mRNAs during "cap-snatching" when 5'meG cap structures on host mRNAs are recognized and bound by PB2 subunit of viral polymerase (Guilligay et al., 2008) and further endonucleolytically cleaved by viral PB1 and PA (Li et al., 2001a; Dias et al., 2009; Yuan et al., 2009). The "snatched" 5'meG-caps also provide 3'-OH ends for viral mRNA chain elongation by PB1 (Poch et al., 1989). The synthesis of viral mRNA ends after reiterative copying of short uridine stretches located at the 5' end of vRNA and the resulting viral mRNA contains 150–200 adenine bases at its 3' end (Plotch and Krug, 1977; Robertson et al., 1981; Poon et al., 1999). Cap-containing polyadenylated viral mRNAs are structurally indistinguishable from host transcripts. They are exported from the nucleus via the cellular mRNA export route and are translated in the cytoplasm (Chen and Krug, 2000; Read and Digard, 2010). Many of the synthesized viral proteins shuttle back to the nucleus to facilitate production of new vRNPs and their export to the cytoplasm (Greenspan et al., 1988; Neumann et al., 1997; Huet et al., 2010; Wang et al., 2013).

Replication of influenza A viral genomes occurs through two steps. First, complementary ribonucleoproteins (cRNPs) containing positive single-stranded cRNA are produced. Next, these cRNPs serve as templates for production of progeny vRNP (Elton et al., 2005). In contrast to mRNA, the synthesis of both cRNA and vRNA is carried out *in trans* by the free viral polymerase available in the nucleus after synthesis and import of new viral proteins (Jorba et al., 2009; Moeller et al., 2012). Moreover, initiation of both cRNA and vRNA synthesis does not require cell- or virus-derived primers and occurs *de novo* resulting in the presence of triphosphates at their 5' ends (Hay et al., 1982; Zhang et al., 2010). The progeny vRNPs are assembled in the nucleus and contain copies of parental vRNA, a single viral polymerase and multiple copies of NP. They can be transcribed and later exported from the nucleus for virion assembly (Resa-Infante et al., 2011).

The ultimate virion assembly and budding occur at the cellular plasma membrane and require transport of essential components of progeny virions to the site of assembly. The vRNPs in a complex with M1 and NEP are exported from the nucleus via cellular Crm1/exportin-1 pathway and then are transported to the site of budding via cellular microtubules (Akarsu et al., 2003; Momose et al., 2007; Kawaguchi et al., 2012). The transport of vRNPs along microtubule tracks is characterized by an intermittent motion typical for microtubule-mediated cargo, although no specific motor complex has yet been identified (Amorim et al., 2011; Momose et al., 2011; Avilov et al., 2012). Viral envelope proteins (HA, NA and M2) obtain specific sorting signals for their targeting to the budding site (Hughey et al., 1992; Kundu et al., 1996; Tall et al., 2003) and are transported there through the Golgi network (Daniels-Holgate and Edwardson, 1989). The virion assembly is localized to specific cholesterol- and sphingolipid-enriched regions of plasma membrane referred to as lipid rafts (Scheiffele et al., 1999). The budding requires HA for initiation of cellular membrane curvature, coordinated interaction of M1 and vRNPs for packaging of viral genomes and M2 for bud scission (Nayak et al., 2009; Rossman and Lamb, 2011). After virion assembly and bud formation NA cleaves the sialic acids off the cellular surface releasing new virions that can initiate another infection cycle (Barman et al., 2004).

1.4 Host factors involved in influenza A virus replication cycle

Because of the limited capacity of influenza A virus to encode its own proteins, its effective replication relies on cellular factors. A large number of such factors has been recently identified using yeast two-hybrid assay, genome-wide RNAi screening, and proteomic approaches (Mayer et al., 2007; Hao et al., 2008; Brass et al., 2009; Shapira et al., 2009; Karlas et al., 2010; König et al., 2010; Shaw, 2011; Song et al., 2011). At least 128 of them were identified in two or more screens simultaneously. Functional clustering of these factors revealed their involvement in essentially all stages of viral replication (Watanabe et al., 2010). A scheme summarizing host factors involved in

different steps of the influenza A replication cycle is presented on Figure 1.

The clathrin-mediated endocytosis of influenza A requires cellular clathrin and epsin-1 (Chen and Zhuang, 2008) and the efficient endosomal transport depends on cellular GTPases Rab5 and Rab7 (Sieczkarski and Whittaker, 2003). Fusion of viral and endosomal membranes is dependent on vacuolar proton-ATPase that acidifies endosomal interior and several subunits of this macromolecular complex have been identified as host factors required for influenza A replication (Watanabe et al., 2010).

Nuclear import of vRNPs occurs in an active way and requires the interaction of NP with importin $\alpha 1$ or importin $\alpha 5$ (Cros et al., 2005). Although viral RdRp is sufficient to transcribe influenza A RNAs and does not require additional factors, it interacts with cellular DNA-dependent RNA polymerase II presumably to facilitate cap-snatching (Engelhardt et al., 2005). Furthermore, influenza A utilizes cellular splicing machinery to process its mRNAs derived from segments 7 and 8 (Dubois et al., 2014) and cellular nuclear export machinery to deliver its transcripts to the cytoplasm (York and Fodor, 2013). As the virus encodes none of the translation machinery components, its protein synthesis completely depends on the host translation machinery and the efficacy of viral protein production is secured via the tight interaction between viral NS1 and cellular translation factors (de la Luna et al., 1995; Aragon et al., 2000; Burgui et al., 2003).

Effective synthesis of vRNPs requires interaction of RdRp with cellular minichromosome maintainance complex (Kawaguchi and Nagata, 2007) and serine/threonine phosphatase 6 (York et al., 2014). The export of vRNPs is dependent on the interaction of M1-vRNP with the cellular nuclear export receptor Crm1, which presumably is bridged by viral NEP (Brunotte et al., 2014) and their further transport to the budding site requires interaction of viral NP with cellular Rab11 GTPase (Eisfeld et al., 2011). Finally, assembly of the virion at the budding site and bud formation requires functional actin microfilaments and cellular energy sources (Nayak et al., 2004). The above mentioned interactions give just a few examples of a complex interactome that

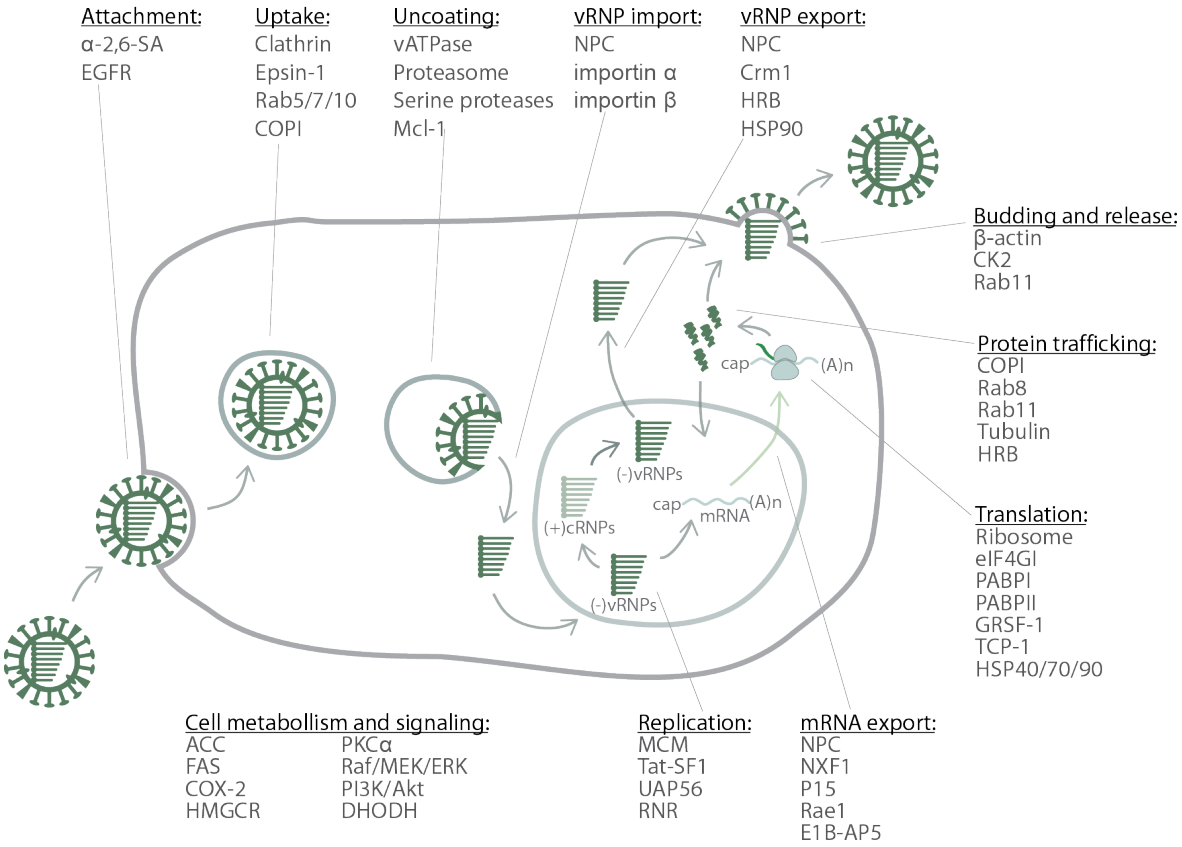

Figure 1. Influenza A virus replication cycle and cellular factors involved in it. The abbreviated host factors are: α-2,6-SA—α-2,6-linked sialic acids; EGFR—epidermal growth factor receptor; COPI—coatomer 1 vesicular transport complex; vATPase, vacuolar H+-ATPase; Rab 5/7/8/10/11—small GTPases; Mcl-1—induced myeloid leukemia cell differentiation protein Mcl-1; NPC—nuclear pore complex; CRM1—exportin-1; HRB — HIV rev-binding protein; HSP40/70/90—heat shock protein 40, 70 or 90 kDa; CK2—casein kinase 2; Rab 8/11—small GTPases; eIF4GI—eukaryotic initiation factor 4 gamma 1; PABPI/II—polyadenylate-binding protein cytoplasmic isoforms I and II; GRSF1—G-rich sequence factor 1; TCP1, T-complex protein 1; NXF1—nuclear mRNA export factor 1; P15—mRNA export factor; Rae1—mRNA export factor 1; E1B-AP5—heterogeneous nuclear ribonucleoprotein U-like protein 1; MCM—minichromosome maintenance complex IREF-1; Tat-SF1—Tat-specific factor 1; UAP56—helicase UAP56; RNR—ribonucleotide reductase; ACC—acetyl-CoA carboxylase; FAS—fatty acid synthase; COX-2—cyclooxygenase 2; HMGCR—3-hydroxy-3-methylglutaryl-coenzyme A reductase; Raf/MEK/ERK—Ras/Raf/mitogen-activated protein kinase/extracellular signal-regulated kinase pathway; PI3K/Akt, phosphatidylinositol-3-kinase/RAC-alpha serine/threonine-protein kinase pathway; DHODH—dihydroorotate dehydrogenase.

the virus establishes during infection: accession of host-pathogen interaction database (Kumar and Nanduri, 2010) yielded published associations of viral proteins with over 400 host factors (www.agbase.msstate.edu, accessed on 10.12.2014).

Influenza A replication triggers innate immune responses and therefore its effectiveness is not limited to recruiting essential host factors but expands far beyond that.

Establishment of tight control over innate immune responses to infection is absolutely critical for successful viral replication in an immune-competent system.

1.5 Innate immune responses to influenza A infection

In mammals influenza A is transmitted mainly through aerosols and droplets and enters the host through the respiratory tract (Brankston et al., 2007). The first line of antiviral defense in the respiratory tract is represented by the airway mucus. It consists mainly of glycoproteins, antimicrobial and antiviral substances and is an essential barrier for virus infection (Thornton et al., 2008; Nicholas et al., 2006). The viruses that penetrate the airway mucus barrier initiate infections of the respiratory tract epithelial cells and can also spread to immune cells of the respiratory tract, mainly macrophages and dendritic cells (DCs) (Perrone et al., 2008; Bender et al., 1998). Infection of susceptible cells with influenza A is rapidly detected by innate immune sensors that trigger induction of antiviral gene expression and activation of pro-inflammatory responses. Antiviral gene products are essential for restriction of viral replication and reduction of virus burden, whereas pro-inflammatory responses are required for establishment of inflammation (Iwasaki and Pillai, 2014). Furthermore, the onset of innate immune responses is required for informing the adaptive immunity that regulates clearance of the infection site and generation of immune memory (Iwasaki and Medzhitov, 2010).

Although innate responses involve a complex network of events that are hard to tackle, in recent years substantial progress has been made towards the understanding of critical processes that regulate virus detection, antiviral signaling and activation of immune-related genes.

1.5.1 Virus recognition by innate immunity

Eukaryotic cells evolved a way to distinguish between "self" and "non-self" via expression of specific detection molecules called pattern recognition receptors (PRRs) (Janeway and Medzhitov, 2002). These PRRs recognize specific molecular signatures

produced by invading microorganisms that are called pathogen-associated molecular patterns (PAMPs) and initiate downstream signaling events to activate innate immune responses (Janeway, 1989). The current paradigm of innate immunity to influenza A virus assumes that during replication the virus produces three types of PAMPs. These PAMPs are single- and double-stranded viral RNA and 5' triphosphates generated during viral genome synthesis by RdRp (Lund et al., 2004; Guillot et al., 2005; Hornung et al., 2006; Kato et al., 2006). They are recognized in the endosome or in the cytoplasm by three major classes of cellular PRRs: NOD-like receptors (NLRs), Toll-like receptors (TLRs), and RIG-I-like receptors (RLRs) (Iwasaki and Pillai, 2014).

Viral recognition in the endosome relies on three different TLR class members: TLR3 which recognizes dsRNA, and TLR7 and TLR8 which recognize ssRNA (Fig. 2) (Iwasaki and Pillai, 2014). TLR3 is constitutively expressed in pulmonary and airway epithelial cells and in DCs (Guillot et al., 2005; Schulz et al., 2005; Ioannidis et al., 2013). It has been initially shown to recognize dsRNA and induce interferon (IFN) production in response to it (Alexopoulou et al., 2001; Guillot et al., 2005). TLR3 signaling is activated in response to replicating influenza A virus (Guillot et al., 2005). TLR7 is expressed by airway epithelial cells and DCs and plasmocytoid DCs (Ioannidis et al., 2013; Lund et al., 2004). TLR7 can be activated by ssRNA and is proposed to recognize genomic vRNA of influenza A virus in the endosome (Diebold et al., 2004). Unlike other TLRs, TLR8 has been so far only found in macrophages and monocytes where it is activated in response to ssRNA and 5' triphosphates (Ablasser et al., 2009). TLR8 signaling is activated upon influenza A infection and leads to production of interleukin (IL)-12, however its distinct role in regulation of innate immunity is yet to be determined (Lee et al., 2013b).

The listed TLRs are expressed in endosomal compartments of the cells, except TLR3, which was also found in the outer membrane (Diebold et al., 2004; Schulz et al., 2005; Ablasser et al., 2009). The proposed mechanisms for their activation in response to influenza A are, however, unclear for several reasons: (i) influenza A does not produce

detectable amounts of dsRNA during its replication due to activity of cellular RNA helicase UAP56 (Wisskirchen et al., 2011); (ii) paired 5' and 3' ends of vRNA are bound to viral RdRp, which can hinder 5' triphosphates from their recognition by TLRs (Arranz et al., 2012); (iii) TLR3 and TLR8 are dispensable for influenza A recognition and only TLR7 seems to be critical for it (Lund et al., 2004); and (iv) it is not clear whether TLR7 recognizes any specific structures or sequences within RNA, as it was shown to become readily activated in response to both "self" and "non-self" RNA (Diebold et al., 2004).

TLR7 and TLR8 interact with their common adapter MyD88 (Figure 2) (Medzhitov et al., 1998). MyD88 recruits IRAK family kinases which mediate phosphorylation and nuclear translocation of interferon regulatory factor (IRF)3 and IRF7—transcription factors that induce *IFN* gene expression (Burns et al., 2003; Honda et al., 2005). In addition, MyD88 activates mitogen-activated protein kinases (MAPKs) signaling and transcription factor activator protein 1 (AP-1), that controls expression of pro-inflammatory genes (Kawai and Akira, 2007). TLR3 induces its signaling via interaction with TIR-domain-containing adapter-inducing interferon-β (TRIF) and its downstream pathways bifurcate (Guillot et al., 2005; Kumar et al., 2009). One of those induces type I IFN production via TBK1 and inhibitor of nuclear factor kappa-B kinase (IKK) kinases and IRF3 and IRF7. Another one stimulates production of pro-inflammatory cytokines via IKK and nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) or via MAPK signaling and transcription factor AP-1 (Guillot et al., 2005; Vercammen et al., 2008).

Viral recognition in the cytoplasm relies on RLRs and NLRs. RLRs is a group of helicases named after its representative retinoic acid inducible gene I (RIG-I). RLRs are constitutively present in low amounts in multiple cell types, but their most prominent location is airway epithelium (Bogefors et al., 2011) where they play an essential role in detection of airborne pathogens. The RLR group consists of three proteins: RIG-I, melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics and

physiology-2 (LGP-2) (Kang et al., 2004; Yoneyama et al., 2004; Yoneyama et al., 2005). They are structurally similar and contain RNA-binding C-terminal domain (CTD) and a DExD/H box helicase domain (Cui et al., 2008; Takahasi et al., 2009). RIG-I and MDA5 also contain two consecutive N-terminal caspase recruitment domains (CARDs) that mediate signaling (Yoneyama et al., 2004; Kang et al., 2004). All RLRs recognize dsRNA, and RIG-I can also recognize ssRNA with 5' triphosphates (Cui et al., 2008).

In the cytoplasm RIG-I is normally present in an inactive autorepressed state in which its CARDs are sequestered by the helical domain, preventing non-specific induction of RIG-I downstream signaling (Figure 2) (Kowalinski et al., 2011). Upon sensing its ligands by CTD, RIG-I undergoes conformational rearrangement which liberates its CARDs for downstream signaling (Kowalinski et al., 2011). Activation of RIG-I is dependent on its ubiquitination by E3 ubiquitin ligases TRIM25 and Riplet or on binding to free polyubiquitin chains generated by TRIM25. Both TRIM25 and Riplet are required for RIG-I signaling *in vitro* and *in vivo* (Gack et al., 2007; Oshiumi et al., 2010; Zeng et al., 2010). The modified RIG-I oligomerizes (Patel et al., 2013) and undergoes additional conformational rearrangements that enable interaction with its adapter mitochondrial antiviral-signaling protein (MAVS) (Kawai et al., 2005; Seth et al., 2005). For this, RIG-I is targeted to mitochondria in a "translocon" complex containing TRIM25 and mitochondrial targeting chaperone 14-3-3ϵ (Liu et al., 2012). Upon binding RIG-I, MAVS oligomerizes and forms a scaffold for a multi-kinase signaling complex which includes c-Jun N-terminal kinase (JNK), TANK-binding kinase 1 and IKKϵ complex, and IKK$\alpha/\beta/\gamma$ complex (McWhirter et al., 2005). These kinases eventually activate transcription factors IRF3, AP-1 and NFkB which regulate type I IFN genes (McWhirter et al., 2005).

The only NLR that detects influenza A is LRR- and pyrin domain-containing protein 3 (NLRP3) found in lung and bronchial epithelial cells, monocytes, macrophages and DCs (Guarda et al., 2011; Kim et al., 2014). It is constitutively present in an inactive form in the cell cytoplasm. During influenza A infection NLRP3 is activated

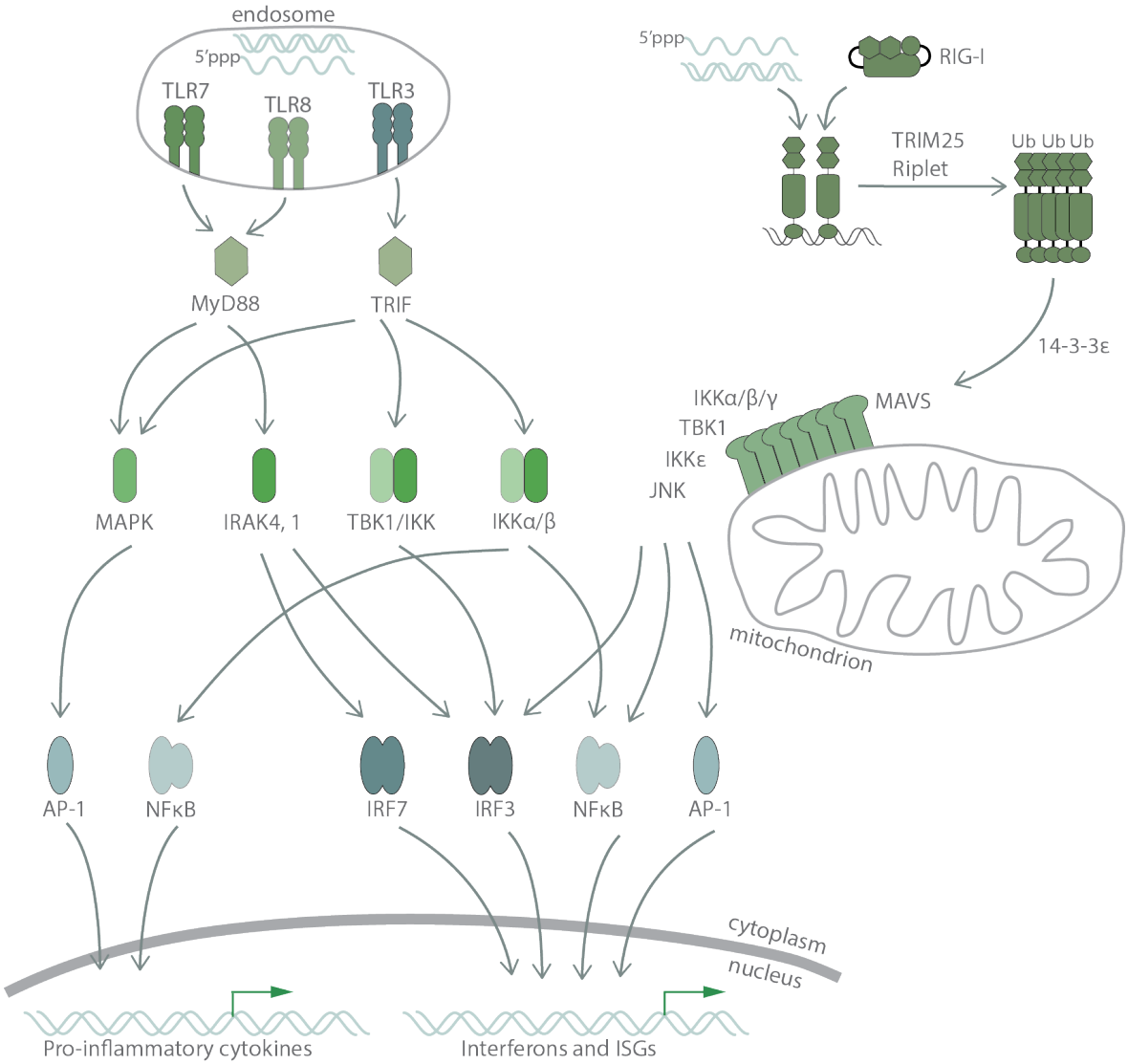

Figure 2. Viral PAMPs detection by endosomal and cytoplasmic PRRs. TLRs 3, 7 and 8 recognize dsRNA in the endosome and induce downstream signaling via the adapter proteins MyD88 or TRIF. The signal transduction is mediated by protein kinases MAPK, IRAK4,1, TBK1/IKK and IKKα/β. RIG-I recognizes dsRNA and 5'triphosphorylated-ssRNA and undergoes conformational changes followed by its uniquitination by TRIM25 and Riplet. The modified RIG-I oligomerizes and translocates to mitochondrion where it triggers oligomerization of the adapter protein MAVS. MAVS facilitates signal transduction by protein kinases JNK, IKKϵ, TBK1 and IKK$\alpha/\beta/\gamma$. TLR and RIG-I signaling activates transcription factors AP-1, NFκB, IRF3 and IRF7 which translocate to the nucleus and transcriptionally induce expression of interferons, interferon-stimulated genes and pro-inflammatory cytokines, thus activating the innate immune responses to influenza A infection.

by sensing viral ssRNA or proton flux mediated by viral M2 in trans Golgi network (Thomas et al., 2009; Allen et al., 2009; Ichinohe et al., 2010). Virus-mediated activation and oligomerization of NLRP3 leads to formation of the inflammasome—a multipro-

tein complex that includes NLRP3, apoptosis-associated speck-like protein containing a CARD domain and pro-caspase 1 (Tschopp and Schroder, 2010). The inflammasome is required for proteolytic self-activation of pro-caspase 1, which afterwards cleaves IL1β and IL18 precursors, resulting in production of IL1β and IL18.

Whereas RIG-I activation results in induction of antiviral responses by IFNs and interferon-stimulated genes (ISGs), signaling by NLRP3 and TLRs can also induce pro-inflammatory responses (see section 1.5.3. for more details) (Le Goffic et al., 2007; Allen et al., 2009; Kawai and Akira, 2007).

1.5.2 Antiviral responses by interferons and interferon-stimulated genes

Following detection of viral PAMPs and establishment of PRR signaling, the infected cells produce and secrete small regulatory proteins known as interferons (Fensterl and Sen, 2009). Interferons are subdivided into three types (I–III) based on their respective receptors (Branca and Baglioni, 1981; Sheppard et al., 2003). Type I and III IFN signaling activates ISGs and antiviral responses in autocrine and paracrine manner (Kotenko et al., 2003; García-Sastre and Biron, 2006), reinforces PRR production (Pothlichet et al., 2013), and regulates adaptive responses via enhancement of antigen presentation to $CD4^+$ and $CD8^+$ T-cells (Zietara et al., 2009).

Both type I and III IFNs are secreted by nearly all cell types, although the majority of them is secreted by DCs (Siegal et al., 1999; Odendall et al., 2014). Type I IFNs include IFNα and IFNβ and utilize dimeric receptor IFNAR1/IFNAR2 on cell surface (Mogensen et al., 1999). Type III IFNs are IFNλ1, IFNλ2, IFNλ3 (also called IL29, IL28A and IL28B, respectively), and IFNλ4. They bind to their heterodimeric receptor IL10R2/IFNLR1 (Kotenko et al., 2003; Sheppard et al., 2003). Both type I and III IFN receptors activate signaling through the Janus kinases (JAKs) / signal transducer and activator of transcription (STAT) pathway (Figure 3). Binding of IFN to its receptor triggers a series of phosphorylation events in which receptor-bound JAKs phosphorylate themselves, the IFN receptor and receptor-associated proteins STAT1 and STAT2

(van Boxel-Dezaire et al., 2006). Phosphorylation of STAT1 and STAT2 triggers their heterodimerization and formation of regulatory complex with IRF9 (Fu et al., 1990). This complex is translocated to the nucleus where it transcriptionally activates ISGs (Levy et al., 1988). A schematic illustration of IFN signaling is presented in Figure 3.

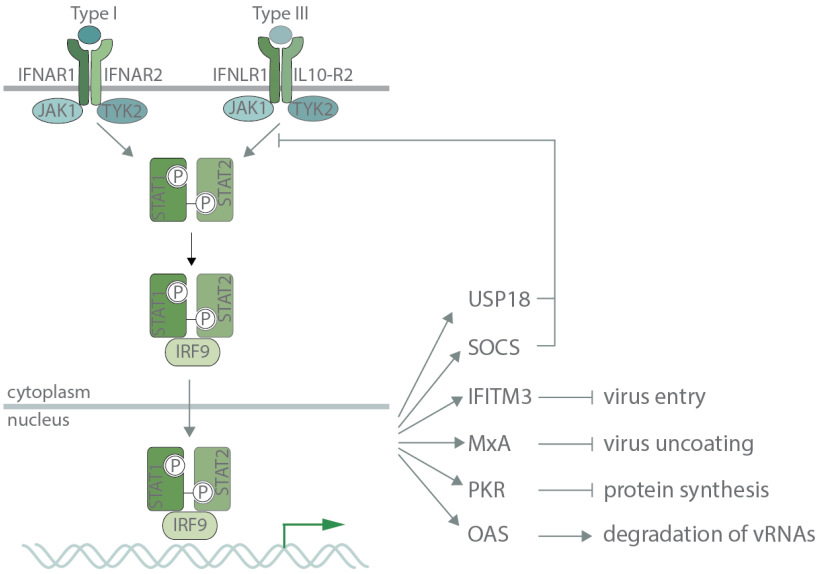

Figure 3. A schematic representation of type I and type III IFN responses. Interaction of type I and type III interferons with their corresponding receptors results in autophosphorylation of receptor-associated JAK kinases JAK1 and TYK2, which phosphorylate STAT1 and STAT2 proteins. Phosphorylated STAT1 and STAT2 form heterodimers that interact with regulatory protein IRF9. The STAT1-STAT2-IRF9 complex translocates to the nucleus where it transcriptionally activates interferon-stimulated genes. Interferon-stimulated gene products inhibit viral entry and uncoating, suppress protein synthesis in infected cell, regulate degradation of vRNAs and control interferon signaling via feedback loops.

ISGs are a diverse group of genes that control multiple cellular processes: they enhance virus sensing by PRRs, target pathways essential for viral replication, upregulate cytokine and chemokine production, control IFN response via positive and negative feedback loops. The best-described ISGs with antiviral action include myxovirus resistance gene product (MxA), interferon-inducible transmembrane protein 3 (IFITM3), cholesterol 25-hydroxylase, ubiquitin-like protein ISG15, protein kinase R (PKR), and 2'-5'-oligoadenylate syntethase (OAS) (Sadler and Williams, 2008).

IFITM3 is localized in late endosomes and exerts its antiviral activity during virus entry. It alters properties of the endosomal membrane and prevents viral fusion (Desai

et al., 2014). Indeed, in cells overexpressing IFITM3 virus-like particles bearing β-lactamase do not fuse in late endosomes and no cytoplasmic β-lactamase activity can be detected (Desai et al., 2014).

Human MxA gene product is a dynamin-like guanosine triphosphatase (Nakayama et al., 1992). It is localized in the cytoplasm of infected cells where it self-assembles into ring-like ordered structures (Gao et al., 2010). Although the detailed mechanism of its antiviral activity is yet do be established, MxA oligomers presumably recognize and bind vRNPs in the cytoplasm, preventing their nuclear import and thus attenuate influenza A replication (Haller et al., 2010).

PKR and OAS act at later stages of viral replication cycle. PKR is a multifunctional serine/threonine kinase that has a critical role in host antiviral responses (García et al., 2006). It is constitutively present in the cytoplasm at low abundance, and its expression is trancsriptionally induced by type I and III IFNs (Meurs et al., 1990). PKR is activated via its interaction with dsRNA or protein activator of the interferon-induced protein kinase (Li et al., 2006b). It phosphorylates and inactivates translation initiation factor 2α (eIF2α) (Levin and London, 1978), which is indispensable for initiation of cap- and often of internal ribosome entry site (IRES)-driven translation. Thus, activation of PKR in response to virus infection shuts down protein synthesis (Kimball, 1999). In addition to controlling translation in infected cells, PKR can also detect dsRNA, mediate IFNγ-induced NFkB activation (Deb et al., 2001), modulate STAT1 signaling (Wong et al., 1997), induce JNK and MAPK signaling in response to viral infection (Chu et al., 1999), induce apoptosis and autophagy following the shut down of protein synthesis (Gil and Esteban, 2000; Tallóczy et al., 2002). PKR mediates death of infected cells unless the virus counteracts its action (Takizawa et al., 1996; Hatada et al., 1999). As no cytoplasmic dsRNA is detectable during influenza A replication (Wisskirchen et al., 2011), PKR activation during infection is likely mediated by TLR signaling (Jiang et al., 2003) or by binding to its protein activator (García et al., 2006).

OAS and ribonuclease L (RNAse L) act together in the antiviral RNA decay path-

way. Both enzymes are upregulated by IFN, but they are also present constitutively in cell cytoplasm (Sadler and Williams, 2008). Like PKR, OAS can be activated by binding to dsRNA (Castelli et al., 1998). Upon its activation, OAS synthesizes 2'-5'-linked adenosine triphosphate oligomers, which, in turn, act as inducers of latent RNAse L (Rebouillat and Hovanessian, 1999). Activated RNAse L catalyzes endonucleolytic degradation of cellular and viral ssRNAs and mRNAs thus contributing to host antiviral responses (Dyer and Rosenberg, 2006). In addition to viral RNA elimination, RNAse L reinforces virus detection by TLRs and RIG-I, supports IFN response, and regulates apoptosis in infected cells (Liang et al., 2006). Replication of influenza A virus that is unable to inhibit OAS/RNAse L pathway is attenuated (Min and Krug, 2006).

Some ISGs are also implicated in interferon response control via negative feedback loops (Schneider et al., 2014). For example, SOCS proteins inhibit JAK/STAT signaling (Hong and Carmichael, 2013) and USP18 binds to IFNAR2 and inhibits it (Ritchie et al., 2004).

1.5.3 Pro-inflammatory responses

Pro-inflammatory responses induced by TLR and NLRP3 signaling activate production of chemokines including IL1-β, IL6, IL8, IL18, RANTES, MCP-1, MCP-3 and MIP-3α (Julkunen et al., 2000; Le Goffic et al., 2007). Unlike type I IFNs, pro-inflammatory cytokines may not induce direct antiviral resistance, but are required for antigen presentation, establishment of inflammation, recruitment of leukocytes to the site of infection and proliferation of $CD8^+$ cytotoxic T lymphocytes (CTLs) (Van Der Sluijs et al., 2005; Schulz et al., 2005; Le Goffic et al., 2006). Consequently, they do not inhibit viral replication, but are essential for the host resistance to infection (Pang et al., 2013). In addition, the correct onset of pro-inflammatory responses regulates adaptive immunity to influenza A infection (Trinchieri, 2003; Ichinohe et al., 2009). However, robust induction of pro-inflammatory response to some influenza A subtypes, e.g. H5N1, can be

detrimental to the host and lead to severe immunopathology, thereby increasing viral pathogenicity (La Gruta et al., 2007).

1.6 Apoptosis

In addition to activating antiviral and pro-inflammatory responses, influenza A was shown to induce apoptosis in infected cells (Fesq et al., 1994; Hinshaw et al., 1994; Mori et al., 1995; Brydon et al., 2005). The central role in apoptotic response belongs to cysteinyl proteases (caspases) which proteolytically inactivate numerous cellular proteins leading to cell death (Cohen, 1997; Thornberry and Lazebnik, 1998)

A variety of cellular signaling pathways, including MAPK, NFkB, PKR and phospho-inisitide-3-kinase/Akt, are implicated in apoptosis regulation during infection (Gil and Esteban, 2000; Xing et al., 2010; Lu et al., 2010). Activation of apoptosis can play a role in host defense via facilitating premature cell death and also via triggering production of pro-inflammatory cytokines (Julkunen et al., 2000). It also contributes to viral clearance in cell culture and *in vivo* being essential for $CD8^+$-mediated killing of infected cells (Ishikawa et al., 2005; Brincks et al., 2008). On the other hand, onset of apoptic responses can be beneficial for the virus and influenza A propagation is attenuated in the presence of caspase inhibitors due to retention of vRNPs in the nucleus (Wurzer et al., 2003). Due to this twofold role of apoptosis in influenza A infection, it is tightly controlled by both cellular and viral factors.

1.7 Viral counteraction to innate responses. NS1.

Successful counteraction to antiviral responses is critical for influenza A replication and several viral proteins support overcoming of intrinsic barriers and replication in the context of immune response. For instance, NA cleaves sialic acids in host mucus to facilitate viral penetration (Cohen et al., 2013), PB1-F2 inactivates RIG-I/MAVS and NFkB signaling (Varga et al., 2011; Dudek et al., 2011; Reis and McCauley, 2013), and

NP mediates resistance to MxA (Dittmann et al., 2008). Both PB1-F2 and M2 can contribute to regulation of apoptosis during infection (Herold et al., 2012).

However, the critical role in inhibition of antiviral responses and regulation of virus-host interactions is assigned to NS1 (Garcia-Sastre et al., 1998). NS1 is a non-structural protein, but it is expressed in high quantities in infected cells (Ritchey et al., 1976). Initial studies indicated that NS1 is essential for viral replication (Koennecke et al., 1981) and further investigations proved that it is a versatile viral protein which is a key regulator of influenza A virus-host interactions (Ayllon and Garcia-Sastre, 2015). Indeed, viruses lacking functional NS1 are severely attenuated, especially in immune-competent systems, and can only replicate in the absence of STAT1 or PKR (Garcia-Sastre et al., 1998; Egorov et al., 1998; Donelan et al., 2003; Falcón et al., 2004). Due to its critical role in viral replication NS1 has been extensively studied and its roles in regulation of virus-host interactions stretch beyond regulation of IFN responses and include regulation of vRNA synthesis, enhancement of viral protein production, regulation of virion assembly, modulation of cellular signaling, apoptosis inhibition, contribution to host range definition and pathogenesis.

1.7.1 NS1 synthesis and localization

The mRNA of NS1 is generated by colinear transcription of the 8^{th} genomic segment (NS). About 10 % of NS transcripts are spliced and generate the mRNA of another viral protein, NEP (Lamb et al., 1980), which shares the first 10 amino acids with NS1 (Inglis et al., 1979; Lamb and Choppin, 1979; Lamb et al., 1980). As NS1 is not found in virions, it appears in infected cells only after viral transcripts have been generated and translated. Although intracellular localization of NS1 may vary depending on its abundance, the virus strain, cell type and polarity, and time post infection, the majority NS1 is localized in the cellular nucleus, but a fraction of it is also present in the cytoplasm (Greenspan et al., 1988; Li et al., 1998; Melen et al., 2007; Newby et al., 2007; Melen et al., 2012).

Generally, proteins of under 30 kDa smoothly diffuse through the nuclear pore complex channel and do not require specific NLSs, whereas proteins of 40–60 kDa usually are delivered to the nucleus via active import (Macara, 2001; Wang and Brattain, 2007; Ma et al., 2012). NS1 is a relatively small protein and its molecular mass is only 26 kDa (Ward et al., 1994), however, its nuclear import occurs in an active way. Depending on the virus subtype, NS1 can contain one or two NLSs which mediate interaction with cellular importin α (Melen et al., 2007), thus securing rapid nuclear import of NS1 (Privalsky and Penhoet, 1981). The monopartite NLS1 is located close to the protein N-terminus, involves amino acids (aas) R35, R38 and K41, and is conserved across most influenza A isolates. The bipartite C-terminal NLS2 is present in a subset of viral strains expressing extended 237 aa NS1. It is located around aa 219–237 and also serves as a nucleolar localization sequence (Melen et al., 2007; Melen et al., 2012). Cytoplasmic localization of NS1 seems to depend on nuclear export sequence (NES) which lies within residues 138–147 (Li et al., 1998). This NES, however, is masked by adjacent residues 148–161 and its activation requires "unmasking" which presumably occurs via the conformational change upon interaction of NS1 with and unidentified protein partner(s).

1.7.2 Post-translational modifications of NS1

Initial studies indicated that a large portion of NS1 is phosphorylated during infection and this phosphorylation occurs in the nucleus (Privalsky and Penhoet, 1981). Four residues within NS1 can be phosphorylated—S42, S48, T197 and T215—although their phosphorylation may be virus subtype specific (Petri et al., 1982). Phosphorylation of NS1 at S48 by protein kinase A, at T197 by an unidentified kinase and at T215 by cyclin-dependent kinase 5 and extracellular signal-regulated kinase 2 do not seem to affect viral replication and roles of these modifications need further elucidation (Hale et al., 2009; Hutchinson et al., 2012; Hsiang et al., 2012). Phosphorylation at S42 by protein kinase C alpha is proposed to attenuate viral replication presumably via

impairing the nucleic acid binding function of NS1 (Hsiang et al., 2012).

NS1 can be also modified by linkage of small ubiquitin-like modifier protein (SUMO)—a small regulatory protein that affects activity, stability, localization and interactions of its targets (Pal et al., 2010; Johnson, 2004). NS1 extensively interacts with the cellular SUMOylation system in the nucleus and can be modified with three SUMO isoforms—SUMO1 and SUMO2/3 (Pal et al., 2011; Santos et al., 2013). This modification is subtype-specific: NS1 from some, but not all H5N1, H9N2 and H1N1 influenza A viruses can be SUMOylated (Xu et al., 2011). SUMOylation sites are lysines 219 and 221 in NS1 C-terminus (Xu et al., 2011). So far only two studies have addressed the functional role of this modification. They indicate that it regulates NS1 stability, abundance of NS1 dimers and trimers and may facilitate immunomodulatory functions of NS1 (Xu et al., 2011; Santos et al., 2013).

Another modification of NS1 that occurs in infected cells is conjugation of a small ubiquitin-like protein ISG15, that is produced in response to various stress stimuli including influenza A infection (Pitha-Rowe and Pitha, 2007; Sadler and Williams, 2008; Hsiang et al., 2009). ISG15 conjugation to NS1 by its IFN-induced ligases Ube1L, UbcH8 and Herc5 occurs primarily at lysines 41, 126, 217 and 219 (Zhao et al., 2010; Tang et al., 2010). It inhibits NS1 dimerization, interaction with PKR and with importin α, alleviates NS1-mediated inhibition of cytokine production by the infected cell and attenuates viral growth kinetics (Zhao et al., 2010; Tang et al., 2010). Like other modifications of NS1, ISGylation is strain-specific: avian NS1s differ from human in their ISGylation profiles (Tang et al., 2010). Experiments with recombinant human H3N2 influenza A virus showed that mutation K41R in NS1 renders ISGylation inefficient without compromising NS1 functions and hence acquisition of such mutations may be beneficial for the virus (Zhao et al., 2010).

1.7.3 Structure of NS1

NS1 is a relatively small protein of 219–237 aa depending on the virus strain (Hale et al., 2008c). It is subdivided in four regions: the N-terminal RNA-binding domain (RBD), the inter-domain linker, the effector domain (ED) and a disordered C-terminal "tail", for which no crystal structure is available (Figure 4A) (Hale, 2014). Several structural studies have provided detailed information on the organization of NS1 domains, the full-length protein and on the structural polymorphisms of NS1s from different viral sybtypes (Chien et al., 1997; Liu et al., 1997; Wang et al., 1999; Bornholdt and Prasad, 2006; Yin et al., 2007b; Hale et al., 2008a; Cheng et al., 2009; Xia et al., 2009; Kerry et al., 2011; Carrillo et al., 2014). As implied by their names, the RBD of NS1 interacts with the RNA, whereas ED accommodates the majority of interaction sites with NS1 cellular partners (Hale et al., 2008c).

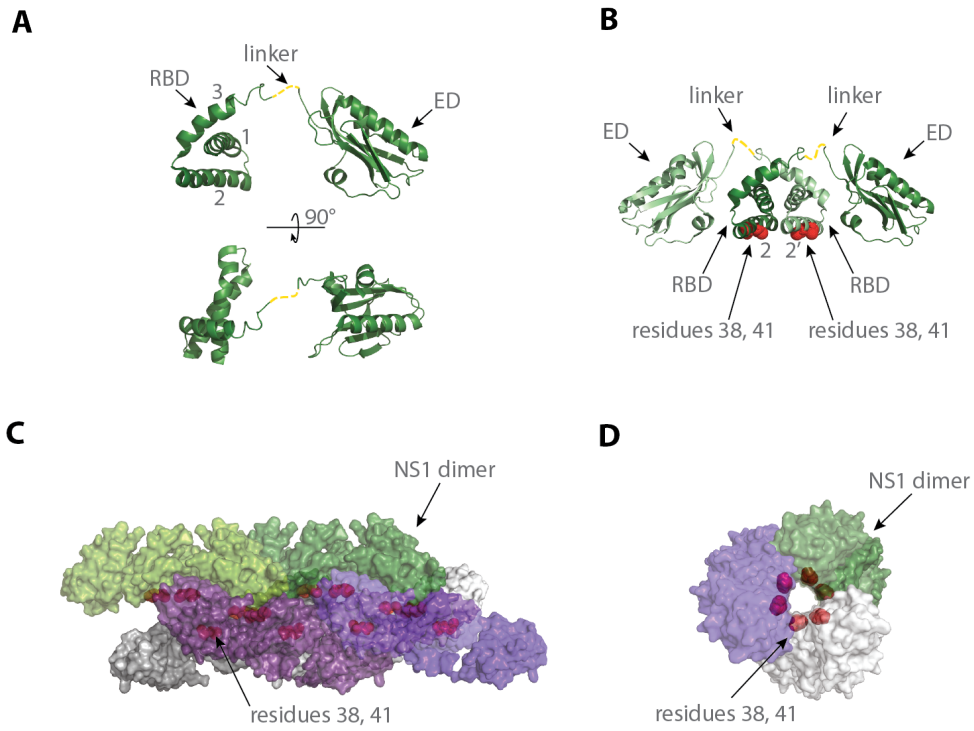

Figure 4. Crystal structure of H5N1 NS1 R38A, K41A mutant protein (Bornholdt and Prasad, 2008). (A) NS1 monomer. The three α-helices of RBD are marked as 1, 2 and 3 (B) NS1 dimer. Groove-forming α-helices of distinct RBD's are marked as 2 and 2'. Residues 38 and 41 are shown in red. (C,D) Oligomerized NS1. The dimers are marked with distinct colors, residues 38 and 41 shown in red can be seen inside the transparent helix.

RBD comprises the first 73 N-terminal aa of NS1 (Qian et al., 1995a; Yin et al., 2007b). About 80 % of its residues are organized into three positively charged α-helices (Qian et al., 1995a; Liu et al., 1997). RBD itself forms highly stable dimers in which anti-parallel α-helices 2 and 2' of corresponding monomers form the groove in which RNA can be accommodated (Figure 4B) (Chien et al., 1997; Wang et al., 1999). As a dimer NS1 can bind ss- and, with higher affinity, dsRNA in a sequence-unspecific manner (Hatada and Fukuda, 1992; Qian et al., 1995b; Chien et al., 1997). Residue R38 is critical and residue K41 is important for both RBD dimerization and NS1 ability to interact with RNA (Hatada and Fukuda, 1992; Wang et al., 1999).

Inter-domain linker in most cases is comprised of residues 74-84, but its length may vary between viral subtypes, contributing to NS1 structural polymorphism (Bornholdt and Prasad, 2006; Carrillo et al., 2014; Kerry et al., 2011).

ED in most viral subtypes encompasses residues 88–202 (Hale, 2014). It comprises seven β-strands and three α-helices and can also homodimerize (Bornholdt and Prasad, 2006; Hale et al., 2008a; Xia et al., 2009). The dimerization occurs primarily via helix-helix interface, in which strictly conserved residue T187 plays a critical role (Hale et al., 2008a; Kerry et al., 2011). Unlike stable RBD dimerization, the interactions between ED monomers are likely to be transient (Kerry et al., 2011; Hale, 2014).

Dimerization is important for NS1 function and NS1 monomers have not been observed *in vitro* or *in vivo* (Hale, 2014). The crystal structure of NS1 dimer suggests that its formation relies on the stable interactions between RBDs of NS1 monomers whereas EDs are not directly involved in dimerization and are probably free for interactions with cellular proteins (Figure 4B) (Bornholdt and Prasad, 2008). Interestingly, the full-length protein not only can dimerize, but also may form hollow helices (Bornholdt and Prasad, 2008). The formation of such oligomers is mediated by inter-NS1 interactions of both RBD and ED (Figure 4C, D) (Bornholdt and Prasad, 2008; Carrillo et al., 2014). In addition, full-length NS1 retains conformational plasticity with three possible orientations of ED to RBD. Preference for certain states is dependent

on NS1 inter-domain linker length, residue 71 and a mechanical hinge, and determines strain-specific variations in NS1 structure and function (Carrillo et al., 2014).

1.7.4 Inhibition of interferon signaling at pre-transcriptional level

Inhibition of the interferon response is a function of NS1. The mechanisms behind this function have been extensively studied over the past two decades and according to the current paradigm NS1 subverts development of immune responses by counteracting PRR signaling, co- and post-transcriptional inhibiting of host gene expression and post-translationally inactivating interferon-stimulated gene products (Ayllon and Garcia-Sastre, 2014). For this, the multi-functional NS1 targets numerous factors, which are discussed below. The mapped interactions of NS1 are schematically shown in Figure 5.

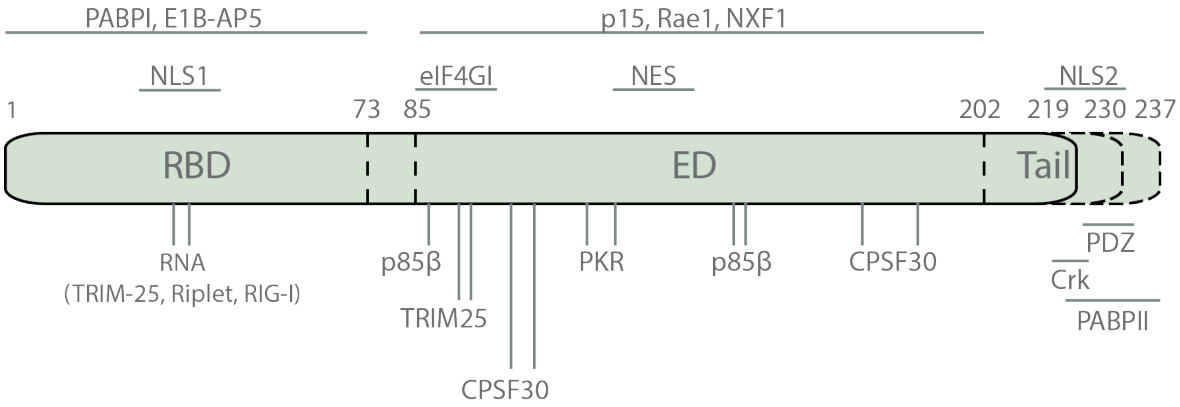

Figure 5. Schematic representation of NS1 and its described interactions. NS1 length is 219–237 amino acids, depending on the viral subtype. Residues 1–73 comprise an RNA-binding domain (RBD), residues 85-202 comprise an effector domain (ED) and residues 202–219/230/237 comprise a C-terminal "tail". NS1 contains two nuclear localization signals (NLS1 and NLS2) and a nuclear export signal (NES). NS1 is thought to interact with RNA, RIG-I, TRIM25 and Riplet to alleviate RIG-I signaling, with PABPII and CPSF30 to inhibit cellular mRNA processing, with PKR, eIF4GI and PABPI to facilitate viral protein synthesis, with nuclear pore complex components NXFI, p15, Rae1 and E1B-AP5 to inhibit nuclear export of cellular mRNAs, with p85β, PDZ-domain containing proteins, Crk and Crk-like proteins to regulate cellular signaling.

NS1 inhibits interferon signaling at the pre-transcriptional level by preventing activation and nuclear translocation of IRF3, AP-1, NFkB mainly via alleviating RIG-I signaling (Talon et al., 2000; Ludwig et al., 2002; Wang et al., 2000; Geiss et al., 2002; Munir et al., 2012). Multiple studies indicate that NS1 employs both its RBD and ED

to subvert RIG-I signaling at multiple steps (Wang et al., 2000; Ludwig et al., 2002; Haye et al., 2009; Tisoncik et al., 2011). NS1 binds RIG-I, although direct inhibitory effects of this interaction have not been reported yet (Opitz et al., 2007; Mibayashi et al., 2007). It also established inhibitory interactions with two indispensable RIG-I regulators: TRIM25 and Riplet (Gack et al., 2009; Rajsbaum et al., 2012). Interaction with TRIM25 requires E96, E97 residues within NS1 ED and RNA-binding residues R38, K41, although it is not clear whether the latter are involved in interaction with TRIM-25 (direct or RNA-mediated) or just support suitable NS1 conformation (Gack et al., 2009). Interaction with Riplet requires R38, K41 although again their exact roles in this interaction still need clarification (Rajsbaum et al., 2012). The involvement of R38 and K41 residues in regulation of RIG-I has raised discussions of another possible mechanism of RIG-I inhibition by NS1 in which NS1 sequesters dsRNA, a known RIG-I inducer, thereby preventing activation of the RIG-I signaling axis. The role of NS1 RNA-binding in pre-transcriptional control of immune responses, however, needs to be elucidated, because (i) influenza A does not seem to generate dsRNA during its replication (Wisskirchen et al., 2011) and (ii) the affinity of NS1 for dsRNA is much lower than that of RIG-I (Chien et al., 2004; Yin et al., 2007a; Vela et al., 2012).

In addition to inhibition of RIG-I signaling, NS1 has evolved several other ways to effectively inhibit interferon induction at the transcriptional level. It subverts both canonical and non-canonical NFkB pathways (Ruckle et al., 2012) and prevents nuclear translocation of NFkB via direct inhibition of alpha and beta subunits of IKK (Gao et al., 2012). NS1 impairs c-Jun and JNK signaling, preventing AP-1-regulated gene expression (Ludwig et al., 2002). It also alleviates IFN response by inducing suppressor of cytokine signaling-3, a negative regulator of JAK-STAT signaling (Pauli et al., 2008).

1.7.5 Inhibition of interferon signaling at post-transcriptional level

NS1 acts beyond pre-transcriptional level and controls development of antiviral responses also post-transcriptionally by targeting pre-mRNA processing and nuclear ex-

port machinery of the host. The majority of cellular mRNAs produced by RNA polymerase II undergo cleavage of their 3' termini and subsequent addition of a polyadenine stretch (poly(A)) which is required for their effective translation and also regulates their nuclear export (Vassalli et al., 1989; Zarkower and Wickens, 1987; Huang and Carmichael, 1996). Pre-mRNA cleavage and polyadenylation are catalyzed by cleavage and polyadenylation specific factor (CPSF)—a polyprotein complex formed by four subunits (Wilusz et al., 1990; Colgan and Manley, 1997). NS1 binds CPSF30, the smallest protein of subunit 4, thereby inhibiting the activity of the whole complex (Nemeroff et al., 1998). The interaction between NS1 ED and CPSF30 has been characterized both biochemically and structurally and the current model proposes interaction of NS1 ED with F2F3 zinc finger pocket of CPSF30 (Noah et al., 2003; Twu et al., 2006; Kochs et al., 2007; Das et al., 2008). Recent crystal studies suggest that ED dimerization is incompatible with CPSF30 interaction (Aramini et al., 2011; Kerry et al., 2011). Hydrophobic residues 184–188 within NS1 ED are essential for this interaction and mutation of G184 prevents NS1-CPSF complex formation (Das et al., 2008). In addition, residues F103 and M106 facilitate NS1-CPSF30 complex formation (Kochs et al., 2007; Das et al., 2008). Residues 223–237 within C-termini of some NS1 proteins comprise a site for interaction with poly(A)-binding protein (PABP)II—another protein essential for effective polyadenylation (Li et al., 2001b). Blocking cellular pre-mRNA processing via interactions with CPSF30 and PABPII provides several advantages for viral replication: (i) it prevents production of functional cellular mRNAs, including those involved in IFN response; (ii) it prevents nuclear export of capped cellular mRNAs providing a pool of 5' caps to be "snatched" by viral polymerase (Nemeroff et al., 1998); (iii) it does not inhibit production and export of viral mRNAs whose polyadenylation is independent of CPSF30 (Plotch and Krug, 1977). The residues involved in CPSF30 binding are highly conserved among human influenza A NS1 proteins (Kochs et al., 2007; Das et al., 2008).

NS1 also inhibits mRNA splicing by binding to the formed spliceosome and sup-

pressing its catalytic activity (Lu et al., 1994; Qiu et al., 1995). Interestingly, this effect is specific to host mRNAs. Although viral mRNAs recruit cellular spliceosome for their post-transcriptional processing, NS1 does not affect splicing of its own mRNA (Robb et al., 2010) and has little, if any, effect on M mRNA splicing (Salvatore et al., 2002; Robb and Fodor, 2012). The possible reason for such selectivity could be recognition of specific motifs within viral mRNAs by NS1, which, however, is questionable, since no sequence specificity is so far known for NS1 RNA binding. Another possibility is recruitment of different spliceosomal factors to viral transcripts by viral polymerase (Fournier et al., 2014).

In addition to its direct effects on mRNA synthesis and processing, NS1 also inhibits nuclear export of cellular mRNAs. It specifically binds to nuclear pore complex components NXF1, p15, Rae1 and E1B-AP5, thus contributing to retention of cellular mRNAs in the nucleus (Satterly et al., 2007). Importantly, inhibition of nuclear pore complex by NS1 does not attenuate viral replication, as export of viral RNAs relies on the alternative Crm1-mediated pathway (Neumann et al., 2000).

The combination of NS1 effects on host mRNAs production, processing and export contributes to host protein synthesis shut-off which is commonly observed during influenza A infection (Beloso et al., 1992).

1.7.6 Direct inhibition of interferon-stimulated gene products

In addition to the control at pre-transcriptional, transcriptional and post-transcriptional level, NS1 antagonizes IFN responses by directly targeting PKR and OAS.

Influenza A mRNAs are structurally indistinguishable from cellular mRNA and hence production of viral proteins requires functional cap-dependent translation. For this, the virus prevents activation of the negative translational regulator PKR (Katze et al., 1986; Katze et al., 1988). Inhibition of PKR is to a large extent a function of NS1, as viruses lacking functional NS1 can only replicate in the absence of PKR (Bergmann et al., 2000). NS1 was proposed to prevent activation of PKR by binding to dsRNA and

thus sequestering it away from PKR (Lu et al., 1995). Such regulation, however, seems controversial for several reasons: (i) while PKR senses dsRNA in the cytoplasm, the site of influenza A RNA transcription and replication is the nucleus, and thus the presence of virus replication intermediates in the cellular cytoplasm seems unlikely (Jackson et al., 1982), (ii) viral genomes are exported to the cytoplasm as vRNPs, and thus base-paired regions of vRNA are likely to be inaccessible to PKR (Coloma et al., 2009), (iii) the affinity of NS1 for dsRNA is much lower than that of PKR (Chien et al., 2004; Husain et al., 2012), and (iv) dsRNA binding function of NS1 is not required for PKR inhibition (Li et al., 2006a). NS1 has been shown to form a complex with PKR which appears to be inhibitory for PKR activation (Tan and Katze, 1998; Li et al., 2006a). It has been shown that residues 123–127 within NS1 are required for interaction with PKR and its inhibition (Min et al., 2007).

Inhibition of OAS/RNAse L pathway is also a function of NS1. So far only one study has described the putative mechanism of NS1 control over the OAS/RNAse L pathway which presumes that dsRNA binding by NS1 is required for sequestration of dsRNA away from OAS (Min and Krug, 2006). This observation is supported by low affinity of OAS to dsRNA (Hartmann et al., 2003), however, the abundancy of influenza-generated dsRNA in cell cytoplasm still remains an open question.

1.7.7 Other pro-viral functions of NS1

In addition to its critical role in control of innate immune responses, NS1 targets a number of other cellular factors to facilitate virus replication. These additional pro-viral functions of NS1 include regulation of production of viral RNA and protein synthesis, control of apoptosis and modulation of cell signaling.

Influenza A virus RNA production occurs in two phases: early, when NS1 and NP vRNAs are preferentially synthesized, and late, when all eight segments are produced in equimolar quantities (Skehel, 1973; Shapiro et al., 1987). This temporal regulation of vRNAs production has been shown to require functional NS1 (Falcón et al., 2004)

and is linked to residues 123 and 124 within its ED (Min et al., 2007). Although these residues overlap with the PKR binding site on NS1, NS1 regulates vRNA independently of its interaction with PKR, which has been proven using PKR-deficient mice (Min et al., 2007). NS1 through an as yet unknown mechanism also specifically regulates production of HA vRNA (Maamary et al., 2012). The regulation of vRNA production by NS1 is likely linked to its interaction with vRNPs which disrupts inhibitory binding of cellular helicase DDX21 to PB1 (Marión et al., 1997; Chen et al., 2014).

NS1 has been also discussed as a factor that regulates protein synthesis in infected cells. As viral mRNAs contain 5'meG "cap" and 3' poly(A), they are structurally indistinguishable from cellular mRNAs and require the same subset of translation factors (Poch et al., 1989; Poon et al., 1999). The bottleneck of the complex translation process is its initiation which requires coordinated assembly of translation factors and 43S pre-initiation complex on mRNA followed by start codon scanning and ribosome assembly (Pestova et al., 2001). In addition to the prevention of eIF2α inhibition by PKR described in section 1.7.6, NS1 contributes to translational control by direct interactions with translation initiation factors. Thus, fragment 1–81 has been shown to bind the translation initiation factor 4GI (eIF4GI) via protein-protein interactions and is supposed to facilitate recruitment of this factor onto viral mRNAs (Aragon et al., 2000). In addition, it interacts with PABPI, another factor essential for translation initiation (Burgui et al., 2003). The proposed mechanism of translational regulation by NS1 suggests preferential recruitment of translation initiation factors on viral mRNAs due to specific recognition of their 5' untranslated region (UTR) by NS1 (de la Luna et al., 1995). Such a mechanism, however, is in controversy with the very low affinity of NS1 to ssRNA and with its proposed nonsequence-specific binding to RNA (Hatada and Fukuda, 1992) and requires further description of NS1 interactions with translation factors and/or search for possible specificity of NS1 towards viral mRNAs.

Influenza A infection induces prolonged activation of PI3K and its downstream effector Akt several hours post infection due to NS1 activity (Ehrhardt et al., 2006; Ehrhardt

and Ludwig, 2009). Structural information suggests that NS1 ED forms heterotrimeric complex with regulatory (p85) and catalytic (p110) subunits of PI3K. Interaction with NS1 alters positions of p85 and p110 in respect to each other and results in PI3K/Akt signaling activation (Hale et al., 2008b; Hale et al., 2010a). The interaction between NS1 and p85 is dependent on the ^{164}PxxP1^{167} Src-homology 3 domain (SH3) motif and a conservative tyrosine 89 within NS1 ED (Hale et al., 2006; Shin et al., 2007a; Shin et al., 2007b). As PI3K/Akt signaling inhibits apoptosis, its activation by NS1 was initially regarded as a mechanism to prevent premature cell death and secure viral replication (Ehrhardt et al., 2007; Zhirnov and Klenk, 2007). However, p85-binding deficient NS1 also exibits anti-apoptotic activity indicating possible redundancy in NS1 approaches to control apoptosis (Jackson et al., 2010a).

In addition to the PI3K/Akt branch, NS1 also controls the JNK branch of apoptosis. This control is realized through binding of SH3 domains present in some NS1 subtypes to cellular adapter proteins Crk and Crk-like (Heikkinen et al., 2008; Hrincius et al., 2010).

Another way by which NS1 protects infected cells from apoptosis could be its ability to bind to signaling proteins that contain a domain called PDZ (**p**ostsynaptic density protein 95, ***D****rosophila* disc large tumor suppressor, and **z**onula occludens 1 protein) (Liu et al., 2010). The C-terminus of 230 aa long avian NS1s can contain a PDZ-binding motif ESEV/EPEV, which mediates interaction with PDZ-domain containing proteins (Golebiewski et al., 2011). In addition to apoptosis regulation, PDZ binding may provide control over formation of cellular junctions and establishment of cell polarity, although these possibilities, as well as the overall effect of PDZ-binding on influenza A virulence are yet to be investigated (Jackson et al., 2010b; Zielecki et al., 2010; Javier and Rice, 2011).

1.7.8 NS1 diversity

NS1 contributes to influenza A variability by displaying a substantial number of sequence, length, structure, modification and functional polymorphisms. Initial studies of sequence similarity revealed two groups of NS1 proteins designated as alleles A and B (Scholtissek and von Hoyningen-Huene, 1980). Allele A NS1s have been found in avian, as well as in mammalian strains, whereas allele B appears to be exclusively avian indicating the contribution of NS1 to host-specificity (Treanor et al., 1989; Ludwig et al., 1991). The percentage of sequence identity is over 90 % within alleles, but between alleles it is below 70 % (Scholtissek and von Hoyningen-Huene, 1980).

Structural polymorphisms of NS1 are mainly dependent on the length of inter-domain linker. A recent study has shown that the linker length and residue 71 determine the preference of RBD orientation towards ED and provide the structural basis for strain-dependent NS1 functions (Carrillo et al., 2014). Interestingly, whereas the inter-domain linker is 12 amino acid long in the majority of NS1s, its length in highly pathogenic avian influenza A viruses is only 7 amino acids, indicating that although there are no known interaction motifs within the linker, it still can determine virulence (Long et al., 2008).

Although functional NS1 is required for replication in immune-competent systems (Garcia-Sastre et al., 1998; Donelan et al., 2003), NS1s from different subtypes may vary in their ability to modulate certain cellular responses. For example, although different NS1s are able to bind components of the RIG-I pathway, the extent to which they inhibit RIG-I signaling varies and some H1N1, H2N2 and human H3N2 viruses fail to pre-transcriptionally block IFN induction (Kochs et al., 2007; Haye et al., 2009; Munir et al., 2011; Munir et al., 2012). These viruses compensate for their inefficiency in pre-transcriptional control with strong CPSF30 binding, whereas the mouse-adapted PR8 strain as well as the pandemic H1N1/2009 (pH1N1/2009) acquired mutations that prevent interactions with CPSF30 (Kochs et al., 2007; Hale et al., 2010c). Human and avian NS1 proteins also markedly differ in their ability to modulate signaling pathways:

instead of functional PDZ-binding motifs ESEV/EPEV found in avian strains human NS1s contain RSKV/RSEV motifs that can not interact with PDZ-domain containing proteins (Liu et al., 2010). Furthermore, interaction with CRK and CRK-like protein adapters is also a characteristic of avian strains and the 1918 pandemic influenza A virus which contain SH3-binding motif (Heikkinen et al., 2008).

Naturally occurring NS1 proteins vary in length due to C-terminal truncations and extensions that arise via appearance or loss of nonsense codons (Parvin et al., 1983). Although selective pressure maintains avian and avian-originated human NS1 at 230 aa and human pH1N1/2009 at 219 aa, the appearance of truncated isolates has been observed (Suarez and Perdue, 1998; Lakspere et al., 2014). The attempts to address the effects of NS1 C-terminal truncations on viral pathogenicity brought controversial results and the significance of these truncations is still unclear (Hale et al., 2010b; Tu et al., 2011). The relatively short NS1 C-terminus can accommodate, lose or acquire a number of binding motifs and modification sites that can mediate virus-host interactions, including PDZ- and Crk/CrkL-binding sites (Heikkinen et al., 2008; Liu et al., 2010), histone mimic motif (Marazzi et al., 2012), PABPII (Li et al., 2001b), nuclear and nucleolar localization sites (Melen et al., 2012), phosphorylation (Hsiang et al., 2012) and ISGylation sites (Hsiang et al., 2009). Therefore addressing the role of NS1 C-terminal polymorphisms could be beneficial for understanding viral evolution and pathogenicity.

2 Objectives of the Present Study

The objective of the present study was to provide novel insights into modulation of influenza A virus-host cell interactions by NS1 and to utilize that knowledge further. The specific aims were:

1. To study the functional role of the NS1 nucleic acid binding in transcriptional control of cellular genes
2. To understand how NS1 regulates protein synthesis
3. To determine the contribution of the NS1 C-terminal "tail" to viral replication and pathogenicity
4. To utilize understanding of translational modulation by NS1 for improvement of a biotechnologically important cell-free protein synthesis system

3 Materials and Methods

The material and methods used in this study are described in details in the original publications and manuscripts (I – IV). This section briefly summarizes them.

All cells used in this study were maintained in their recommended culture media containing required supplements at 37 °C at 5 % CO_2. The cells were regularly checked for *Mycoplasma* contamination. The list of established cell lines and primary cells used in this study is presented in Table 1.

Table 1. Cells used in the study

Cells	**Study**
Madin-Darby Canine Kidney cell line (MDCK)	I, IV
Human retinal pigment epithelium cell line (RPE)	I
Adenocarcinomic human alveolar basal epithelium cell line (A549)	IV
African green monkey kidney cell line (Vero)	I, III
Human embryonic kindey 293 cells (HEK)	I, III
Primary human macrophages	I, III

The plasmids used in the study are summarized in Table 2. Their detailed description can be found in corresponding articles.

Table 2. Plasmids used in the study

Plasmid	**Application**	**Source**	**Study**
pHW181-PB2	influenza A reverse genetics	(Hoffmann et al., 2000)	I, III
pHW182-PB1	influenza A reverse genetics	(Hoffmann et al., 2000)	I, III
pHW183-PA	influenza A reverse genetics	(Hoffmann et al., 2000)	I, III

Table 2. Plasmids used in the study

Plasmid	Application	Source	Study
pHW184-HA	influenza A reverse genetics	(Hoffmann et al., 2000)	I, III
pHW185-NA	influenza A reverse genetics	(Hoffmann et al., 2000)	I, III
pHW186-NP	influenza A reverse genetics	(Hoffmann et al., 2000)	I, III
pHW187-M	influenza A reverse genetics	(Hoffmann et al., 2000)	I, III
pHW188-NS	influenza A reverse genetics	(Hoffmann et al., 2000)	I, III
pHW188-NS1-RK/AA	influenza A reverse genetics	in-house	I, III
pHW188-NS1-202	influenza A reverse genetics	in-house	III
pHW188-NS1-220	influenza A reverse genetics	in-house	III
pCMV-tag4A	transfection experiments	Agilent Technologies	I
pCMV-tag4A-NS1-WT	NS1 expression in cells	in-house	I
pCMV-tag4A-NS1-RK/AA	NS1 expression in cells	in-house	I
pNic28-H5N1-NS1	NS1 protein production	Dr. Denis Kainov	II
pNic28-H5N1-NS1-RK/AA	NS1 protein production	Dr. Denis Kainov	II, IV
pNic28-H5N1-NS1-RK/AA-25,26	NS1 protein production	in-house	II
pNic28-H5N1-NS1-RK/AA-48	NS1 protein production	in-house	II

Table 2. Plasmids used in the study

Plasmid	Application	Source	Study
pNic28-H5N1-NS1-RK/AA-67	NS1 protein production	in-house	II
pNic28-H5N1-NS1-RK/AA-25,26,48,67	NS1 protein production	in-house	II
pNic28-H5N1-RBD	NS1 protein production	in-house	II
pNic28-H5N2-NS1	NS1 protein production	Dr. Denis Kainov	II
pNic28-H5N2-NS1-RK/AA	NS1 protein production	Dr. Denis Kainov	II
pNic28-H5N2-NS1-delC	NS1 protein production	Dr. Denis Kainov	II
pET151-H1N1-NS1-RK/AA	NS1 protein production	Dr. Denis Kainov	II
pET151-H1N1-NS1-RK/AA-25,26	NS1 protein production	MA	II
pET151-H1N1-NS1-RK/AA-48	NS1 protein production	in-house	II
pET151-H1N1-NS1-RK/AA-67	NS1 protein production	in-house	II
pET151-H1N1-NS1-RK/AA-25,26,48,67	NS1 protein production	in-house	II
Luciferase control DNA	mRNA synthesis	Promega	II, IV
pGL-HCV-poly(A)	mRNA synthesis	Dr. Ilya Terenin	IV
pGL-HCV-poly(A)	mRNA synthesis	Dr. Ilya Terenin	IV
pGL-globin-SV40	mRNA synthesis	Dr. Ilya Terenin	IV
pGL-globin-poly(A)	mRNA synthesis	Dr. Ilya Terenin	IV
pGL-EMCV-poly(A)	mRNA synthesis	Dr. Ilya Terenin	IV

The viruses used in this study were generated using a reverse genetics system for

influenza A (Hoffmann et al., 2000) and propagated in MDCK or Vero cells. Wild type and mutant viruses were sequenced to ensure the absence of undesirable substitutions and the proper introduction of required mutations. Titers of viral stocks were determined by plaque assay on MDCK cells. The viruses used in the study are summarized in Table 3.

Table 3. Recombinant viruses used in the study

Virus	**Description**	**Study**
WSN-WT	Wild type influenza A/WSN/33 (H1N1) virus	I, III, IV
WSN-RK/AA	Influenza A/WSN/33 (H1N1) virus expressing R38A, K41A mutant NS1 protein	I
WSN-202	Influenza A/WSN/33 (H1N1) virus expressing 202 amino acids long truncated NS1 protein	III
WSN-220	Influenza A/WSN/33 (H1N1) virus expressing 220 amino acids long truncated NS1 protein	III

The list of antibodies used in the study is presented in Table 4.

Table 4. Antibodies used in the study

Antibody	**Source**	**Study**
primary rabbit polyclonal anti-NS1	Prof. Ilkka Julkunen	I
primary mouse monoclonal anti-phospho-eIF2α (Ser51)	Cell signaling	unpublished
primary goat polyclonal anti-luciferase	Promega	II
secondary IRDye680-conjugated donkey anti-mouse	Li-Cor	unpublished
secondary Alexa594-conjugated goat anti-rabbit	Invitrogen	I
secondary Cy5-conjugated donkey anti-goat	Abcam	II

The methods used in the current study are listed in Table 5.

Table 5. Methods used in the study

Method	**Study**
Mammalian cell culture	I, III, IV
Isolation of human peripheral blood mononuclear cells	I, III
DNA-mediated transformation of mammalian cells	I, III
Plasmid-based reverse genetics system for Influenza A	I, III
Virus culture and purification	I, III
Plaque assay	I, III
Influenza A infections	I, III, IV
RNA isolation	I, III, IV
Reverse transcription	I, III
Polymerase chain reaction	I, III
One step reverse transcription and polymerase chain reaction	I, III
Quantitative polymerase chain reaction	I, II
Nuclear protein isolation	I
Cytokine profiling	I, III
Phosphoprotein profiling	III
Enzyme-linked immunosorbent assay	I
Immunofluorescence	I
Gene expression profiling	I, III
Chromatin immunoprecipitation (ChIP)	I
Chomatin immunoprecipitation followed by next generation sequencing (ChIPseq)	I
Recombinant protein production and purification	II, IV
Electrophoretic mobility shift assay	I, II
In vitro transcription	I, II, IV
Sodium dodecyl sulphate polyacrylamide gel electrophoresis	I, II, III

Table 5. Methods used in the study

Method	**Study**
Molecular cloning	I, III, II
Agarose gel electrophoresis	I, IV
Cell free protein synthesis	II, IV
Luciferase reporter assay	II, IV
Microscale thermophoresis	I
Density centrifugation in sucrose gradient	IV
Determination of influenza A morbidity and mortality *in vivo*	III

4 Results and Discussion

4.1 Conserved residues within NS1 bind dsDNA and control transcription of cellular genes (Study I)

The nucleic acid binding function of NS1 is extremely important for influenza A replication and residues R38 and K41 are conserved across viral subtypes (Hatada and Fukuda, 1992; Zohari et al., 2008). The mutations R38A and K41A were shown to prevent dsRNA binding by NS1, and result in increased IFN production by infected cells and severely attenuated viral phenotype (Donelan et al., 2003). Although the proposed role of nucleic acid binding by NS1 is sequestration of viral replication intermediates away from cellular PRRs, it is controversial as stated in Section 1.7.4. We addressed the role of nucleic acid binding function of NS1 using the recombinant influenza A/WSN/33 (WSN) viruses expressing wild type NS1 or its R38A, K41A (RK/AA) mutant which can not bind nucleic acids. In agreement with previous studies (Min and Krug, 2006), RK/AA WSN virus exhibited severely attenuated phenotype in cell culture.

Gene expression profiling of human macrophages infected with RK/AA viruses resulted in strong up-regulation of a large set of cellular genes (**I**, Fig. 1A and B). Gene set enrichment analysis (Subramanian et al., 2005) indicated that these genes were involved in the regulation of innate and adaptive immune responses and belonged to TLR and Jak/STAT signaling pathways, cytokine-cytokine receptor interaction and natural killer cell mediated cytotoxicity pathways. Accordingly, RK/AA infection resulted in increased production of both antiviral and pro-inflammatory cytokines (**I**, Fig. 1C). Importantly, the effect of RK/AA mutations on cellular gene expression and cytokine production was not specific to immune cells and was also observed in a retinal pigment epithelium (RPE) cell line (**I**, suppl. Fig. 1A and B).

Wild type NS1 is abundant in the insoluble fraction of cell lysate (**I**, Fig. 2A), where it associates with cellular chromatin, presumably via protein-DNA, protein-protein or both interactions (**I**, Fig. 2B). Although the direct binding of NS1 to dsDNA has

not been shown previously, the structure of NS1 RBD and full-length dimer does not exclude its interaction with dsDNA (Bornholdt and Prasad, 2008; Cheng et al., 2009). Therefore, we tested this possibility in an electophoretic mobility shift assay using purified recombinant wild type and RK/AA NS1 proteins and synthetic DNA. Indeed, purified wild-type NS1 did interact with 190 bp long linear dsDNA and the dissociation constant (K_d) was 11.1 $\pm$ 0.7 μM, indicative of a probably nonspecific binding. The residues R38 and K41 were absolutely required for dsDNA interaction (**I**, Fig. 3B, C).

The ability of NS1 to associate with cellular chromatin and to bind dsDNA prompted us to test its effects on transcription. To dissect effects of NS1-dsDNA binding and upstream events we addressed this question in a cell-free run-off transcription assay using purified wild type and RK/AA NS1 proteins. Binding of wt NS1 to DNA had inhibitory effects on RNA synthesis by RNA polymerase II and time of addition experiment suggested that NS1 inhibited transcription at its initiation step (**I**, Fig. 3D, E). Interestingly, NS1 prevented RNA synthesis after preincubation with dsDNA or with transcription factors and RNA polymerase II (**I**, Fig. 3F). This finding indicates that NS1 binds dsDNA and inhibits transcription initiation by preventing pre-initiation complex and RNA polymerase II loading on dsDNA. Alternatively, dsDNA binding could coordinate inhibitory protein-protein interactions between NS1 and components of transcription initiation machinery.

Is the dsDNA binding by NS1 that we observed *in vitro* relevant to infection? To answer this question we addressed NS1 interaction with dsDNA in infected cells. Chromatin immunoprecipitation (ChIP) experiment in infected cells revealed the presence of NS1 on the promoter and exon regions of the *IFNB1* gene which is strongly induced upon influenza A infection. Interaction of NS1 with *IFNB1* DNA was R38-, K41-dependent, suggesting direct dsDNA binding as was observed in an *in vitro* assay (**I**, Fig. 4A). The pattern of NS1 association with *IFNB1* promoter and exon regions was opposite to that of RNA polymerase II, which may indicate possible inhibition of *IFNB1* gene expression due to NS1 DNA-binding.

Functional versatility of NS1 complicates studying the role of its dsDNA binding in the context of viral infection, because in addition to nucleic acid binding residues R38 and K41 are involved in inhibitory interactions with TRIM25, RIPLET and RIG-I, which also prevent transcriptional activation of *IFNB1* (Gack et al., 2009; Rajsbaum et al., 2012). To separate NS1 transcriptional effects from its role in virus-induced RIG-I signaling, we addressed the function of residues R38 and K41 in cells overexpressing NS1, taking advantage of the absence of RIG-I activation in such a set up. In this system the wild type NS1 was detected on promoter and exon regions of housekeeping gene *EML4*, indicating its ability to interact with cellular dsDNA (**I**, Fig. 4C). As expected, the RK/AA mutant NS1 was not able to do so. To address direct effects of NS1 dsDNA binding on transcriptional regulation of cellular genes we exposed cells expressing wild type or RK/AA mutant NS1 to polyinosinic:polycytidylic acid (poly(I:C)). External poly(I:C) is detected by cell surface TLR3 and thus IFN responses are induced without activation of dsRNA signaling (Karpala et al., 2005). As expected, the wild type NS1 capable of dsDNA binding strongly inhibited poly(I:C)-induced activation of *IFNB1*, *IFNA1* and *IFNA16*, supporting our hypothesis of its role in direct transcriptional control of cellular immune-related genes (**I**, fig. 4D).

In summary, these results provide a novel insight into IFN response control by NS1, suggesting direct binding of NS1 to promoter and exon regions of DNA and prevention of effective transcription of cellular genes. The K_d of NS1-dsDNA binding was 11.1 ± 0.7 μM and is comparable to the K_d of NS1-dsRNA binding, which is also in micromolar range (Yin et al., 2007a). Provided this, and also given that the NS1 is predominantly localized in cellular nucleus, where its concentration is very high (Li et al., 2010; Marazzi et al., 2012), we can propose that the dsDNA-binding of NS1 is as important as its well-described dsRNA-binding. Further studies in RIG-I-, PKR- and OAS-deficient systems would be helpful for dissecting these two essential functions of NS1.

From our studies we can not determine, whether NS1 interacts with dsDNA as a

dimer or as an oligomer. Interestingly, the tunnel diameter inside the NS1 oligomer was reported to be 20 Ångström (Å) (Bornholdt and Prasad, 2008), and, if no conformational changes occur during binding, it is more likely to accommodate a 20 Å B-form DNA double helix, rather than 26 Å RNA double helix. This question could be addressed in detail with further biophysical and structural studies of NS1-dsDNA complex.

With regards sequence specificity of NS1 dsDNA binding, our results suggest the lack of it, however, one can speculate that during infection transcriptionally active chromatin, i.e. *IFN* genes, is more accessible to NS1. This question, however, needs to be addressed in detail using genome-wide studies, for example, ChIP combined with sequencing. Furthermore, our *in vitro* results suggest that NS1 is able to bind dsDNA *per se*, however it would be interesting to investigate whether NS1 interacts with possible cellular factors to facilitate its targeting to dsDNA in a crowded nucleus environment.

When this study was initiated, NS1 had been thought to control activation of antiviral genes at pre-transcriptional level (see section 1.7.5). However, later it was shown that NS1 also inhibits cellular transcription by targeting histone H3-interacting transcription elongation complex PAF1 (Marazzi et al., 2012). Such an inhibition, however, required histone-mimic sequence ^{226}ARSK^{229} in NS1 C-terminus and was specific to viruses of H3N2 subtype (Marazzi et al., 2012). The mechanism of transcriptional control by NS1 discovered in our study, in contrast, seems to be general across different influenza A subtypes and involves conserved residues R38, K41. The importance of NS1 dsDNA binding for viral replication and conservation of R38 and K41 suggest that this function of NS1 could be an interesting target for development of antivirals. Indeed, a recent study utilized SELEX approach to select ssDNA aptamers that are bound by NS1 with high affinity and suppress its anti-interferon functions (Woo et al., 2013).

4.2 Regulation of general protein synthesis by NS1 (Studies II, IV)

Another essential function of NS1 is to support effective viral protein synthesis in the context of infection and thus secure viral replication. Influenza A infection activates cellular PKR which phosphorylates and inactivates translation initiation factor eIF2α, thus inhibiting protein synthesis and inducing death of infected cells (Levin and London, 1978). NS1 regulates general protein synthesis by preventing PKR activation and its residues 123–127 are thought to be essential for this (Lu et al., 1995; Min et al., 2007). In addition, NS1 interacts with translation initiation factors eIF4GI and PABPI and few studies proposed that it recruits these factors to viral mRNAs thus specifically enhancing viral protein synthesis (de la Luna et al., 1995; Aragon et al., 2000; Burgui et al., 2003). Such recruitment, however, would require specific binding of NS1 to distinct sequences on viral mRNAs.

We addressed regulation of protein synthesis by NS1. To separate the effects of NS1 on protein synthesis from its other functions we used a rabbit reticulocyte lysate (RRL) cell-free translation system.

Avian high pathogenic H5N1 NS1 enhanced translation of reporter mRNA and protein production by RRL over six fold (**II**, Fig. 2A and C). Importantly, the RNA-binding function of NS1 was not required for translation regulation in RRL and the RK/AA mutant protein was as efficient enhancer of translation as the wild type NS1 (**II**, Fig. 2A). This observation allowed us to use the RK/AA NS1 proteins in further experiments as they show remarkably better solubility compared to the wild type proteins (Bornholdt and Prasad, 2008).

NS1 effects on translation in RRL were subtype specific: avian H5N1 and H5N2 NS1s were efficient translational enhancers, whereas pH1N1/2009 NS1 did not affect protein synthesis (**II**, Fig. 2B and C).

Translational enhancement by NS1 was not limited to viral mRNAs. In RRL pro-

grammed with RNAs extracted from infected cells at different time points post infection NS1 promoted production of both viral and cellular proteins (**IV**, Fig. 1A). This finding was supported by our observation that RNA-binding was not required for NS1 translational control in RRL and hence in this system the protein synthesis rate does not seem to depend on virus-specific sequences on RNA with which NS1 could interact, meaning that one should observe enhancement of both cellular and viral mRNA translation.

In addition to its interactions with translation factors, NS1 has been shown to associate with human homolog of *Drosophila melanogaster* Staufen protein (hStaufen) on polysomes in infected cells (Falcón et al., 1999). As the hStaufen has been implicated in regulation of mRNA stability, we addressed if translational enhancement in RRL in the presence of NS1 was a result of increased mRNA stability. This appeared not to be the case and NS1 did not affect the mRNA degradation rate (**II**, Fig. 2E and F). Instead, NS1 enhanced association of mRNA with ribosomes and polysomes in RRL reactions which suggested its possible role in translation initiation (**IV**, Fig. 1B).

We utilized differential effects of H5N1, H5N2 and pH1N1/2009 NS1 proteins on RRL protein synthesis to map NS1 residues essential for translation enhancement. The NS1 protein of pH1N1/2009 is 11 aa shorter than H5N1 and H5N2 NS1s due to a C-terminal truncation. However, neither 11 C-terminal residues, nor the whole ED was essential for translation modulation by NS1. RBD alone was sufficient for this function (**II**, Fig. 2G and H). Importantly, the RBD of NS1 can be involved in protein-protein interactions and therefore we searched to potential determinants of those. The RBD of pH1N1/2009 NS1 contained several unique amino acids compared to H5N1 and H5N2: N25, G26, N48 and W67 (**II**, Fig. 1A). Introduction of these residues singularly or all at once into H5N1 NS1 resulted in loss of its function in RRL protein synthesis. In a reciprocal experiment introduction of Q25/N26 and R67 from H5N1 in pH1N1/2009 NS1 resulted in its gain of function confirming that these residues are essential for translational control in RRL (**II**, Fig. 2I). In the 3D structure of NS1 the side chains of these amino acids are exposed to solvent, indicating their possible involvement in

interaction(s) between NS1 and its protein partner(s) (**II**, Fig. 4B). Thus, our results clearly indicate that in RRL NS1 non-specifically enhances production of both viral and cellular proteins and critical residues for this function are Q25, E26, S48 and R67 with NS1 RBD.

In the system that we used, the stimulation of protein synthesis in RRL was not specific to viral mRNAs and also did not require NS1 RNA-binding function. Therefore, this approach can not address the ability of NS1 to target translation factors to viral mRNAs and our data neither contradict, nor support previous studies.

Translational enhancement by NS1 in RRL occurred due to prevention of eIF2α phosphorylation (Fig. 6). There are four kinases that are known to phosphorylate eIF2α: PKR, PKR-like endoplasmic reticulum kinase (PERK), general control non-derepressible kinase 2 (GCN2) and heme-regulated inhibitor (HRI) (Donnelly et al., 2013). Of these, only PKR has an assigned role in influenza infection. NS1 is proposed to prevent activation of the PKR in infected cells by two mechanisms: (i) by binding to dsRNA with its R38 and K41 and sequestering it away from PKR (Lu et al., 1995) or (ii) by direct interaction with PKR via its residues 123–127 (Min et al., 2007) and the latter activity is proposed to be critical for PKR inactivation. It is possible that endogenous PKR is activated in RRL and NS1 prevents this activation thus securing protein synthesis. However, three observations are not in line with this mechanism: (i) RNA-binding function of NS1 was not required for its function in RRL. Therefore, NS1 could not prevent eIF2α phosphorylation by sequestering possible dsRNA substrates of PKR; (ii) in our experiments NS1 RBD alone was sufficient to prevent eIF2α phosphorylation in RRL via its residues Q25, E26, S48 and R67, whereas previously published data suggests that PKR inhibition by NS1 requires aas 123–127 within its ED; and (iii) a previous study by Min et al. reported that RBD of A/Udorn/72 H3N2 which is highly similar to H5N1 and H5N2 NS1 RBDs and contains residues Q25, E26, S48 and K67 did not inhibit PKR (Min et al., 2007). Therefore, it is possible that the protein target of NS1 in RRL is not PKR. The other kinases possibly inhibited by NS1 are

HRI, GCN2 and PERK. Although their role in influenza A infection are not known, all of them are induced in response to various stress stimuli (Donnelly et al., 2013). The possible inhibition of one of these kinases by NS1 could represent an additional approach by which influenza A secures protein synthesis during cellular stress. However, the exact target is yet to be identified.

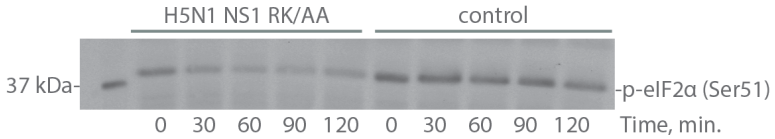

Figure 6. NS1 inhibits eIF2α phosphorylation in RRL. RRL reactions were supplied with H5N1 RK/AA NS1 or control buffer and incubated for the time indicated. Equal amounts of reaction mixes were loaded on the SDS-polyacrylamide gel and phosphorylation status of eIF2α was assayed using immunoblot.

Because NS1 proteins used in our work were deficient of RNA binding, our results do not address possible targeting of NS1 on specific sequences on mRNA. However, in a recent study Marc et al. have identified virus-specific motifs on RNAs to which NS1 bound with nanomolar K_d (Marc et al., 2013). Biologically, such binding could be related to several essential processes in virus life cycle, including enhanced translation of viral mRNAs, however, it is still yet to be confirmed in infected cells. Altogether, our results do not contradict possible targeting of NS1 to viral mRNAs, but demonstrate a strategy of general protein synthesis stabilisation during infection employed by the virus.

4.3 C-terminus of NS1 contributes to modulation of host antiviral responses (Study III)

Although NS1 is a well structured protein, its C-terminus is likely to be unstructured (Hale et al., 2008c). Nevertheless, it can play an essential regulatory role in infection, as it accommodates numerous interaction motifs and modification sites that can modulate virus-host interactions, ofter in strain-specific manner (Li et al., 2001a; Hsiang

et al., 2009; Liu et al., 2010; Hsiang et al., 2012; Marazzi et al., 2012; Melen et al., 2012). Naturally occurring truncations and extensions of NS1 C-terminus are have been described in influenza A viruses of different genetic backgrounds (Suarez and Perdue, 1998). The NS1 protein of H1N1 viruses isolated in 2009–2014 is typically 219 aa long, and H1N1 viruses encoding NS1 of 202 and 230 aa have been recently reported in Finnish patients (Lakspere et al., 2014). However, the relevance of NS1 C-terminal truncations and extensions to viral replication and virulence is unclear.

We aimed to address the functional role of NS1 C-terminus length using influenza A viruses of well-characterized A/WSN/33 background that express NS1 proteins of 202, 220 and 230 amino acids (**III**, Fig. 1B). All viruses were able to propagate in immune-competent MDCK cells, but the virus with the shortest NS1 (202 aa) displayed delayed growth kinetics (**III**, suppl. Fig. 1A).

We addressed the effects of NS1 C-terminus length in human macrophages. Sequential truncations of NS1 C-terminus increased transcriptional activation of cellular genes and the virus expressing the shortest NS1 was least successful in control of host gene expression (**III**, Fig. 2A). The majority of genes differentially regulated by NS1 C-terminus length, e.g. *MX1*, *ISG15*, *OASL*, *CCL8*, *IL6*, and *IL8*, was involved in the regulation of innate and adaptive immune responses (**III**, Fig. 2B). This effect was to some extent reflected on the level of cytokine secretion and amounts of IL-6 and IL-8 secreted by macrophages were increased upon NS1 truncations (**III**, Fig. 2C and D).

Differential regulation of immune-related gene expression and cytokine production prompted us to address the role of NS1 C-terminus in cellular signaling pathways. We observed that C-terminal truncations of NS1 upregulated phosphorylation of several phosphoproteins involved in control of immune responses. The shorter the NS1 C-terminus was, the stronger was observed phosphorylation of STAT3, JNK, HSP60 and AMPKα1 (**III**, Fig. 3).

We used the viruses that express 230 and 202 aa long NS1 to address the effect of NS1 C-terminus *in vivo*. NS1 C-terminus deletion reduced influenza A virulence

and pathogenicity in mice (**III**, Fig. 4A and B), suggesting the regulatory role of NS1 C-terminus in infection. Thus, our results indicate that the length of NS1 C-terminus is essential for interactions with the host cell, control of antiviral responses and modulation of viral pathogenicity.

The C-terminus of A/WSN/33 NS1 contains human-like PDZ-binding motif for which no interactions have yet been identified and a phosphorylation site T215 for which the effect is also unclear (Jackson et al., 2010b; Hsiang et al., 2012). Nevertheless, our data indicate the functional role of NS1 C-terminus length in the regulation of the host immune responses and in viral pathogenicity and virulence. Sporadic truncations and extensions of NS1 are quite seldom events, but they could provide opportunities to lose, acquire or activate functional motifs within NS1 C-terminus. For example, despite that in initial experiments appearance of functional Crk/CrkL-binding motif in H1N1 NS1 C-terminus did not bring on notable effects (Hale et al., 2010b), it seems that a combination of Crk/CrkL and NS1 C-terminus extension could promote viral replication and increase virulence (Dr. Eike Hrincius, personal communication). Further modeling of C-terminal polymorphisms on pH1N1/2009 genetic background could provide deeper insights in virus-host interactions and influenza A adaptation to host.

The H1N1 influenza A viruses isolated from 2009 demonstrate the highest substitution rate in NS1 compared to other known viral subtypes (Xu et al., 2011). A recent study conducted in UK indeed reported that currently circulating pH1N1/2009 virus rapidly adapted to human, and acquired mutations in multiple viral genes, including NS1, that increase viral replication and limit host cytokine production (Elderfield et al., 2014). Several isolates of pH1N1/2009 influenza A have acquired a functional Crk/CrkL-binding domain or an extended C-terminus (**III**, Fig. 1A). Although such viruses may not be of immediate concern, monitoring of changes within NS1 and its C-terminus is worth undertaking, as while amino acid variations in NS1 C-terminus are not detrimental, they provide the opportunity for influenza A viruses to probe additional ways of virus-host interactions under human selection pressure.

4.4 NS1 as a tool to improve cell-free protein synthesis system (Study IV)

The effects of NS1 on translation in RRL observed in Study II prompted us to utilize NS1 for improvement of RRL cell-free translation system. RRL is widely used for translation of specific templates, membrane protein studies, to study post-translational events in folding and protein modification, and high-throughput screening for protein-protein interactions (Fuller and Cuthbert, 2000; Douthwaite, 2012; Fixsen and Howard, 2010; Wang et al., 2011). However, low protein yield is a considerable limitation of RRL, which should be overcome to improve its current applications (Carlson et al., 2012). As viruses have evolved multiple ways to facilitate protein synthesis, a possible way to improve cell-free protein synthesis is addition of viral factors which regulate translation. These include viral proteins and specific regulatory structures on mRNA such as UTRs or IRESs.

The NS1-mediated enhancement of RRL translation occurred through inhibition of eIF2α phosphorylation (Fig. 6) and stabilization of translation initiation and was not specific to viral mRNAs (**IV**, fig. 1). We reasoned that RRL translation could be reinforced using mRNAs with specific 5' and 3' regulatory structures which together with NS1 would exert a cumulative effect. For this several reporter mRNAs with 5'meG or IRES and with different 3' UTRs were tested in RRL at their optimal concentration (**II**, Fig. 2D; **IV**, Fig. 2A). We observed the most robust enhancement of translation when mRNA containing 5' encephalomyocarditis virus (EMCV) IRES and 3' poly(A) sequence was used together with NS1. The protein production by RRL in this case was increased over 11 fold (**IV**, Fig. 2B). The EMCV IRES is known to initiate translation with limited initiation factor requirement and without ribosome scanning for AUG codon (Pestova et al., 2001). However, it is still dependent on non-phosphorylated eIF2α (Terenin et al., 2008), which in our system is controlled by NS1.

In summary, our strategy to use viral enhancers of translation provides an easy and

efficient way to improve cell-free translation in RRL and enhance its protein yield. This strategy appears to be more effective compared to traditional usage of small molecular inhibitors of eIF2α kinases (Jammi et al., 2003). It also provides a proof-of-concept for generally utilizing viral proteins to modify cell-free translation systems from different origins.

5 Conclusions and Future Perspectives

NS1 is an extremely versatile protein that controls influenza A virus-host interactions at multiple levels. It has been extensively studied over the past few decades, but despite a great deal of information obtained, the understanding of multiple functions of NS1 is still far from being complete. For example, nucleic acid binding by R38 and K41 of NS1 has been well-described, but its role in the viral replication cycle still needs elucidation. NS1 interactions with cellular factors of translation might facilitate selective synthesis of viral proteins, but the exact mechanism needs to be described. Furthermore, a C-terminal disordered "tail" of NS1 is a key contributor to NS1 diversity and length polymorphisms, but its role in viral replication is far from being understood.

In this work I set specific questions aiming to provide novel insights into modulation of influenza A virus-host interactions by NS1: What is the role of NS1 nucleic acid binding and residues R38 and K41 in infection? Does NS1 specifically or non-specifically enhance translation? What is the contribution of NS1 C-terminus in modulation of virus-host interactions?

We approached of nucleic acid binding by NS1 in our study I and demonstrated that NS1 can directly bind dsDNA and inhibit cellular gene expression at transcriptional level. This does not exclude the RNA-binding—a well-characterized function of NS1—but seems to be as important and provides additional level of NS1 control over host immune responses. As it often occurs in science, our observation not only uncovers a previously undescribed function of NS1, but also brings up several questions for further elucidation.

How does NS1 inhibit transcription in the cell, without leading to its premature death? Does it target specific cellular gene subsets? Are there any cellular factors hijacked by the virus to secure this targeting? Addressing these questions in genome-wide distribution of NS1 on cellular dsDNA using a high-throughput method such as ChIP-seq, and identification of NS1 interacting partners using immunoprecipitation coupled

with mass-spectrometry analysis could contribute to understanding the mechanistic details of transcriptional control by NS1. As the NS1 residues involved in this function are conserved, one would expect that the transcriptional control by dsDNA binding is a common function of NS1 proteins. Is it so? This can be addressed in question with similar studies using other influenza A viruses, such as A/Udorn/72 (H3N2), A/PR8/34 (H1N1) and A/California/07/2009 (pH1N1/2009) for which reverse genetics systems are available. Furthermore, as the NS1 nucleic acid binding function(s) appear to be critical for influenza A replication in immune competent systems and residues R38 and K41 are conserved, targeting this function with an inhibitor molecules may provide a promising antiviral, to which resistance is less likely to develop.

In our study II we addressed the translation modulation by NS1 in a cell-free translation system. We observed that NS1 non-specifically enhanced translation of viral and cellular mRNAs. The mapped residues within NS1 that secure this function indicate that it occurs via protein-protein interactions and RNA binding is not involved. Thus, our results did not disprove or support previous suggestions that NS1 specifically targets viral mRNAs and recruits translation factors therein. We rather added a new level of translational control by NS1. Although our studies of translational control by NS1 might be limited by the system used, our results provoke further investigation of cellular responses to influenza A infection. Are there translational regulators, other than PKR, induced by viral infection and the linked cellular stress? Answering this question by specific studies of activation of eIF2α kinases upon influenza A infection and probing possible protein-protein interactions between NS1 and these kinases can bring new insights in understanding of complex control over cellular protein synthesis during infection.

Interestingly, our observation that NS1 prevents eIF2α phosphorylation in RRL allowed us to develop an approach to utilize NS1 to improve biotechnologically important cell-free translation system and to increase the protein yield. Our study IV has laid a proof-of-principle for using viral translational enhancers in biotechnological systems.

As Influenza A infects mammals, our method improved mammalian cell-free translation system based on RRL. Viruses infect all forms of life and are obligate parasites of host translation, hence further studies may identify viral enhancers for any given cell-free translation system.

Finally, in our study III we showed that the length of NS1 C-terminal tail contributes to control over cellular antiviral responses and regulates virus pathogenicity. These findings suggests that further monitoring of NS1 length polymorphisms should be undertaken. As human infections with influenza A of pH1N1/2009 origin are still a cause of concern, it would be important to address the role of NS1 C-terminal truncations and extensions using reverse genetics system for pH1N1/2009. Furthermore, influenza A strains lacking NS1 protein are considered as potential vaccine candidates (Mossler et al., 2013), however, in my experience propagation of such viruses in cell culture is extremely challenging. As viruses expressing C-terminally truncated NS1 induce stronger antiviral responses and their virulence is limited, such viruses could be investigated further as potential candidates for vaccine development.

Altogether, this work added three new functions of different NS1 determinants in regulation of several aspects of virus host interactions. Although it would be interesting to study each of these functions in more detail, it reminds us of a bigger question to address: How does such a small protein like NS1 retain its functional versatility? Such a versatility is apparently controlled at the level of protein structure, modifications, and temporal and spatial regulation. Unfortunately, multiple studies addressingJAKAKA these issues, were performed using different viral strains and experimental approaches and, although they provided a huge amount of information, the detailed control of NS1 multifunctionality in a cell during infection still remains largely elusive. Undoubtedly, further studies of influenza A virus-host interactions and their modulation by NS1 will bring deeper understanding of multiple functions of this extremely interesting protein and hopefully will contribute to improvement of current means to control influenza A.

Acknowledgements

I have been working on this dissertation for five years at the Institute for Molecular Medicine Finland, FIMM. This has been an exciting time but also a long way which could not be completed without assistance, support and friendship of people that I was lucky to have around.

I thank my supervisor Dr. Denis Kainov for providing me with never-ending opportunities to develop, helping to find ways out of what could seem to be a dead end and for being an example of real enthusiasm and determination. I clearly learned a lot while working with you, Denis.

I am grateful to my co-supervisor Prof. Sarah Butcher for her never-failing support, wise advices on every issue, and hours of inspiring discussions. Sarah, your door was always open for me and your energizing laughter and *"It's gonna be fine!"* helped me keep on going when I could not believe that this dissertation would ever be completed. Thank you.

Special thanks to my thesis advisory board Prof. Kalle Saksela and Prof. Dennis Bamford for their guidance and advices during all five years.

I am grateful to Docent Maija Vihinen-Ranta and to Docent Thedi Ziegler who kindly agreed to serve as pre-examiners of my dissertation and provided useful comments that helped me improve this manuscript. I also thank Maija and Thedi for doing a great job in a very short time. I am honored to have Prof. Stephan Ludwig as an examiner of my dissertation and thank him for accepting the invitation to be my opponent.

I would like to thank my colleagues and collaborators that brought their expertise and best efforts to facilitate my work: Prof. Jean-Marc Egly, Dr. Nicolas Le May, Prof. Xavier Saelens, Dr. Bert Schepens, Docent Tuula Nyman, Dr. Tiina Öhman, Docent Sampsa Matikainen, Sandra Söderholm, Dr. Ilya Terenin, Prof. Ilkka Julkunen, Janne Tynell, Dr. Maxim Bespalov, Laura Turunen, Lin Feng and Dr. Jakob Stenman. I would

like to express special gratitude to Prof. Jean-Marc Egly and Dr. Nicolas Le May for an exciting opportunity to work with them during my lab visit to IGBMC which was an invaluable experience during my PhD studies.

I thank former members of Denis Kainov group Andrey, Laura, Johanna, Oxana, Minttu, Petri, Triin, Al, Maarten and Jens. Thank you for being excellent colleagues, great office neighbors and fun company outside the lab. Special thanks Andrey and Al for their friendship and for deep discussions about everything. Laura, you mentioned recently that there would be no words to thank you, but *seriosno*, I am very grateful to you for your rock-solid support and friendship over these almost five years that I know you. Thank you for your help in the lab (and for donating macrophages), for advising on all kinds of matters (good that you know everything), for proofreading whatever I have ever written (even this thesis, at least twice), and for a great company on any occasion.

I thank current and former members of Sarah's lab with whom I had a chance to interact: Lassi, Pasi, Ari, Shabih, Aušra, James, Jani, Veli-Pekka, Sergei, Noha and Benita. Thank you guys for welcoming me in your lab, you've always been very friendly and helpful.

I thank people from Sergey's and Emmy's groups at FIMM with whom I have been working side-by-side for these years. Dasha, Pauliina, Manu, Yuexi, Sharif, Sergey, Katja, Rita, Ashwini, Dat, Jenni, Annabrita, Emmy for a great working environment and friendly atmosphere. I also thank JP, the most patient bioinformatician, for helping with data analysis. I thank FIMM personnel for stimulating atmosphere during seminars and retreats and for many bright moments. I thank FIMM director Prof. Olli Kallioniemi and administrative manager Reetta Niemelä also for the back-up during some of the not-so-bright moments. Gretchen, thank you for your excellent work to manage the information flow and make whichever tricky organizational issues become crystal clear. I am grateful to Helsinki Biomedical Graduate School (now Doctoral Program in Biomedicine).

I thank my friends Zaza, Steffi, Masha, Nitai, Alla, Ira, Asja, Shvilya and Kristiina. Despite some of you live miles away you all have stayed close by and I appreciate sharing happy moments with you and knowing that at darker days you are also there for me. I want to express my special gratitude to my friends Dima M. and Karina. Guys, you've always been around, you've seen all my ups and downs and I thank you for your support, wisdom and occasional magic kicks. You helped me enjoy the happy moments and overcome incidental asperities.

I thank Dima whose path happened to join mine at this exciting time of thesis finalization. Thank you for the patience, understanding, courage and for staying calm even during my "now-panic-and-freak-out" moments. And a special thank you for your extraordinary ability to listen.

This work would have been be absolutely impossible without my family, which is my true fortress at all times. I thank my grandmas, my siblings and my parents for their never ending support. I am extremely grateful to my parents for their unconditional love, care and the whole world of opportunities that they brought to me (and also for the good genes). I thank mom for her patience, wisdom, understanding and for that unbelievable source of energy which she generously shares with her children.

My research has been financially supported by the Finnish Committee for International Mobility (CIMO), Helsinki Graduate Program in Biomedicine, University of Helsinki, European Molecular Biology Organization (EMBO) and Finnish Cultural Foundation, to which I am very grateful.

Helsinki, March 2015

References

Ablasser A, Poeck H, Anz D, Berger M, Schlee M, Kim S, Bourquin C, Goutagny N, Jiang Z, Fitzgerald KA, Rothenfusser S, Endres S, Hartmann G, and Hornung V. "Selection of molecular structure and delivery of RNA oligonucleotides to activate TLR7 versus TLR8 and to induce high amounts of IL-12p70 in primary human monocytes." In: *Journal of Immunology* 182 (2009), pp. 6824–6833.

Akarsu H, Burmeister WP, Petosa C, Petit I, Müller CW, Ruigrok RWH, and Baudin F. "Crystal structure of the M1 protein-binding domain of the influenza A virus nuclear export protein (NEP/NS2)". In: *EMBO Journal* 22 (2003), pp. 4646–4655.

Alexopoulou L, Holt AC, Medzhitov R, and Flavell RA. "Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3." In: *Nature* 413 (2001), pp. 732–738.

Allen IC, Scull MA, Moore CB, Holl EK, McElvania-TeKippe E, Taxman DJ, Guthrie EH, Pickles RJ, and Ting JPY. "The NLRP3 Inflammasome Mediates In Vivo Innate Immunity to Influenza A Virus through Recognition of Viral RNA". In: *Immunity* 30 (2009), pp. 556–565.

Amorim MJ, Bruce EA, Read EKC, Foeglein A, Mahen R, Stuart AD, and Digard P. "A Rab11- and microtubule-dependent mechanism for cytoplasmic transport of influenza A virus viral RNA." In: *Journal of Virology* 85 (2011), pp. 4143–4156.

Aragon T, de la Luna S, Novoa I, Carrasco L, Ortin J, and Nieto A. "Eukaryotic translation initiation factor 4GI is a cellular target for NS1 protein, a translational activator of influenza virus". In: *Molecular and Cellular Biology* 20.17 (2000), pp. 6259–6268.

Aramini JM, Ma LC, Zhou L, Schauder CM, Hamilton K, Amer BR, Mack TR, Lee HW, Ciccosanti CT, Zhao L, Xiao R, Krug RM, and Montelione GT. "Dimer interface of the effector domain of non-structural protein 1 from influenza A virus: An interface with multiple functions". In: *Journal of Biological Chemistry* 286 (2011), pp. 26050–26060.

Arranz R, Coloma R, Chichón FJ, Conesa JJ, Carrascosa JL, Valpuesta JM, Ortín J, and Martín-Benito J. "The structure of native influenza virion ribonucleoproteins." In: *Science* 338 (2012), pp. 1634–7.

Avilov SV, Moisy D, Naffakh N, and Cusack S. "Influenza A virus progeny vRNP trafficking in live infected cells studied with the virus-encoded fluorescently tagged PB2 protein". In: *Vaccine* 30 (2012), pp. 7411–7417.

Ayllon J and Garcia-Sastre A. "The NS1 Protein: A Multitasking Virulence Factor". In: *Current Topics in Microbiology and Immunology* (2014).

Ayllon J and Garcia-Sastre A. "The NS1 Protein: A Multitasking Virulence Factor." In: *Current Topics in Microbiology and Immunology* 386 (2015), pp. 73–107.

Babcock HP, Chen C, and Zhuang X. "Using single-particle tracking to study nuclear trafficking of viral genes." In: *Biophysical journal* 87 (2004), pp. 2749–2758.

Baltimore D. "Expression of animal virus genomes." In: *Bacteriological Reviews* 35 (1971), pp. 235–241.

Barman S, Adhikary L, Chakrabarti AK, Bernas C, Kawaoka Y, and Nayak DP. "Role of transmembrane domain and cytoplasmic tail amino acid sequences of influenza a

virus neuraminidase in raft association and virus budding." In: *Journal of Virology* 78.10 (2004), pp. 5258–5269.

Bartenschlager R, Penin F, Lohmann V, and André P. "Assembly of infectious hepatitis C virus particles". In: *Trends in Microbiology* 19 (2011), pp. 95–103.

Beaton AR and Krug RM. "Selected host cell capped RNA fragments prime influenza viral RNA transcription in vivo." In: *Nucleic Acids Research* 9 (1981), pp. 4423–4436.

Beloso A, Martinez C, Valcarcel J, Santaren JF, and Ortin J. "Degradation of cellular mRNA during influenza virus infection: Its possible role in protein synthesis shutoff". In: *Journal of General Virology* 73 (1992), pp. 575–581.

Bender A, Albert M, Reddy A, Feldman M, Sauter B, Kaplan G, Hellman W, and Bhardwaj N. "The Distinctive Features of Influenza Virus Infection of Dendritic Cells". In: *Immunobiology* 198.5 (1998), pp. 552–567.

Bergmann M, Garcia-Sastre A, Carnero E, Pehamberger H, Wolff K, Palese P, and Muster T. "Influenza virus NS1 protein counteracts PKR-mediated inhibition of replication." In: *Journal of Virology* 74 (2000), pp. 6203–6206.

Bogefors J, Kvarnhammar AM, Latif L, Petterson T, Uddman R, and Cardell LO. "Retinoic acid-inducible gene 1-like receptors in the upper respiratory tract." In: *American Journal of Rhinology & Allergy* 25.6 (2011), e262–7.

Bornholdt ZA and Prasad BVV. "X-ray structure of influenza virus NS1 effector domain." In: *Nature Structural & Molecular Biology* 13 (2006), pp. 559–560.

Bornholdt ZA and Prasad BVV. "X-ray structure of NS1 from a highly pathogenic H5N1 influenza virus." In: *Nature* 456 (2008), pp. 985–988.

Branca AA and Baglioni C. "Evidence that types I and II interferons have different receptors." In: *Nature* 294 (1981), pp. 768–770.

Brankston G, Gitterman L, Hirji Z, Lemieux C, and Gardam M. "Transmission of influenza A in human beings". In: *Lancet Infectious Diseases* 7 (2007), pp. 257–265.

Brass AL, Huang IC, Benita Y, John SP, Krishnan MN, Feeley EM, Ryan BJ, Weyer JL, van der Weyden L, Fikrig E, Adams DJ, Xavier RJ, Farzan M, and Elledge SJ. "The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus". In: *Cell* 139 (2009), pp. 1243–1254.

Brincks EL, Kucaba TA, Legge KL, and Griffith TS. "Influenza-induced expression of functional tumor necrosis factor-related apoptosis-inducing ligand on human peripheral blood mononuclear cells". In: *Human Immunology* 69 (2008), pp. 634–646.

Brunotte L, Flies J, Bolte H, Reuther P, Vreede F, and Schwemmle M. "The nuclear export protein of H5N1 influenza A viruses recruits M1 to the viral ribonucleoprotein to mediate nuclear export." In: *Journal of Biological Chemistry* (2014).

Brydon EWA, Morris SJ, and Sweet C. "Role of apoptosis and cytokines in influenza virus morbidity". In: *FEMS Microbiology Reviews* 29 (2005), pp. 837–850.

Bui M, Whittaker G, and Helenius A. "Effect of M1 protein and low pH on nuclear transport of influenza virus ribonucleoproteins." In: *Journal of Virology* 70 (1996), pp. 8391–8401.

Burgui I, Aragon T, Ortin J, and Nieto A. "PABP1 and eIF4GI associate with influenza virus NS1 protein in viral mRNA translation initiation complexes". In: *Journal of General Virology* 84.Pt 12 (2003), pp. 3263–3274.

Burns K, Janssens S, Brissoni B, Olivos N, Beyaert R, and Tschopp J. "Inhibition of Interleukin 1 Receptor/Toll-like Receptor Signaling through the Alternatively Spliced, Short Form of MyD88 Is Due to Its Failure to Recruit IRAK-4". In: *The Journal of Experimental Medicine* 197.2 (2003), pp. 263–268.

Carlson ED, Gan R, Hodgman CE, and Jewett MC. "Cell-free protein synthesis: applications come of age". In: *Biotechnology Advances* 30.5 (2012), pp. 1185–1194.

Carr CM and Kim PS. "A spring-loaded mechanism for the conformational change of influenza hemagglutinin". In: *Cell* 73 (1993), pp. 823–832.

Carrillo B, Choi JM, Bornholdt ZA, Sankaran B, Rice AP, and Prasad BV. "The influenza A virus protein NS1 displays structural polymorphism". In: *Journal of Virology* 88.8 (2014), pp. 4113–4122.

Castelli J, Wood KA, and Youle RJ. "The 2-5A system in viral infection and apoptosis". In: *Biomedicine and Pharmacotherapy* 52 (1998), pp. 386–390.

Chen C and Zhuang X. "Epsin 1 is a cargo-specific adaptor for the clathrin-mediated endocytosis of the influenza virus." In: *Proceedings of the National Academy of Sciences of the USA* 105 (2008), pp. 11790–11795.

Chen G, Liu CH, Zhou L, and Krug RM. "Cellular DDX21 RNA Helicase Inhibits Influenza A Virus Replication but Is Counteracted by the Viral NS1 Protein". In: *Cell Host and Microbe* 15.4 (2014), pp. 484–493.

Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, Bacik I, Basta S, O'Neill R, Schickli J, Palese P, Henklein P, Bennink JR, and Yewdell JW. "A novel influenza A virus mitochondrial protein that induces cell death." In: *Nature Medicine* 7 (2001), pp. 1306–1312.

Chen Z and Krug RM. "Selective nuclear export of viral mRNAs in influenza-virus-infected cells". In: *Trends in Microbiology* 8 (2000), pp. 376–383.

Cheng A, Wong SM, and Yuan YA. "Structural basis for dsRNA recognition by NS1 protein of influenza A virus." In: *Cell Research* 19.2 (2009), pp. 187–95.

Chien CY, Tejero R, Huang YP, Zimmerman DE, Rios CB, Krug RM, and Montelione GT. "A novel RNA-binding motif in influenza A virus non-structural protein 1". In: *Nature Structural Biology* 4 (1997), pp. 891–895.

Chien CY, Xu Y, Xiao R, Aramini JM, Sahasrabudhe PV, Krug RM, and Montelione GT. "Biophysical characterization of the complex between double-stranded RNA and the N-terminal domain of the NS1 protein from influenza A virus: evidence for a novel RNA-binding mode". In: *Biochemistry* 43.7 (2004), pp. 1950–1962.

Chu WM, Ostertag D, Li ZW, Chang L, Chen Y, Hu Y, Williams B, Perrault J, and Karin M. "JNK2 and IKKbeta are required for activating the innate response to viral infection." In: *Immunity* 11 (1999), pp. 721–731.

Cohen GM. "Caspases: the executioners of apoptosis." In: *The Biochemical Journal* 326 (Pt 1 (1997), pp. 1–16.

Cohen M, Zhang XQ, Senaati HP, Chen HW, Varki NM, Schooley RT, and Gagneux P. "Influenza A penetrates host mucus by cleaving sialic acids with neuraminidase." In: *Virology Journal* 10 (2013), p. 321.

Colgan DF and Manley JL. "Mechanism and regulation of mRNA polyadenylation." In: *Genes & Development* 11.21 (1997), pp. 2755–2766.

Coloma R, Valpuesta JM, Arranz R, Carrascosa JL, Ortín J, and Martín-Benito J. "The structure of a biologically active influenza virus ribonucleoprotein complex". In: *PLoS Pathogens* 5 (2009).

Connor RJ, Kawaoka Y, Webster RG, and Paulson JC. "Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates." In: *Virology* 205 (1994), pp. 17–23.

Cros JF, Garcia-Sastre A, and Palese P. "An unconventional NLS is critical for the nuclear import of the influenza A virus nucleoprotein and ribonucleoprotein." In: *Traffic* 6.3 (2005), pp. 205–213.

Cui S, Eisenächer K, Kirchhofer A, Brzózka K, Lammens A, Lammens K, Fujita T, Conzelmann KK, Krug A, and Hopfner KP. "The C-Terminal Regulatory Domain Is the RNA 5'-Triphosphate Sensor of RIG-I". In: *Molecular Cell* 29 (2008), pp. 169–179.

Daniels-Holgate PU and Edwardson JM. "Transport of influenza virus envelope proteins from the Golgi complex to the apical plasma membrane in MDCK cells: pH-controlled interaction with a cycling receptor is not involved." In: *FEBS Letters* 249 (1989), pp. 407–410.

Das K, Ma LC, Xiao R, Radvansky B, Aramini J, Zhao L, Marklund J, Kuo RL, Twu KY, Arnold E, Krug RM, and Montelione GT. "Structural basis for suppression of a host antiviral response by influenza A virus." In: *Proceedings of the National Academy of Sciences of the USA* 105.35 (2008), pp. 13093–8.

De la Luna S, Fortes P, Beloso A, and Ortin J. "Influenza virus NS1 protein enhances the rate of translation initiation of viral mRNAs". In: *Journal of Virology* 69.4 (1995), pp. 2427–2433.

Deb A, Haque SJ, Mogensen T, Silverman RH, and Williams BR. "RNA-dependent protein kinase PKR is required for activation of NF-kappa B by IFN-gamma in a STAT1-independent pathway." In: *Journal of Immunology* 166 (2001), pp. 6170–6180.

Desai TM, Marin M, Chin CR, Savidis G, Brass AL, and Melikyan GB. "IFITM3 Restricts Influenza A Virus Entry by Blocking the Formation of Fusion Pores following Virus-Endosome Hemifusion". In: *PLoS Pathogens* 10 (2014).

Desselberger U, Nakajima K, Alfino P, Pedersen FS, Haseltine WA, Hannoun C, and Palese P. "Biochemical evidence that "new" influenza virus strains in nature may arise by recombination (reassortment)." In: *Proceedings of the National Academy of Sciences of the USA* 75 (1978), pp. 3341–3345.

Dias A, Bouvier D, Crépin T, McCarthy AA, Hart DJ, Baudin F, Cusack S, and Ruigrok RWH. "The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit." In: *Nature* 458 (2009), pp. 914–918.

Diebold SS, Kaisho T, Hemmi H, Akira S, and Reis e Sousa C. "Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA." In: *Science* 303 (2004), pp. 1529–1531.

Dimitrov DS. "Virus entry: molecular mechanisms and biomedical applications." In: *Nature Reviews. Microbiology* 2 (2004), pp. 109–122.

Dittmann J, Stertz S, Grimm D, Steel J, García-Sastre A, Haller O, and Kochs G. "Influenza A virus strains differ in sensitivity to the antiviral action of Mx-GTPase." In: *Journal of Virology* 82 (2008), pp. 3624–3631.

Donelan NR, Basler CF, and Garcia-Sastre A. "A recombinant influenza A virus expressing an RNA-binding-defective NS1 protein induces high levels of beta interferon and is attenuated in mice". In: *Journal of Virology* 77.24 (2003), pp. 13257–13266.

Donnelly N, Gorman AM, Gupta S, and Samali A. "The eIF2alpha kinases: their structures and functions". In: *Cellular and Molecular Life Sciences* 70.19 (2013), pp. 3493–3511.

Dourmashkin RR and Tyrrell DA. "Electron microscopic observations on the entry of influenza virus into susceptible cells." In: *The Journal of General Virology* 24.1 (1974), pp. 129–141.

Douthwaite JA. "Eukaryotic ribosome display selection using rabbit reticulocyte lysate". In: *Methods in Molecular Biology* 805 (2012), pp. 45–57.

Drake JW. "Rates of spontaneous mutation among RNA viruses." In: *Proceedings of the National Academy of Sciences of the USA* 90 (1993), pp. 4171–4175.

Dubois J, Terrier O, and Rosa-Calatrava M. "Influenza viruses and mRNA splicing: Doing more with less". In: *mBio* 5 (2014).

Dudek SE, Wixler L, Nordhoff C, Nordmann A, Anhlan D, Wixler V, and Ludwig S. "The influenza virus PB1-F2 protein has interferon antagonistic activity". In: *Biological Chemistry* 392 (2011), pp. 1135–1144.

Dyer KD and Rosenberg HF. "The RNase a superfamily: Generation of diversity and innate host defense". In: *Molecular Diversity* 10 (2006), pp. 585–597.

Egorov A, Brandt S, Sereinig S, Romanova J, Ferko B, Katinger D, Grassauer A, Alexandrova G, Katinger H, and Muster T. "Transfectant influenza A viruses with long deletions in the NS1 protein grow efficiently in Vero cells." In: *Journal of Virology* 72 (1998), pp. 6437–6441.

Ehrhardt C and Ludwig S. "A new player in a deadly game: Influenza viruses and the PI3K/Akt signalling pathway". In: *Cellular Microbiology* 11 (2009), pp. 863–871.

Ehrhardt C, Marjuki H, Wolff T, Nurnberg B, Planz O, Pleschka S, and Ludwig S. "Bivalent role of the phosphatidylinositol-3-kinase (PI3K) during influenza virus infection and host cell defence." In: *Cellular Microbiology* 8.8 (2006), pp. 1336–1348.

Ehrhardt C, Wolff T, Pleschka S, Planz O, Beermann W, Bode JG, Schmolke M, and Ludwig S. "Influenza A virus NS1 protein activates the PI3K/Akt pathway to mediate antiapoptotic signaling responses." In: *Journal of Virology* 81 (2007), pp. 3058–3067.

Eisfeld AJ, Kawakami E, Watanabe T, Neumann G, and Kawaoka Y. "RAB11A is essential for transport of the influenza virus genome to the plasma membrane." In: *Journal of Virology* 85 (2011), pp. 6117–6126.

Elderfield RA, Watson SJ, Godlee A, Adamson WE, Thompson CI, Dunning J, Fernandez-Alonso M, Blumenkrantz D, Hussell T, Zambon M, Openshaw P, Kellam P, and Barclay WS. "Accumulation of human-adapting mutations during circulation of A(H1N1)pdm09 influenza virus in humans in the United Kingdom." In: *Journal of Virology* 88.22 (2014), pp. 13269–13283.

Elton D, Digard P, Tiley L, and Ortin J. "Structure and function of the influenza virus RNP". In: *Current Topics in Influenza Virology* (2005), pp. 1–92.

Engelhardt OGO, Smith M, and Fodor E. “Association of the Influenza A Virus RNA-Dependent RNA Polymerase with Cellular RNA Polymerase II”. In: *Journal of Virology* 79 (2005), pp. 5812–8.

Falcón AM, Fortes P, Marión RM, Beloso A, and Ortín J. “Interaction of influenza virus NS1 protein and the human homologue of Staufen in vivo and in vitro.” In: *Nucleic Acids Research* 27 (1999), pp. 2241–2247.

Falcón AM, Marión RM, Zürcher T, Gómez P, Portela A, Nieto A, and Ortín J. “Defective RNA replication and late gene expression in temperature-sensitive influenza viruses expressing deleted forms of the NS1 protein.” In: *Journal of Virology* 78 (2004), pp. 3880–3888.

Fensterl V and Sen GC. “Interferons and viral infections”. In: *BioFactors* 35 (2009), pp. 14–20.

Fesq H, Bacher M, Nain M, and Gemsa D. “Programmed cell death (apoptosis) in human monocytes infected by influenza A virus.” In: *Immunobiology* 190 (1994), pp. 175–182.

Fineberg HV. “Pandemic Preparedness and Response — Lessons from the H1N1 Influenza of 2009”. In: *New England Journal of Medicine* 370.14 (2014), pp. 1335–1342.

Fixsen SM and Howard MT. “Processive selenocysteine incorporation during synthesis of eukaryotic selenoproteins”. In: *Journal of Molecular Biology* 399.3 (2010), pp. 385–396.

Forrest HL and Webster RG. “Perspectives on influenza evolution and the role of research.” In: *Animal Health Research Reviews* 11 (2010), pp. 3–18.

Fournier G, Chiang C, Munier S, Tomoiu A, Demeret C, Vidalain PO, Jacob Y, and Naffakh N. “Recruitment of RED-SMU1 Complex by Influenza A Virus RNA Polymerase to Control Ciral mRNA Splicing”. In: *PLoS Pathogens* 10 (2014).

Fu XY, Kessler DS, Veals SA, Levy DE, and Darnell JE. “ISGF3, the transcriptional activator induced by interferon alpha, consists of multiple interacting polypeptide chains.” In: *Proceedings of the National Academy of Sciences of the USA* 87 (1990), pp. 8555–8559.

Fujiyoshi Y, Kume NP, Sakata K, and Sato SB. “Fine structure of influenza A virus observed by electron cryo-microscopy.” In: *The EMBO journal* 13 (1994), pp. 318–326.

Fuller W and Cuthbert AW. “Post-translational disruption of the delta F508 cystic fibrosis transmembrane conductance regulator (CFTR)-molecular chaperone complex with geldanamycin stabilizes delta F508 CFTR in the rabbit reticulocyte lysate”. In: *Journal of Biological Chemistry* 275.48 (2000), pp. 37462–37468.

Gack MU, Shin YC, Joo CH, Urano T, Liang C, Sun L, Takeuchi O, Akira S, Chen Z, Inoue S, and Jung JU. “TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity.” In: *Nature* 446 (2007), pp. 916–920.

Gack MU, Albrecht RA, Urano T, Inn KS, Huang IC, Carnero E, Farzan M, Inoue S, Jung JU, and Garcia-Sastre A. “Influenza A virus NS1 targets the ubiquitin ligase TRIM25 to evade recognition by the host viral RNA sensor RIG-I.” In: *Cell Host and Microbe* 5.5 (2009), pp. 439–449.

Galluzzi L, Brenner C, Morselli E, Touat Z, and Kroemer G. “Viral control of mitochondrial apoptosis”. In: *PLoS Pathogens* 4 (2008).

Gao S, Song L, Li J, Zhang Z, Peng H, Jiang W, Wang Q, Kang T, Chen S, and Huang W. "Influenza A virus-encoded NS1 virulence factor protein inhibits innate immune response by targeting IKK." In: *Cellular Microbiology* 14.12 (2012), pp. 1849–1866.

Gao S, von der Malsburg A, Paeschke S, Behlke J, Haller O, Kochs G, and Daumke O. "Structural basis of oligomerization in the stalk region of dynamin-like MxA." In: *Nature* 465 (2010), pp. 502–506.

García MA, Gil J, Ventoso I, Guerra S, Domingo E, Rivas C, and Esteban M. "Impact of protein kinase PKR in cell biology: from antiviral to antiproliferative action." In: *Microbiology and Molecular Biology Reviews* 70 (2006), pp. 1032–1060.

Garcia-Sastre A, Egorov A, Matassov D, Brandt S, Levy DE, Durbin JE, Palese P, and Muster T. "Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems". In: *Virology* 252.2 (1998), pp. 324–330.

García-Sastre A and Biron CA. "Type 1 interferons and the virus-host relationship: a lesson in détente ." In: *Science* 312 (2006), pp. 879–882.

Geiss GK, Salvatore M, Tumpey TM, Carter VS, Wang X, Basler CF, Taubenberger JK, Bumgarner RE, Palese P, Katze MG, and Garcia-Sastre A. "Cellular transcriptional profiling in influenza A virus-infected lung epithelial cells: the role of the nonstructural NS1 protein in the evasion of the host innate defense and its potential contribution to pandemic influenza". In: *Proceedings of the National Academy of Sciences of the USA* 99.16 (2002), pp. 10736–10741.

Gil J and Esteban M. "Induction of apoptosis by the dsRNA-dependent protein kinase (PKR): mechanism of action." In: *Apoptosis: an International Journal on Programmed Cell Death* 5.2 (2000), pp. 107–114.

Golebiewski L, Liu H, Javier RT, and Rice AP. "The Avian Influenza Virus NS1 ESEV PDZ Binding Motif Associates with Dlg1 and Scribble To Disrupt Cellular Tight Junctions". In: *Journal of Virology* 85 (2011), pp. 10639–10648.

Greenspan D, Palese P, and Krystal M. "Two nuclear location signals in the influenza virus NS1 nonstructural protein." In: *Journal of Virology* 62 (1988), pp. 3020–3026.

Grove J and Marsh M. "The cell biology of receptor-mediated virus entry". In: *Journal of Cell Biology* 195 (2011), pp. 1071–1082.

Guarda G, Zenger M, Yazdi AS, Schroder K, Ferrero I, Menu P, Tardivel A, Mattmann C, and Tschopp J. "Differential expression of NLRP3 among hematopoietic cells." In: *Journal of Immunology* 186 (2011), pp. 2529–2534.

Guilligay D, Tarendeau F, Resa-Infante P, Coloma R, Crepin T, Sehr P, Lewis J, Ruigrok RWH, Ortin J, Hart DJ, and Cusack S. "The structural basis for cap binding by influenza virus polymerase subunit PB2." In: *Nature Structural and Molecular Biology* 15 (2008), pp. 500–506.

Guillot L, Le Goffic R, Bloch S, Escriou N, Akira S, Chignard M, and Si-Tahar M. "Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus." In: *Journal of Biological Chemistry* 280 (2005), pp. 5571–5580.

Hale BG. "Conformational plasticity of the influenza A virus NS1 protein." In: *Journal of General Virology* 95.Pt 10 (2014), pp. 2099–105.

Hale BG, Barclay WS, Randall RE, and Russell RJ. "Structure of an avian influenza A virus NS1 protein effector domain." In: *Virology* 378.1 (2008), pp. 1–5.

Hale BG, Batty IH, Downes CP, and Randall RE. "Binding of influenza a virus NS1 protein to the inter-SH2 domain of p85?? suggests a novel mechanism for phosphoinositide 3-kinase activation". In: *Journal of Biological Chemistry* 283 (2008), pp. 1372–1380.

Hale BG, Jackson D, Chen YH, Lamb RA, and Randall RE. "Influenza A virus NS1 protein binds p85beta and activates phosphatidylinositol-3-kinase signaling." In: *Proceedings of the National Academy of Sciences of the USA* 103 (2006), pp. 14194–14199.

Hale BG, Kerry PS, Jackson D, Precious BL, Gray A, Killip MJ, Randall RE, and Russell RJ. "Structural insights into phosphoinositide 3-kinase activation by the influenza A virus NS1 protein". In: *Proceedings of the National Academy of Sciences of the USA* 107 (2010), pp. 1954–1959.

Hale BG, Knebel A, Botting CH, Galloway CS, Precious BL, Jackson D, Elliott RM, and Randall RE. "CDK/ERK-mediated phosphorylation of the human influenza A virus NS1 protein at threonine-215". In: *Virology* 383 (2009), pp. 6–11.

Hale BG, Randall RE, Ortin J, and Jackson D. "The multifunctional NS1 protein of influenza A viruses". In: *Journal of General Virology* 89 (2008), pp. 2359–2376.

Hale BG, Steel J, Manicassamy B, Medina RA, Ye J, Hickman D, Lowen AC, Perez DR, and García-Sastre A. "Mutations in the NS1 C-terminal tail do not enhance replication or virulence of the 2009 pandemic H1N1 influenza A virus". In: *Journal of General Virology* 91 (2010), pp. 1737–1742.

Hale BG, Steel J, Medina RA, Manicassamy B, Ye J, Hickman D, Hai R, Schmolke M, Lowen AC, Perez DR, and García-Sastre A. "Inefficient control of host gene expression by the 2009 pandemic H1N1 influenza A virus NS1 protein." In: *Journal of Virology* 84 (2010), pp. 6909–6922.

Haller O, Gao S, Von Der Malsburg A, Daumke O, and Kochs G. "Dynamin-like MxA GTPase: Structural insights into oligomerization and implications for antiviral activity". In: *Journal of Biological Chemistry* 285 (2010), pp. 28419–28424.

Hao L, Sakurai A, Watanabe T, Sorensen E, Nidom CA, Newton MA, Ahlquist P, and Kawaoka Y. "Drosophila RNAi screen identifies host genes important for influenza virus replication." In: *Nature* 454 (2008), pp. 890–3.

Harris A, Cardone G, Winkler DC, Heymann JB, Brecher M, White JM, and Steven AC. "Influenza virus pleiomorphy characterized by cryoelectron tomography." In: *Proceedings of the National Academy of Sciences of the USA* 103 (2006), pp. 19123–19127.

Hartmann R, Justesen J, Sarkar SN, Sen GC, and Yee VC. "Crystal structure of the 2'-specific and double-stranded RNA-activated interferon-induced antiviral protein 2'-5'-oligoadenylate synthetase." In: *Molecular Cell* 12.5 (2003), pp. 1173–1185.

Hatada E and Fukuda R. "Binding of influenza A virus NS1 protein to dsRNA in vitro". In: *Journal of General Virology* 73 (Pt 12 (1992), pp. 3325–3329.

Hatada E, Saito S, and Fukuda R. "Mutant influenza viruses with a defective NS1 protein cannot block the activation of PKR in infected cells". In: *Journal of Virology* 73.3 (1999), pp. 2425–2433.

Hay AJ, Skehel JJ, and McCauley J. "Characterization of influenza virus RNA complete transcripts." In: *Virology* 116.2 (1982), pp. 517–522.

Haye K, Burmakina S, Moran T, García-Sastre A, and Fernandez-Sesma A. "The NS1 protein of a human influenza virus inhibits type I interferon production and the induction of antiviral responses in primary human dendritic and respiratory epithelial cells." In: *Journal of Virology* 83 (2009), pp. 6849–6862.

Haywood AM. "Membrane uncoating of intact enveloped viruses." In: *Journal of Virology* 84 (2010), pp. 10946–10955.

Heikkinen LS, Kazlauskas A, Melen K, Wagner R, Ziegler T, Julkunen I, and Saksela K. "Avian and 1918 Spanish influenza a virus NS1 proteins bind to Crk/CrkL Src homology 3 domains to activate host cell signaling". In: *Journal of Biological Chemistry* 283.9 (2008), pp. 5719–5727.

Herold S, Ludwig S, Pleschka S, and Wolff T. "Apoptosis signaling in influenza virus propagation, innate host defense, and lung injury". In: *Journal of Leukocyte Biology* 92 (2012), pp. 75–82.

Hershey A and Chase M. "Independent functions of viral protein and nucleic acid in growth of bacteriophage". In: *The Journal of General Physiology* 36 (1952), pp. 39–56.

Hinshaw VS, Olsen CW, Dybdahl-sissoko N, and Evans D. "Apoptosis: a mechanism of cell killing by influenza A and B viruses." In: *Journal of Virology* 68 (1994), pp. 3667–73.

Hoffmann E, Neumann G, Kawaoka Y, Hobom G, and Webster RG. "A DNA transfection system for generation of influenza A virus from eight plasmids". In: *Proceedings of the National Academy of Sciences of the USA* 97.11 (2000), pp. 6108–6113.

Honda K, Yanai H, Negishi H, Asagiri M, Sato M, Mizutani T, Shimada N, Ohba Y, Takaoka A, Yoshida N, and Taniguchi T. "IRF-7 is the master regulator of type-I interferon-dependent immune responses". In: *Nature* 434.7034 (2005), pp. 772–777.

Hong XX and Carmichael GG. "Innate immunity in pluripotent human cells: Attenuated response to interferon-beta". In: *Journal of Biological Chemistry* 288 (2013), pp. 16196–16205.

Hornung V, Ellegast J, Kim S, Brzózka K, Jung A, Kato H, Poeck H, Akira S, Conzelmann KK, Schlee M, Endres S, and Hartmann G. "5'-Triphosphate RNA is the ligand for RIG-I." In: *Science* 314 (2006), pp. 994–997.

Hrincius ER, Wixler V, Wolff T, Wagner R, Ludwig S, and Ehrhardt C. "CRK adaptor protein expression is required for efficient replication of avian influenza A viruses and controls JNK-mediated apoptotic responses". In: *Cellular Microbiology* 12 (2010), pp. 831–843.

Hsiang TY, Zhou L, and Krug RM. "Roles of the phosphorylation of specific serines and threonines in the NS1 protein of human influenza A viruses". In: *Journal of Virology* 86.19 (2012), pp. 10370–10376.

Hsiang TY, Zhao C, and Krug RM. "Interferon-induced ISG15 conjugation inhibits influenza A virus gene expression and replication in human cells." In: *Journal of Virology* 83 (2009), pp. 5971–5977.

Huang Y and Carmichael GG. "Role of polyadenylation in nucleocytoplasmic transport of mRNA." In: *Molecular and Cellular Biology* 16 (1996), pp. 1534–1542.

Huet S, Avilov S, Ferbitz L, Daigle N, Cusack S, and Ellenberg J. "Nuclear import and assembly of the influenza A virus RNA polymerase studied in live cells by

Fluorescence Cross Correlation Spectroscopy". In: *Journal of Virology* 84 (2010), pp. 1254–1264.

Hughey PG, Compans RW, Zebedee SL, and Lamb RA. "Expression of the influenza A virus M2 protein is restricted to apical surfaces of polarized epithelial cells." In: *Journal of Virology* 66.9 (1992), pp. 5542–5552.

Husain B, Mukerji I, and Cole JL. "Analysis of high-affinity binding of protein kinase R to double-stranded RNA." In: *Biochemistry* 51.44 (2012), pp. 8764–8770.

Hutchinson EC, Denham EM, Thomas B, Trudgian DC, Hester SS, Ridlova G, York A, Turrell L, and Fodor E. "Mapping the Phosphoproteome of Influenza A and B Viruses by Mass Spectrometry". In: *PLoS Pathogens* 8 (2012).

Ichinohe T, Lee HK, Ogura Y, Flavell R, and Iwasaki A. "Inflammasome recognition of influenza virus is essential for adaptive immune responses." In: *The Journal of Experimental Medicine* 206 (2009), pp. 79–87.

Ichinohe T, Pang IK, and Iwasaki A. "Influenza virus activates inflammasomes via its intracellular M2 ion channel." In: *Nature Immunology* 11 (2010), pp. 404–410.

Inglis SC, Barrett T, Brown CM, and Almond JW. "The smallest genome RNA segment of influenza virus contains two genes that may overlap." In: *Proceedings of the National Academy of Sciences of the USA* 76 (1979), pp. 3790–3794.

Ioannidis I, Ye F, McNally B, Willette M, and Flaño E. "Toll-like receptor expression and induction of type I and type III interferons in primary airway epithelial cells." In: *Journal of Virology* 87 (2013), pp. 3261–70.

Ishikawa E, Nakazawa M, Yoshinari M, and Minami M. "Role of tumor necrosis factor-related apoptosis-inducing ligand in immune response to influenza virus infection in mice". In: *Journal of Virology* 79 (2005), pp. 7658–7663.

Iwasaki A and Medzhitov R. "Regulation of adaptive immunity by the innate immune system." In: *Science* 327 (2010), pp. 291–295.

Iwasaki A and Pillai PS. "Innate immunity to influenza virus infection". In: *Nature Reviews Microbiology Immunol* 14.5 (2014), pp. 315–328.

Jackson DA, Caton AJ, McCready SJ, and Cook PR. "Influenza virus RNA is synthesized at fixed sites in the nucleus." In: *Nature* 296 (1982), pp. 366–368.

Jackson D, Killip MJ, Galloway CS, Russell RJ, and Randall RE. "Loss of function of the influenza A virus NS1 protein promotes apoptosis but this is not due to a failure to activate phosphatidylinositol 3-kinase (PI3K)". In: *Virology* 396 (2010), pp. 94–105.

Jackson RJ, Hellen CU, and Pestova TV. "The mechanism of eukaryotic translation initiation and principles of its regulation". In: *Nature Reviews Molecular and Cellular Biology* 11.2 (2010), pp. 113–127.

Jagger BW, Wise HM, Kash JC, Walters KA, Wills NM, Xiao YL, Dunfee RL, Schwartzman LM, Ozinsky A, Bell GL, Dalton RM, Lo A, Efstathiou S, Atkins JF, Firth AE, Taubenberger JK, and Digard P. "An Overlapping Protein-Coding Region in Influenza A Virus Segment 3 Modulates the Host Response". In: *Science* 337 (2012), pp. 199–204.

Jammi NV, Whitby LR, and Beal PA. "Small molecule inhibitors of the RNA-dependent protein kinase". In: *Biochemical and Biophysical Research Communications* 308.1 (2003), pp. 50–57.

Janeway CA. "Approaching the asymptote? Evolution and revolution in immunology". In: *Cold Spring Harbor Symposia on Quantitative Biology*. Vol. 54. 1989, pp. 1–13.

Janeway CA and Medzhitov R. "Innate immune recognition." In: *Annual Review of Immunology* 20 (2002), pp. 197–216.

Javier RT and Rice AP. "Emerging Theme: Cellular PDZ Proteins as Common Targets of Pathogenic Viruses". In: *Journal of Virology* 85 (2011), pp. 11544–11556.

Jiang H, White EJ, Ríos-Vicil CI, Xu J, Gomez-Manzano C, and Fueyo J. "Human adenovirus type 5 induces cell lysis through autophagy and autophagy-triggered caspase activity." In: *Journal of Virology* 85 (2011), pp. 4720–4729.

Jiang Z, Zamanian-Daryoush M, Nie H, Silva AM, Williams BRG, and Li X. "Poly(I-C)-induced Toll-like receptor 3 (TLR3)-mediated activation of NFkappa B and MAP kinase is through an interleukin-1 receptor-associated kinase (IRAK)-independent pathway employing the signaling components TLR3-TRAF6-TAK1-TAB2-PKR ." In: *Journal of Biological Chemistry* 278.19 (2003), pp. 16713–16719.

Johnson ES. "Protein modification by SUMO." In: *Annual Review of Biochemistry* 73 (2004), pp. 355–382.

Jorba N, Coloma R, and Ortín J. "Genetic trans-complementation establishes a new model for influenza virus RNA transcription and replication". In: *PLoS Pathogens* 5 (2009).

Julkunen I, Melén K, Nyqvist M, Pirhonen J, Sareneva T, and Matikainen S. "Inflammatory responses in influenza A virus infection." In: *Vaccine* 19 Suppl 1 (2000), S32–S37.

Kang DC, Gopalkrishnan RV, Lin L, Randolph A, Valerie K, Pestka S, and Fisher PB. "Expression analysis and genomic characterization of human melanoma differentiation associated gene-5, mda-5: a novel type I interferon-responsive apoptosis-inducing gene." In: *Oncogene* 23 (2004), pp. 1789–1800.

Karlas A, Machuy N, Shin Y, Pleissner KP, Artarini A, Heuer D, Becker D, Khalil H, Ogilvie LA, Hess S, Mäurer AP, Müller E, Wolff T, Rudel T, and Meyer TF. "Genome-wide RNAi screen identifies human host factors crucial for influenza virus replication." In: *Nature* 463 (2010), pp. 818–822.

Karpala AJ, Doran TJ, and Bean AGD. "Immune responses to dsRNA: Implications for gene silencing technologies". In: *Immunology and Cell Biology* 83 (2005), pp. 211–216.

Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ, Yamaguchi O, Otsu K, Tsujimura T, Koh CS, Reis e Sousa C, Matsuura Y, Fujita T, and Akira S. "Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses." In: *Nature* 441 (2006), pp. 101–105.

Katze MG, Detjen BM, Safer B, and Krug RM. "Translational control by influenza virus: suppression of the kinase that phosphorylates the alpha subunit of initiation factor eIF-2 and selective translation of influenza viral mRNAs". In: *Molecular and Cellular Biology* 6.5 (1986), pp. 1741–1750.

Katze MG, Tomita J, Black T, Krug RM, Safer B, and Hovanessian A. "Influenza virus regulates protein synthesis during infection by repressing autophosphorylation and activity of the cellular 68,000-Mr protein kinase." In: *Journal of Virology* 62.10 (1988), pp. 3710–3717.

Kawaguchi A, Matsumoto K, and Nagata K. "YB-1 Functions as a Porter To Lead Influenza Virus Ribonucleoprotein Complexes to Microtubules". In: *Journal of Virology* 86 (2012), pp. 11086–11095.

Kawaguchi A and Nagata K. "De novo replication of the influenza virus RNA genome is regulated by DNA replicative helicase, MCM." In: *The EMBO Journal* 26 (2007), pp. 4566–4575.

Kawai T and Akira S. "TLR signaling". In: *Seminars in Immunology* 19 (2007), pp. 24–32.

Kawai T, Takahashi K, Sato S, Coban C, Kumar H, Kato H, Ishii KJ, Takeuchi O, and Akira S. "IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction." In: *Nature Immunology* 6 (2005), pp. 981–988.

Kemler I, Whittaker G, and Helenius A. "Nuclear import of microinjected influenza virus ribonucleoproteins." In: *Virology* 202 (1994), pp. 1028–1033.

Kerry PS, Ayllon J, Taylor Ma, Hass C, Lewis A, García-Sastre A, Randall RE, Hale BG, and Russell RJ. "A transient homotypic interaction model for the influenza A virus NS1 protein effector domain." In: *PloS One* 6.3 (2011), e17946.

Kim SR, Kim DI, Kim SH, Lee H, Lee KS, Cho SH, and Lee YC. "NLRP3 inflammasome activation by mitochondrial ROS in bronchial epithelial cells is required for allergic inflammation". In: *Cell Death and Disease* 5 (2014), e1498.

Kimball SR. "Eukaryotic initiation factor eIF2." In: *The International Journal of Biochemistry & Cell Biology* 31 (1999), pp. 25–29.

Kobiler O, Drayman N, Butin-Israeli V, and Oppenheim A. "Virus strategies for passing the nuclear envelope barrier." In: *Nucleus* 3 (2012), pp. 526–39.

Kochs G, García-Sastre A, and Martínez-Sobrido L. "Multiple anti-interferon actions of the influenza A virus NS1 protein." In: *Journal of Virology* 81 (2007), pp. 7011–7021.

Koennecke I, Boschek C, and Scholtissek C. "Isolation and properties of a temperature-sensitive mutant (ts 412) of an influenza A virus recombinant with a is lesion in the gene coding for the nonstructural protein". In: *Virology* 110.1 (1981), pp. 16–25.

König R, Stertz S, Zhou Y, Inoue A, Hoffmann HH, Bhattacharyya S, Alamares JG, Tscherne DM, Ortigoza MB, Liang Y, Gao Q, Andrews SE, Bandyopadhyay S, De Jesus P, Tu BP, Pache L, Shih C, Orth A, Bonamy G, Miraglia L, Ideker T, García-Sastre A, Young JAT, Palese P, Shaw ML, and Chanda SK. "Human host factors required for influenza virus replication." In: *Nature* 463 (2010), pp. 813–817.

Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, Langer JA, Sheikh F, Dickensheets H, and Donnelly RP. "IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex." In: *Nature Immunology* 4 (2003), pp. 69–77.

Kowalinski E, Lunardi T, McCarthy AA, Louber J, Brunel J, Grigorov B, Gerlier D, and Cusack S. "Structural basis for the activation of innate immune pattern-recognition receptor RIG-I by viral RNA". In: *Cell* 147 (2011), pp. 423–435.

Kumar H, Kawai T, and Akira S. "Toll-like receptors and innate immunity". In: *Biochemical and Biophysical Research Communications* 388 (2009), pp. 621–625.

Kumar R and Nanduri B. "HPIDB–a unified resource for host-pathogen interactions." In: *BMC Bioinformatics* 11 Suppl 6 (2010), S16.

Kundu A, Avalos RT, Sanderson CM, and Nayak DP. "Transmembrane domain of influenza virus neuraminidase, a type II protein, possesses an apical sorting signal in polarized MDCK cells." In: *Journal of Virology* 70 (1996), pp. 6508–6515.

Kundu P, Raychaudhuri S, Tsai W, and Dasgupta A. "Shutoff of RNA polymerase II transcription by poliovirus involves 3C protease-mediated cleavage of the TATA-binding protein at an alternative site: incomplete shutoff of transcription interferes with efficient viral replication." In: *Journal of Virology* 79.15 (2005), pp. 9702–9713.

La Gruta NL, Kedzierska K, Stambas J, and Doherty PC. "A question of self-preservation: immunopathology in influenza virus infection". In: *Immunology & Cell Biology* 85.2 (2007), pp. 85–92.

Lagacé-Wiens PRS, Rubinstein E, and Gumel A. "Influenza epidemiology–past, present, and future." In: *Critical care medicine* 38 (2010), e1–e9.

Lakadamyali M, Rust MJ, Babcock HP, and Zhuang X. "Visualizing infection of individual influenza viruses." In: *Proceedings of the National Academy of Sciences of the USA* 100 (2003), pp. 9280–9285.

Lakspere T, Tynell J, Kaloinen M, Vanlede M, Parsons A, Ikonen N, Kallio-Kokko H, Kantele A, Mattila P, Almusa H, Julkunen I, Kainov D, and Kakkola L. "Full-Genome Sequences of Influenza A(H1N1)pdm09 Viruses Isolated from Finnish Patients from 2009 to 2013". In: *Genome Announcements* 2.1 (2014).

Lamb RA. "The Influenza Virus RNA Segments and Their Encoded Proteins". In: *Genetics of Influenza Viruses SE - 2*. Ed. by Palese P and Kingsbury D. Springer Vienna, 1983, pp. 21–69.

Lamb RA and Choppin PW. "Segment 8 of the influenza virus genome is unique in coding for two polypeptides." In: *Proceedings of the National Academy of Sciences of the USA* 76 (1979), pp. 4908–4912.

Lamb RA, Choppin PW, Chanock RM, and Lai CJ. "Mapping of the two overlapping genes for polypeptides NS1 and NS2 on RNA segment 8 of influenza virus genome." In: *Proceedings of the National Academy of Sciences of the USA* 77 (1980), pp. 1857–1861.

Le Goffic R, Balloy V, Lagranderie M, Alexopoulou L, Escriou N, Flavell R, Chignard M, and Si-Tahar M. "Detrimental contribution of the Toll-like receptor (TLR)3 to influenza A virus-induced acute pneumonia". In: *PLoS Pathogens* 2 (2006), pp. 0526–0535.

Le Goffic R, Pothlichet J, Vitour D, Fujita T, Meurs E, Chignard M, and Si-Tahar M. "Cutting Edge: Influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells." In: *Journal of Immunology* 178 (2007), pp. 3368–3372.

Le May N, Dubaele S, De Santis LP, Billecocq A, Bouloy M, and Egly JM. "TFIIH Transcription Factor, a Target for the Rift Valley Hemorrhagic Fever Virus". In: *Cell* 116 (2004), pp. 541–550.

Lee ASy, Burdeinick-Kerr R, and Whelan SPJ. "A ribosome-specialized translation initiation pathway is required for cap-dependent translation of vesicular stomatitis virus mRNAs." In: *Proceedings of the National Academy of Sciences of the USA* 110 (2013), pp. 324–9.

Lee N, Wong CK, Hui DSC, Lee SKW, Wong RYK, Ngai KLK, Chan MCW, Chu YJ, Ho AWY, Lui GCY, Wong BCK, Wong SH, Yip SP, and Chan PKS. "Role of human

Toll-like receptors in naturally occurring influenza A infections". In: *Influenza and other Respiratory Viruses* 7 (2013), pp. 666–675.

Levin D and London IM. "Regulation of protein synthesis: activation by double-stranded RNA of a protein kinase that phosphorylates eukaryotic initiation factor 2". In: *Proceedings of the National Academy of Sciences of the USA* 75.3 (1978), pp. 1121–1125.

Levy DE, Kessler DS, Pine R, Reich N, and Darnell JE. "Interferon-induced nuclear factors that bind a shared promoter element correlate with positive and negative transcriptional control." In: *Genes & Development* 2 (1988), pp. 383–393.

Li ML, Rao P, and Krug RM. "The active sites of the influenza cap-dependent endonuclease are on different polymerase subunits". In: *EMBO Journal* 20 (2001), pp. 2078–2086.

Li S, Min JY, Krug RM, and Sen GC. "Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA". In: *Virology* 349.1 (2006), pp. 13–21.

Li S, Peters GA, Ding K, Zhang X, Qin J, and Sen GC. "Molecular basis for PKR activation by PACT or dsRNA." In: *Proceedings of the National Academy of Sciences of the USA* 103 (2006), pp. 10005–10010.

Li Y, Chen ZY, Wang W, Baker CC, and Krug RM. "The 3'-end-processing factor CPSF is required for the splicing of single-intron pre-mRNAs in vivo." In: *RNA* 7.6 (2001), pp. 920–931.

Li Y, Yamakita Y, and Krug RM. "Regulation of a nuclear export signal by an adjacent inhibitory sequence: the effector domain of the influenza virus NS1 protein." In: *Proceedings of the National Academy of Sciences of the USA* 95 (1998), pp. 4864–4869.

Li Y, Lu X, Li J, Bérubé N, Giest KL, Liu Q, Anderson DH, and Zhou Y. "Genetically engineered, biarsenically labeled influenza virus allows visualization of viral NS1 protein in living cells." In: *Journal of Virology* 84 (2010), pp. 7204–7213.

Liang SL, Quirk D, and Zhou A. "RNase L: its biological roles and regulation." In: *IUBMB Life* 58 (2006), pp. 508–514.

Liu HM, Loo YM, Horner SM, Zornetzer GA, Katze MG, and Gale M. "The mitochondrial targeting chaperone 14-3-3ϵ regulates a RIG-I translocon that mediates membrane association and innate antiviral immunity". In: *Cell Host and Microbe* 11 (2012), pp. 528–537.

Liu H, Golebiewski L, Dow EC, Krug RM, Javier RT, and Rice AP. "The ESEV PDZ Binding-Motif of the Avian Influenza A Virus NS1 Protein Protects Infected Cells from Apoptosis through Directly Targeting Scribble." In: *Journal of Virology* 84 (2010), pp. 11164–11174.

Liu J, Lynch PA, Chien CY, Montelione GT, Krug RM, and Berman HM. "Crystal structure of the unique RNA-binding domain of the influenza virus NS1 protein." In: *Nature Structural Biology* 4 (1997), pp. 896–899.

Long JX, Peng DX, Liu YL, Wu YT, and Liu XF. "Virulence of H5N1 avian influenza virus enhanced by a 15-nucleotide deletion in the viral nonstructural gene". In: *Virus Genes* 36 (2008), pp. 471–478.

Lu X, Masic A, Li Y, Shin Y, Liu Q, and Zhou Y. "The PI3K/Akt pathway inhibits influenza A virus-induced Bax-mediated apoptosis by negatively regulating the JNK pathway via ASK1". In: *Journal of General Virology* 91 (2010), pp. 1439–1449.

Lu Y, Qian XY, and Krug RM. "The influenza virus NS1 protein: a novel inhibitor of pre-mRNA splicing." In: *Genes & Development* 8 (1994), pp. 1817–1828.

Lu Y, Wambach M, Katze MG, and Krug RM. "Binding of the influenza virus NS1 protein to double-stranded RNA inhibits the activation of the protein kinase that phosphorylates the elF-2 translation initiation factor". In: *Virology* 214.1 (1995), pp. 222–228.

Ludwig S, Schultz U, Mandler J, Fitch WM, and Scholtissek C. "Phylogenetic relationship of the nonstructural (NS) genes of influenza A viruses." In: *Virology* 183 (1991), pp. 566–577.

Ludwig S, Wang X, Ehrhardt C, Zheng H, Donelan N, Planz O, Pleschka S, Garcia-Sastre A, Heins G, and Wolff T. "The influenza A virus NS1 protein inhibits activation of Jun N-terminal kinase and AP-1 transcription factors". In: *Journal of Virology* 76.21 (2002), pp. 11166–11171.

Lund JM, Alexopoulou L, Sato A, Karow M, Adams NC, Gale NW, Iwasaki A, and Flavell RA. "Recognition of single-stranded RNA viruses by Toll-like receptor 7." In: *Proceedings of the National Academy of Sciences of the USA* 101 (2004), pp. 5598–5603.

Lyles DS. "Assembly and Budding of Negative-Strand RNA Viruses". In: *Advances in Virus Research* 85 (2013), pp. 57–90.

Ma J, Goryaynov A, Sarma A, and Yang W. "Self-regulated viscous channel in the nuclear pore complex". In: *Proceedings of the National Academy of Sciences of the USA* 109 (2012), pp. 7326–7331.

Maamary J, Pica N, Belicha-Villanueva A, Chou Yy, Krammer F, Gao Q, Garcia-Sastre A, and Palese P. "Attenuated Influenza Virus Construct with Enhanced Hemagglutinin Protein Expression". In: *Journal of Virology* 86 (2012), pp. 5782–5790.

Macara IG. "Transport into and out of the nucleus." In: *Microbiology and Molecular Biology Reviews* 65 (2001), 570–594, table of contents.

Marazzi I, Ho JS, Kim J, Manicassamy B, Dewell S, Albrecht RA, Seibert CW, Schaefer U, Jeffrey KL, Prinjha RK, Lee K, Garcia-Sastre A, Roeder RG, and Tarakhovsky A. "Suppression of the antiviral response by an influenza histone mimic". In: *Nature* 483.7390 (2012), pp. 428–433.

Marc D, Barbachou S, and Soubieux D. "The RNA-binding domain of influenzavirus non-structural protein-1 cooperatively binds to virus-specific RNA sequences in a structure-dependent manner." In: *Nucleic acids research* 41.1 (2013), pp. 434–449.

Marión RM, Zürcher T, De La Luna S, and Ortín J. "Influenza virus NS1 protein interacts with viral transcription-replication complexes in vivo". In: *Journal of General Virology* 78 (1997), pp. 2447–2451.

Martín J, Wharton SA, Lin YP, Takemoto DK, Skehel JJ, Wiley DC, and Steinhauer DA. "Studies of the binding properties of influenza hemagglutinin receptor-site mutants." In: *Virology* 241 (1998), pp. 101–111.

Martin K and Helenius A. "Transport of incoming influenza virus nucleocapsids into the nucleus." In: *Journal of Virology* 65 (1991), pp. 232–244.

Matlin KS, Reggio H, Helenius A, and Simons K. "Infectious entry pathway of influenza virus in a canine kidney cell line". In: *Journal of Cell Biology* 91 (1981), pp. 601–613.

Mayer D, Molawi K, Martínez-Sobrido L, Ghanem A, Thomas S, Baginsky S, Grossmann J, García-Sastre A, and Schwemmle M. "Identification of cellular interaction partners of the influenza virus ribonucleoprotein complex and polymerase complex using proteomic-based approaches". In: *Journal of Proteome Research* 6 (2007), pp. 672–682.

McGeoch D, Fellner P, and Newton C. "Influenza virus genome consists of eight distinct RNA species." In: *Proceedings of the National Academy of Sciences of the USA* 73 (1976), pp. 3045–3049.

McWhirter SM, TenOever BR, and Maniatis T. "Connecting mitochondria and innate immunity". In: *Cell* 122 (2005), pp. 645–647.

Medzhitov R, Preston-Hurlburt P, Kopp E, Stadlen A, Chen C, Ghosh S, and Janeway Jr. CA. "MyD88 Is an Adaptor Protein in the hToll/IL-1 Receptor Family Signaling Pathways". In: *Molecular Cell* 2.2 (1998), pp. 253–258.

Melen K, Kinnunen L, Fagerlund R, Ikonen N, Twu KY, Krug RM, and Julkunen I. "Nuclear and nucleolar targeting of influenza A virus NS1 protein: striking differences between different virus subtypes". In: *Journal of Virology* 81.11 (2007), pp. 5995–6006.

Melen K, Tynell J, Fagerlund R, Roussel P, Hernandez-Verdun D, and Julkunen I. "Influenza A H3N2 subtype virus NS1 protein targets into the nucleus and binds primarily via its C-terminal NLS2/NoLS to nucleolin and fibrillarin". In: *Virology Journal* 9 (2012), p. 167.

Meurs E, Chong K, Galabru J, Thomas NS, Kerr IM, Williams BR, and Hovanessian AG. "Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon". In: *Cell* 62.2 (1990), pp. 379–390.

Mibayashi M, Martínez-Sobrido L, Loo YM, Cárdenas WB, Gale M, and García-Sastre A. "Inhibition of retinoic acid-inducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus." In: *Journal of Virology* 81 (2007), pp. 514–524.

Min JY and Krug RM. "The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: Inhibiting the 2'-5' oligo (A) synthetase/RNase L pathway". In: *Proceedings of the National Academy of Sciences of the USA* 103.18 (2006), pp. 7100–7105.

Min JY, Li S, Sen GC, and Krug RM. "A site on the influenza A virus NS1 protein mediates both inhibition of PKR activation and temporal regulation of viral RNA synthesis". In: *Virology* 363.1 (2007), pp. 236–243.

Moeller A, Kirchdoerfer RN, Potter CS, Carragher B, and Wilson Ia. "Organization of the influenza virus replication machinery." In: *Science* 338 (2012), pp. 1631–4.

Mogensen KE, Lewerenz M, Reboul J, Lutfalla G, and Uzé G. "The type I interferon receptor: structure, function, and evolution of a family business." In: *Journal of Interferon & Cytokine Research* 19 (1999), pp. 1069–1098.

Momose F, Kikuchi Y, Komase K, and Morikawa Y. "Visualization of microtubule-mediated transport of influenza viral progeny ribonucleoprotein". In: *Microbes and Infection* 9 (2007), pp. 1422–1433.

Momose F, Sekimoto T, Ohkura T, Jo S, Kawaguchi A, Nagata K, and Morikawa Y. "Apical transport of influenza A virus ribonucleoprotein requires Rab11-positive recycling endosome". In: *PLoS ONE* 6 (2011).

Mori I, Komatsu T, Takeuchi K, Nakakuki K, Sudo M, and Kimura Y. "In vivo induction of apoptosis by influenza virus." In: *Journal of General Virology* 76 (Pt 11 (1995), pp. 2869–2873.

Morita E, Sandrin V, McCullough J, Katsuyama A, Baci Hamilton I, and Sundquist WI. "ESCRT-III protein requirements for HIV-1 budding". In: *Cell Host and Microbe* 9 (2011), pp. 235–242.

Mossler C, Groiss F, Wolzt M, Wolschek M, Seipelt J, and Muster T. "Phase I/II trial of a replication-deficient trivalent influenza virus vaccine lacking NS1". In: *Vaccine* 31.52 (2013), pp. 6194–6200.

Munir M, Zohari S, Belák S, and Berg M. "Double-Stranded RNA-Induced Activation of Activating Protein-1 Promoter Is Differentially Regulated by the Nonstructural Protein 1 of Avian Influenza A Viruses". In: *Viral Immunology* (2012), p. 120112065140000.

Munir M, Zohari S, and Berg M. "Non-structural protein 1 of avian influenza A viruses differentially inhibit NF-kappaB promoter activation." In: *Virology Journal* 8 (2011), p. 383.

Muramoto Y, Noda T, Kawakami E, Akkina R, and Kawaoka Y. "Identification of novel influenza A virus proteins translated from PA mRNA." In: *Journal of Virology* 87 (2013), pp. 2455–62.

Nakayama M, Nagata K, and Ishihama A. "Enzymatic properties of the mouse Mx1 protein-associated GTPase." In: *Virus Research* 22 (1992), pp. 227–234.

Nayak DP, Balogun RA, Yamada H, Zhou ZH, and Barman S. *Influenza virus morphogenesis and budding*. 2009.

Nayak DP, Hui EKW, and Barman S. "Assembly and budding of influenza virus". In: *Virus Research* 106 (2004), pp. 147–165.

Nemeroff ME, Barabino SM, Li Y, Keller W, and Krug RM. "Influenza virus NS1 protein interacts with the cellular 30 kDa subunit of CPSF and inhibits 3'end formation of cellular pre-mRNAs." In: *Molecular Cell* 1 (1998), pp. 991–1000.

Neumann G, Castrucci MR, and Kawaoka Y. "Nuclear import and export of influenza virus nucleoprotein." In: *Journal of Virology* 71 (1997), pp. 9690–9700.

Neumann G, Hughes MT, and Kawaoka Y. "Influenza A virus NS2 protein mediates vRNP nuclear export through NES-independent interaction with hCRM1". In: *EMBO Journal* 19 (2000), pp. 6751–6758.

Newby CM, Sabin L, and Pekosz A. "The RNA binding domain of influenza A virus NS1 protein affects secretion of tumor necrosis factor alpha, interleukin-6, and interferon in primary murine tracheal epithelial cells". In: *Journal of Virology* 81.17 (2007), pp. 9469–9480.

Nicholas B, Skipp P, Mould R, Rennard S, Davies DE, O'Connor CD, and Djukanović R. "Shotgun proteomic analysis of human-induced sputum". In: *Proteomics* 6 (2006), pp. 4390–4401.

Noah D, Twu K, and Krug R. "Cellular antiviral responses against influenza A virus are countered at the posttranscriptional level by the viral NS1A protein via its binding

to a cellular protein required for the 3' end processing of cellular pre-mRNAS." In: *Virology* 307.2 (2003), pp. 386–395.

Noah DL and Noah JW. "Adapting global influenza management strategies to address emerging viruses." In: *American journal of physiology. Lung cellular and molecular physiology* 305 (2013), pp. L108–17.

Odendall C, Dixit E, Stavru F, Bierne H, Franz KM, Durbin AF, Boulant S, Gehrke L, Cossart P, and Kagan JC. "Diverse intracellular pathogens activate type III interferon expression from peroxisomes." In: *Nature Immunology* 15 (2014), pp. 717–728.

O'Neill RE, Jaskunas R, Blobel G, Palese P, and Moroianu J. "Nuclear import of influenza virus RNA can be mediated by viral nucleoprotein and transport factors required for protein import". In: *Journal of Biological Chemistry* 270 (1995), pp. 22701–22704.

Opitz B, Rejaibi A, Dauber B, Eckhard J, Vinzing M, Schmeck B, Hippenstiel S, Suttorp N, and Wolff T. "IFNbeta induction by influenza A virus is mediated by RIG-I which is regulated by the viral NS1 protein." In: *Cellular Microbiology* 9.4 (2007), pp. 930–938.

Oshiumi H, Miyashita M, Inoue N, Okabe M, Matsumoto M, and Seya T. "The ubiquitin ligase riplet is essential for RIG-I-dependent innate immune responses to RNA virus infection". In: *Cell Host and Microbe* 8 (2010), pp. 496–509.

Pal S, Rosas JM, and Rosas-Acosta G. "Identification of the non-structural influenza A viral protein NS1A as a bona fide target of the Small Ubiquitin-like MOdifier by the use of dicistronic expression constructs". In: *Journal of Virological Methods* 163 (2010), pp. 498–504.

Pal S, Santos A, Rosas JM, Ortiz-Guzman J, and Rosas-Acosta G. "Influenza A virus interacts extensively with the cellular SUMOylation system during infection". In: *Virus Research* 158 (2011), pp. 12–27.

Palese P. "The genes of influenza virus". In: *Cell* 10.1 (1977), pp. 1–10.

Pang IK, Ichinohe T, and Iwasaki A. "IL-1R signaling in dendritic cells replaces pattern-recognition receptors in promoting CD8 T cell responses to influenza A virus." In: *Nature Immunology* 14 (2013), pp. 246–53.

Parvin JD, Moscona A, Pan WT, Leider JM, and Palese P. "Measurement of the mutation rates of animal viruses: influenza A virus and poliovirus type 1." In: *Journal of Virology* 59 (1986), pp. 377–383.

Parvin JD, Young JF, and Palese P. "Nonsense mutations affecting the lengths of the NS1 nonstructural proteins of influenza A virus isolates". In: *Virology* 128.2 (1983), pp. 512–517.

Patel JR, Jain A, Chou YY, Baum A, Ha T, and García-Sastre A. "ATPase-driven oligomerization of RIG-I on RNA allows optimal activation of type-I interferon." In: *EMBO Reports* 14 (2013), pp. 1–8.

Pauli EK, Schmolke M, Wolff T, Viemann D, Roth J, Bode JG, and Ludwig S. "Influenza A virus inhibits type I IFN signaling via NF-kappaB-dependent induction of SOCS-3 expression." In: *PLoS Pathogens* 4.11 (2008), e1000196.

Pelkmans L and Helenius A. "Insider information: What viruses tell us about endocytosis". In: *Current Opinion in Cell Biology* 15 (2003), pp. 414–422.

Perrone LA, Plowden JK, García-Sastre A, Katz JM, and Tumpey TM. "H5N1 and 1918 Pandemic Influenza Virus Infection Results in Early and Excessive Infiltration of Macrophages and Neutrophils in the Lungs of Mice". In: *PLoS Pathogens* 4.8 (2008), e1000115.

Pestova TV, Kolupaeva VG, Lomakin IB, Pilipenko EV, Shatsky IN, Agol VI, and Hellen CU. "Molecular mechanisms of translation initiation in eukaryotes." In: *Proceedings of the National Academy of Sciences of the USA* 98 (2001), pp. 7029–7036.

Petri T, Patterson S, and Dimmock NJ. "Polymorphism of the NS1 proteins of type A influenza virus." In: *Journal of General Virology* 61 (Pt 2) (1982), pp. 217–231.

Philippe N, Legendre M, Doutre G, Couté Y, Poirot O, Lescot M, Arslan D, Seltzer V, Bertaux L, Bruley C, Garin J, Claverie JM, and Abergel C. "Pandoraviruses: amoeba viruses with genomes up to 2.5 Mb reaching that of parasitic eukaryotes." In: *Science* 341 (2013), pp. 281–6.

Pitha-Rowe IF and Pitha PM. "Viral defense, carcinogenesis and ISG15: Novel roles for an old ISG". In: *Cytokine and Growth Factor Reviews* 18 (2007), pp. 409–417.

Plotch SJ, Bouloy M, Ulmanen I, and Krug RM. "A unique cap(m7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription." In: *Cell* 23 (1981), pp. 847–858.

Plotch SJ and Krug RM. "Influenza virion transcriptase: synthesis in vitro of large, polyadenylic acid-containing complementary RNA." In: *Journal of Virology* 21 (1977), pp. 24–34.

Ploubidou A and Way M. "Viral transport and the cytoskeleton". In: *Current Opinion in Cell Biology* 13 (2001), pp. 97–105.

Poch O, Sauvaget I, Delarue M, and Tordo N. "Identification of four conserved motifs among the RNA-dependent polymerase encoding elements." In: *The EMBO Journal* 8 (1989), pp. 3867–3874.

Poon LL, Pritlove DC, Fodor E, and Brownlee GG. "Direct evidence that the poly(A) tail of influenza A virus mRNA is synthesized by reiterative copying of a U track in the virion RNA template". In: *Journal of Virology* 73.4 (1999), pp. 3473–3476.

Pothlichet J, Meunier I, Davis BK, Ting JPY, Skamene E, von Messling V, and Vidal SM. "Type I IFN triggers RIG-I/TLR3/NLRP3-dependent inflammasome activation in influenza A virus infected cells." In: *PLoS Pathogens* 9 (2013), e1003256.

Potter CW. "A history of influenza". In: *Journal of Applied Microbiology*. Vol. 91. 2001, pp. 572–579.

Privalsky ML and Penhoet EE. "The structure and synthesis of influenza virus phosphoproteins". In: *Journal of Biological Chemistry* 256 (1981), pp. 5368–5376.

Qian XY, Chien CY, Lu Y, Montelione GT, and Krug RM. "An amino-terminal polypeptide fragment of the influenza virus NS1 protein possesses specific RNA-binding activity and largely helical backbone structure." In: *RNA* 1 (1995), pp. 948–956.

Qian XY, Chien CY, Lu Y, Montelione GT, and Krug RM. "An amino-terminal polypeptide fragment of the influenza virus NS1 protein possesses specific RNA-binding activity and largely helical backbone structure". In: *RNA* 1.9 (1995), pp. 948–956.

Qiu Y, Nemeroff M, and Krug RM. "The influenza virus NS1 protein binds to a specific region in human U6 snRNA and inhibits U6-U2 and U6-U4 snRNA interactions during splicing." In: *RNA* 1 (1995), pp. 304–316.

Rajsbaum R, Albrecht RA, Wang MK, Maharaj NP, Versteeg GA, Nistal-Villan E, Garcia-Sastre A, and Gack MU. "Species-specific inhibition of RIG-I ubiquitination and IFN induction by the influenza A virus NS1 protein." In: *PLoS Pathogens* 8.11 (2012), e1003059.

Read EKC and Digard P. "Individual influenza A virus mRNAs show differential dependence on cellular NXF1/TAP for their nuclear export". In: *Journal of General Virology* 91 (2010), pp. 1290–1301.

Rebouillat D and Hovanessian AG. "The human 2',5'-oligoadenylate synthetase family: interferon-induced proteins with unique enzymatic properties." In: *Journal of Interferon & Cytokine Research* 19 (1999), pp. 295–308.

Rees PJ and Dimmock NJ. "Kinetics of synthesis of influenza virus ribonucleoprotein structures". In: *Journal of General Virology* 59 (1982), pp. 403–408.

Reis AL and McCauley JW. "The Influenza Virus Protein PB1-F2 Interacts with IKKβ and Modulates NF-κB Signalling". In: *PLoS ONE* 8 (2013).

Resa-Infante P, Jorba N, Coloma R, and Ortin J. "The influenza virus RNA synthesis machine: Advances in its structure and function". In: *RNA Biology* 8 (2011), pp. 207–215.

Ritchey MB, Palese P, and Schulman JL. "Mapping of the influenza virus genome. III. Identification of genes coding for nucleoprotein, membrane protein, and nonstructural protein." In: *Journal of Virology* 20 (1976), pp. 307–313.

Ritchie KJ, Hahn CS, Kim KI, Yan M, Rosario D, Li L, de la Torre JC, and Zhang DE. "Role of ISG15 protease UBP43 (USP18) in innate immunity to viral infection." In: *Nature Medicine* 10 (2004), pp. 1374–1378.

Robb NC and Fodor E. "The accumulation of influenza A virus segment 7 spliced mRNAs is regulated by the NS1 protein". In: *Journal of General Virology* 93 (2012), pp. 113–118.

Robb NC, Jackson D, Vreede FT, and Fodor E. "Splicing of influenza A virus NS1 mRNA is independent of the viral NS1 protein". In: *Journal of General Virology* 91 (2010), pp. 2331–2340.

Robertson JS, Schubert M, and Lazzarini RA. "Polyadenylation sites for influenza virus mRNA." In: *Journal of Virology* 38 (1981), pp. 157–163.

Rossman JS and Lamb RA. "Influenza virus assembly and budding". In: *Virology* 411 (2011), pp. 229–236.

Ruckle A, Haasbach E, Julkunen I, Planz O, Ehrhardt C, and Ludwig S. "The NS1 protein of influenza A virus blocks RIG-I-mediated activation of the noncanonical NF-kappaB pathway and p52/RelB-dependent gene expression in lung epithelial cells." In: *Journal of Virology* 86.18 (2012), pp. 10211–10217.

Sadler AJ and Williams BRG. "Interferon-inducible antiviral effectors." In: *Nature Reviews Immunology* 8 (2008), pp. 559–568.

Salvatore M, Basler CF, Parisien JP, Horvath CM, Bourmakina S, Zheng H, Muster T, Palese P, and Garcia-Sastre A. "Effects of influenza A virus NS1 protein on protein expression: the NS1 protein enhances translation and is not required for shutoff of host protein synthesis". In: *Journal of Virology* 76.3 (2002), pp. 1206–1212.

Santos A, Pal S, Chacón J, Meraz K, Gonzalez J, Prieto K, and Rosas-Acosta G. "SUMOylation affects the interferon blocking activity of the influenza A nonstructural protein NS1 without affecting its stability or cellular localization." In: *Journal of Virology* 87 (2013), pp. 5602–20.

Satterly N, Tsai PL, van Deursen J, Nussenzveig DR, Wang Y, Faria PA, Levay A, Levy DE, and Fontoura BMA. "Influenza virus targets the mRNA export machinery and the nuclear pore complex." In: *Proceedings of the National Academy of Sciences of the USA* 104 (2007), pp. 1853–1858.

Scheiffele P, Rietveld A, Wilk T, and Simons K. "Influenza viruses select ordered lipid domains during budding from the plasma membrane". In: *Journal of Biological Chemistry* 274 (1999), pp. 2038–2044.

Schneider WM, Chevillotte MD, and Rice CM. "Interferon-stimulated genes: a complex web of host defenses." In: *Annual Review of Immunology* 32 (2014), pp. 513–45.

Scholtissek C and von Hoyningen-Huene V. "Genetic relatedness of the gene which codes for the nonstructural (NS) protein of different influenza A strains." In: *Virology* 102.1 (1980), pp. 13–20.

Schulz O, Diebold SS, Chen M, Näslund TI, Nolte MA, Alexopoulou L, Azuma YT, Flavell RA, Liljeström P, and Reis e Sousa C. "Toll-like receptor 3 promotes cross-priming to virus-infected cells." In: *Nature* 433 (2005), pp. 887–892.

Schwegmann A and Brombacher F. "Host-directed drug targeting of factors hijacked by pathogens." In: *Science Signaling* 1 (2008), re8.

Seth RB, Sun L, Ea CK, and Chen ZJ. "Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3." In: *Cell* 122 (2005), pp. 669–682.

Shapira SD, Gat-Viks I, Shum BOV, Dricot A, de Grace MM, Wu L, Gupta PB, Hao T, Silver SJ, Root DE, Hill DE, Regev A, and Hacohen N. "A Physical and Regulatory Map of Host-Influenza Interactions Reveals Pathways in H1N1 Infection". In: *Cell* 139 (2009), pp. 1255–1267.

Shapiro GI, Gurney T, and Krug RM. "Influenza virus gene expression: control mechanisms at early and late times of infection and nuclear-cytoplasmic transport of virus-specific RNAs." In: *Journal of Virology* 61 (1987), pp. 764–773.

Shaw ML. "The host interactome of influenza virus presents new potential targets for antiviral drugs". In: *Reviews in Medical Virology* 21 (2011), pp. 358–369.

Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE, Kuestner R, Garrigues U, Birks C, Roraback J, Ostrander C, Dong D, Shin J, Presnell S, Fox B, Haldeman B, Cooper E, Taft D, Gilbert T, Grant FJ, Tackett M, Krivan W, McKnight G, Clegg C, Foster D, and Klucher KM. "IL-28, IL-29 and their class II cytokine receptor IL-28R." In: *Nature Immunology* 4 (2003), pp. 63–68.

Shin YK, Li Y, Liu Q, Anderson DH, Babiuk LA, and Zhou Y. "SH3 binding motif 1 in influenza A virus NS1 protein is essential for PI3K/Akt signaling pathway activation". In: *Journal of Virology* 81.23 (2007), pp. 12730–12739.

Shin YK, Liu Q, Tikoo SK, Babiuk LA, and Zhou Y. "Influenza A virus NS1 protein activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway by direct interaction with the p85 subunit of PI3K". In: *Journal of General Virology* 88.Pt 1 (2007), pp. 13–18.

Short JAL. “Viral evasion of interferon stimulated genes”. In: *Bioscience Horizons* 2 (2009), pp. 212–224.

Sieczkarski SB and Whittaker GR. “Differential requirements of Rab5 and Rab7 for endocytosis of influenza and other enveloped viruses.” In: *Traffic* 4 (2003), pp. 333–343.

Sieczkarski SB and Whittaker GR. “Influenza virus can enter and infect cells in the absence of clathrin-mediated endocytosis.” In: *Journal of Virology* 76 (2002), pp. 10455–10464.

Siegal FP, Kadowaki N, Shodell M, Fitzgerald-Bocarsly PA, Shah K, Ho S, Antonenko S, and Liu YJ. “The nature of the principal type 1 interferon-producing cells in human blood.” In: *Science* 284 (1999), pp. 1835–1837.

Skehel JJ. “Early polypeptide synthesis in influenza virus-infected cells.” In: *Virology* 56.1 (1973), pp. 394–399.

Skehel JJ and Wiley DC. “Receptor Binding And Membrane Fusion In Virus Entry: The Influenza Hemagglutinin”. In: *Annual Review of Biochemistry* 69 (2000), pp. 531–569.

Smith W, Andrewes C, and Laidlaw P. “A virus obtained from influenza patients”. In: *Lancet* 222 (1933), pp. 66–68.

Song JM, Choi CW, Kwon SO, Compans RW, Kang SM, and Kim SI. “Proteomic characterization of influenza H5N1 virus-like particles and their protective immunogenicity”. In: *Journal of Proteome Research* 10 (2011), pp. 3450–3459.

Stallknecht DE and Brown JD. “Wild birds and the epidemiology of avian influenza.” In: *Journal of Wildlife Diseases* 43 (2007), S15–S20.

Stauffer S, Feng Y, Nebioglu F, Heilig R, Picotti P, and Helenius A. “Stepwise priming by acidic pH and a high K+ concentration is required for efficient uncoating of influenza A virus cores after penetration.” In: *Journal of Virology* 88.22 (2014), pp. 13029–13046.

Stray SJ, Cummings RD, and Air GM. “Influenza virus infection of desialylated cells.” In: *Glycobiology* 10 (2000), pp. 649–658.

Suarez DL and Perdue ML. “Multiple alignment comparison of the non-structural genes of influenza A viruses”. In: *Virus Research* 54 (1998).

Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, and Mesirov JP. “Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles.” In: *Proceedings of the National Academy of Sciences of the USA* 102 (2005), pp. 15545–15550.

Suomalainen M and Greber UF. “Uncoating of non-enveloped viruses”. In: *Current Opinion in Virology* 3 (2013), pp. 27–33.

Szucs T. “The socio-economic burden of influenza.” In: *The Journal of Antimicrobial Chemotherapy* 44 Suppl B (1999), pp. 11–15.

Takahasi K, Kumeta H, Tsuduki N, Narita R, Shigemoto T, Hirai R, Yoneyama M, Horiuchi M, Ogura K, Fujita T, and Inagaki F. “Solution structures of cytosolic RNA sensor MDA5 and LGP2 C-terminal domains: Identification of the RNA recognition loop in RIG-I-like receptors”. In: *Journal of Biological Chemistry* 284 (2009), pp. 17465–17474.

Takizawa T, Ohashi K, and Nakanishi Y. "Possible involvement of double-stranded RNA-activated protein kinase in cell death by influenza virus infection." In: *Journal of virology* 70 (1996), pp. 8128–8132.

Tall RD, Alonso MA, and Roth MG. "Features of influenza HA required for apical sorting differ from those required for association with DRMS or MAL". In: *Traffic* 4 (2003), pp. 838–849.

Tallóczy Z, Jiang W, Virgin HW, Leib DA, Scheuner D, Kaufman RJ, Eskelinen EL, and Levine B. "Regulation of starvation- and virus-induced autophagy by the eIF2alpha kinase signaling pathway." In: *Proceedings of the National Academy of Sciences of the USA* 99 (2002), pp. 190–195.

Talon J, Horvath CM, Polley R, Basler CF, Muster T, Palese P, and García-Sastre A. "Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein." In: *Journal of Virology* 74 (2000), pp. 7989–7996.

Tan SL and Katze MG. "Biochemical and genetic evidence for complex formation between the influenza A virus NS1 protein and the interferon-induced PKR protein kinase". In: *Journal of Interferon & Cytokine Research* 18.9 (1998), pp. 757–766.

Tang Y, Zhong G, Zhu L, Liu X, Shan Y, Feng H, Bu Z, Chen H, and Wang C. "Herc5 attenuates influenza A virus by catalyzing ISGylation of viral NS1 protein." In: *Journal of Immunology* 184 (2010), pp. 5777–5790.

Taubenberger JK, Reid AH, Krafft AE, Bijwaard KE, and Fanning TG. "Initial genetic characterization of the 1918 "Spanish" influenza virus." In: *Science* 275 (1997), pp. 1793–1796.

Terenin IM, Dmitriev SE, Andreev DE, and Shatsky IN. "Eukaryotic translation initiation machinery can operate in a bacterial-like mode without eIF2." In: *Nature Structural and Molecular Biology* 15 (2008), pp. 836–841.

Thomas PG, Dash P, Aldridge JR, Ellebedy AH, Reynolds C, Funk AJ, Martin WJ, Lamkanfi M, Webby RJ, Boyd KL, Doherty PC, and Kanneganti TD. "The Intracellular Sensor NLRP3 Mediates Key Innate and Healing Responses to Influenza A Virus via the Regulation of Caspase-1". In: *Immunity* 30 (2009), pp. 566–575.

Thornberry NA and Lazebnik Y. "Caspases: enemies within." In: *Science* 281 (1998), pp. 1312–1316.

Thornton DJ, Rousseau K, and McGuckin MA. "Structure and function of the polymeric mucins in airways mucus." In: *Annual Review of Physiology* 70 (2008), pp. 459–486.

Tisoncik JR, Billharz R, Burmakina S, Belisle SE, Proll SC, Korth MJ, Garcia-Sastre A, and Katze MG. "The NS1 protein of influenza A virus suppresses interferon-regulated activation of antigen-presentation and immune-proteasome pathways." In: *Journal of General Virology* 92.Pt 9 (2011), pp. 2093–2104.

Tong S, Li Y, Rivailler P, Conrardy C, Castillo DAA, Chen LM, Recuenco S, Ellison JA, Davis CT, York IA, Turmelle AS, Moran D, Rogers S, Shi M, Tao Y, Weil MR, Tang K, Rowe LA, Sammons S, Xu X, Frace M, Lindblade KA, Cox NJ, Anderson LJ, Rupprecht CE, and Donis RO. "A distinct lineage of influenza A virus from bats". In: *Proceedings of the National Academy of Sciences* 109.11 (2012), pp. 4269–4274.

Tong S, Zhu X, Li Y, Shi M, Zhang J, Bourgeois M, Yang H, Chen X, Recuenco S, Gomez J, Chen LM, Johnson A, Tao Y, Dreyfus C, Yu W, McBride R, Carney PJ,

Gilbert AT, Chang J, Guo Z, Davis CT, Paulson JC, Stevens J, Rupprecht CE, Holmes EC, Wilson Ia, and Donis RO. "New World Bats Harbor Diverse Influenza A Viruses". In: *PLoS Pathogens* 9.10 (2013).

Treanor JJ, Snyder MH, London WT, and Murphy BR. "The B allele of the NS gene of avian influenza viruses, but not the A allele, attenuates a human influenza A virus for squirrel monkeys." In: *Virology* 171.1 (1989), pp. 1–9.

Trinchieri G. "Interleukin-12 and the regulation of innate resistance and adaptive immunity." In: *Nature Reviews Immunology* 3 (2003), pp. 133–146.

Tschopp J and Schroder K. "NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production?" In: *Nature Reviews Immunology* 10 (2010), pp. 210–215.

Tu J, Guo J, Zhang A, Zhang W, Zhao Z, Zhou H, Liu C, Chen H, and Jin M. "Effects of the C-terminal truncation in NS1 protein of the 2009 pandemic H1N1 influenza virus on host gene expression". In: *PLoS One* 6 (2011).

Twu KY, Noah DL, Rao P, Kuo RL, and Krug RM. "The CPSF30 binding site on the NS1A protein of influenza A virus is a potential antiviral target." In: *Journal of Virology* 80 (2006), pp. 3957–3965.

Van Der Sluijs KF, Van Elden LJR, Arens R, Nijhuis M, Schuurman R, Florquin S, Kwakkel J, Akira S, Jansen HM, Lutter R, and Van Der Polls T. "Enhanced viral clearance in interleukin-18 gene-deficient mice after pulmonary infection with influenza A virus". In: *Immunology* 114 (2005), pp. 112–120.

Van Boxel-Dezaire AHH, Rani MRS, and Stark GR. "Complex Modulation of Cell Type-Specific Signaling in Response to Type I Interferons". In: *Immunity* 25 (2006), pp. 361–372.

Van Oers MM. "Opportunities and challenges for the baculovirus expression system". In: *Journal of Invertebrate Pathology* 107 (2011).

Van Riel D, den Bakker MA, Leijten LME, Chutinimitkul S, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RAM, Osterhaus ADME, and Kuiken T. "Seasonal and pandemic human influenza viruses attach better to human upper respiratory tract epithelium than avian influenza viruses." In: *American Journal of Pathology* 176 (2010), pp. 1614–1618.

Vannucci L, Lai M, Chiuppesi F, Ceccherini-Nelli L, and Pistello M. "Viral vectors: a look back and ahead on gene transfer technology." In: *The New Microbiologica* 36 (2013), pp. 1–22.

Varga ZT, Ramos I, Hai R, Schmolke M, García-Sastre A, Fernandez-Sesma A, and Palese P. "The influenza virus protein PB1-F2 inhibits the induction of type i interferon at the level of the MAVS adaptor protein". In: *PLoS Pathogens* 7 (2011).

Vassalli JD, Huarte J, Belin D, Gubler P, Vassalli A, O'Connell ML, Parton LA, Rickles RJ, and Strickland S. "Regulated polyadenylation controls mRNA translation during meiotic maturation of mouse oocytes". In: *Genes and Development* 3 (1989), pp. 2163–2171.

Vela A, Fedorova O, Ding SC, and Pyle AM. "The thermodynamic basis for viral RNA detection by the RIG-I innate immune sensor". In: *Journal of Biological Chemistry* 287 (2012), pp. 42564–42573.

Vercammen E, Staal J, and Beyaert R. "Sensing of viral infection and activation of innate immunity by toll-like receptor 3". In: *Clinical Microbiology Reviews* 21 (2008), pp. 13–25.

Walsh D and Mohr I. "Viral subversion of the host protein synthesis machinery". In: *Nature Reviews Microbiology* 9.12 (2011), pp. 860–875.

Wang R, Iwakura Y, Araki K, Sotoyama H, Takei N, and Nawa H. "In vitro production of an active neurotrophic factor, neuregulin-1: qualitative comparison of different cell-free translation systems". In: *Neuroscience Letters* 497.2 (2011), pp. 90–93.

Wang R and Brattain MG. "The maximal size of protein to diffuse through the nuclear pore is larger than 60 kDa". In: *FEBS Letters* 581 (2007), pp. 3164–3170.

Wang S, Zhao Z, Bi Y, Sun L, Liu X, and Liu W. "Tyrosine 132 phosphorylation of influenza A virus M1 protein is crucial for virus replication by controlling the nuclear import of M1." In: *Journal of Virology* 87 (2013), pp. 6182–91.

Wang W, Riedel K, Lynch P, Chien CY, Montelione GT, and Krug RM. "RNA binding by the novel helical domain of the influenza virus NS1 protein requires its dimer structure and a small number of specific basic amino acids". In: *RNA* 5.2 (1999), pp. 195–205.

Wang X, Li M, Zheng H, Muster T, Palese P, Beg AA, and Garcia-Sastre A. "Influenza A virus NS1 protein prevents activation of NF-kappaB and induction of alpha/beta interferon." In: *Journal of Virology* 74.24 (2000), pp. 11566–11573.

Ward AC, Azad AA, and Macreadie IG. "Expression and characterisation of the influenza A virus non-structural protein NS1 in yeast". In: *Archives of Virology* 138 (1994), pp. 299–314.

Watanabe T, Watanabe S, and Kawaoka Y. "Cellular networks involved in the influenza virus life cycle". In: *Cell Host and Microbe* 7 (2010), pp. 427–439.

Webby RJ and Webster RG. "Are we ready for pandemic influenza?" In: *Science* 302 (2003), pp. 1519–1522.

Webster RG, Bean WJ, Gorman OT, Chambers TM, and Kawaoka Y. "Evolution and ecology of influenza A viruses." In: *Microbiological reviews* 56 (1992), pp. 152–179.

Whitlow ZW, Connor JH, and Lyles DS. "Preferential translation of vesicular stomatitis virus mRNAs is conferred by transcription from the viral genome." In: *Journal of Virology* 80 (2006), pp. 11733–11742.

Wilusz J, Shenk T, Takagaki Y, and Manley JL. "A multicomponent complex is required for the AAUAAA-dependent cross-linking of a 64-kilodalton protein to polyadenylation substrates." In: *Molecular and Cellular Biology* 10 (1990), pp. 1244–1248.

Wise HM, Foeglein A, Sun J, Dalton RM, Patel S, Howard W, Anderson EC, Barclay WS, and Digard P. "A complicated message: Identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA." In: *Journal of Virology* 83 (2009), pp. 8021–8031.

Wisskirchen C, Ludersdorfer TH, Müller DA, Moritz E, and Pavlovic J. "The Cellular RNA Helicase UAP56 Is Required for Prevention of Double-Stranded RNA Formation during Influenza A Virus Infection." In: *Journal of Virology* 85 (2011), pp. 8646–8655.

Wong AHT, Ning Tam NW, Yang YL, Cuddihy AR, Li S, Kirchhoff S, Hauser H, Decker T, and Koromilas AE. "Physical association between STAT1 and the interferon-

inducible protein kinase PKR and implications for interferon and double-stranded RNA signaling pathways". In: *EMBO Journal* 16 (1997), pp. 1291–1304.

Woo HM, Kim KS, Lee JM, Shim HS, Cho SJ, Lee WK, Ko HW, Keum YS, Kim SY, Pathinayake P, Kim CJ, and Jeong YJ. "Single-stranded DNA aptamer that specifically binds to the influenza virus NS1 protein suppresses interferon antagonism". In: *Antiviral Research* 100 (2013), pp. 337–345.

Wurzer WJ, Planz O, Ehrhardt C, Giner M, Silberzahn T, Pleschka S, and Ludwig S. "Caspase 3 activation is essential for efficient influenza virus propagation". In: *EMBO Journal* 22 (2003), pp. 2717–2728.

Wysocka J and Herr W. "The herpes simplex virus VP16-induced complex: The makings of a regulatory switch". In: *Trends in Biochemical Sciences* 28 (2003), pp. 294–304.

Xia S, Monzingo AF, and Robertus JD. "Structure of NS1A effector domain from the influenza A/Udorn/72 virus." In: *Acta Crystallographica. Section D, Biological Crystallography* 65.Pt 1 (2009), pp. 11–7.

Xing Z, Cardona CJ, Anunciacion J, Adams S, and Dao N. "Roles of the ERK MAPK in the regulation of proinflammatory and apoptotic responses in chicken macrophages infected with H9N2 avian influenza virus." In: *The Journal of General Virology* 91.Pt 2 (2010), pp. 343–351.

Xu K, Klenk C, Liu B, Keiner B, Cheng J, Zheng BJ, Li L, Han Q, Wang C, Li T, Chen Z, Shu Y, Liu J, Klenk HD, and Sun B. "Modification of nonstructural protein 1 of influenza A virus by SUMO1." In: *Journal of Virology* 85 (2011), pp. 1086–1098.

Yamauchi Y and Helenius A. "Virus entry at a glance." In: *Journal of Cell Science* 126.Pt 6 (2013), pp. 1289–1295.

Ye Z, Liu T, Offringa DP, McInnis J, and Levandowski RA. "Association of influenza virus matrix protein with ribonucleoproteins." In: *Journal of Virology* 73 (1999), pp. 7467–7473.

Yin C, Khan JA, Swapna GV, Ertekin A, Krug RM, Tong L, and Montelione GT. "Conserved surface features form the double-stranded RNA binding site of nonstructural protein 1 (NS1) from influenza A and B viruses". In: *Journal of Biological Chemistry* 282.28 (2007), pp. 20584–20592.

Yin C, Khan Ja, Swapna GVT, Ertekin A, Krug RM, Tong L, and Montelione GT. "Conserved surface features form the double-stranded RNA binding site of nonstructural protein 1 (NS1) from influenza A and B viruses." In: *Journal of Biological Chemistry* 282.28 (2007), pp. 20584–92.

Ying Chou Y, Heaton NS, Gao Q, Palese P, Singer R, and Lionnet T. "Colocalization of Different Influenza Viral RNA Segments in the Cytoplasm before Viral Budding as Shown by Single-molecule Sensitivity FISH Analysis". In: *PLoS Pathogens* 9 (2013).

Yoneyama M, Kikuchi M, Matsumoto K, Imaizumi T, Miyagishi M, Taira K, Foy E, Loo YM, Gale MJ, Akira S, Yonehara S, Kato A, and Fujita T. "Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity." In: *Journal of Immunology* 175.5 (2005), pp. 2851–2858.

Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, and Fujita T. "The RNA helicase RIG-I has an essential function in

double-stranded RNA-induced innate antiviral responses." In: *Nature Immunology* 5 (2004), pp. 730–737.

York A and Fodor E. "Biogenesis, assembly, and export of viral messenger ribonucleoproteins in the influenza A virus infected cell." In: *RNA Biology* 10 (2013), pp. 1274–82.

York A, Hutchinson EC, and Fodor E. "Interactome analysis of the influenza a virus transcription/replication machinery identifies protein phosphatase 6 as a cellular factor required for efficient virus replication." In: *Journal of Virology* 88.22 (2014), pp. 13284–13299.

Yuan P, Bartlam M, Lou Z, Chen S, Zhou J, He X, Lv Z, Ge R, Li X, Deng T, Fodor E, Rao Z, and Liu Y. "Crystal structure of an avian influenza polymerase PA(N) reveals an endonuclease active site." In: *Nature* 458 (2009), pp. 909–913.

Zarkower D and Wickens M. "Formation of mRNA 3' termini: stability and dissociation of a complex involving the AAUAAA sequence." In: *The EMBO Journal* 6 (1987), pp. 177–186.

Zeng W, Sun L, Jiang X, Chen X, Hou F, Adhikari A, Xu M, and Chen ZJ. "Reconstitution of the RIG-I pathway reveals a signaling role of unanchored polyubiquitin chains in innate immunity." In: *Cell* 141.2 (2010), pp. 315–330.

Zhang S, Wang J, Wang Q, and Toyoda T. "Internal initiation of influenza virus replication of viral RNA and complementary RNA in vitro". In: *Journal of Biological Chemistry* 285 (2010), pp. 41194–41201.

Zhao C, Hsiang TY, Kuo RL, and Krug RM. "ISG15 conjugation system targets the viral NS1 protein in influenza A virus-infected cells". In: *Proceedings of the National Academy of Sciences of the USA* 107.5 (2010), pp. 2253–2258.

Zhirnov OP and Klenk HD. "Control of apoptosis in influenza virus-infected cells by up-regulation of Akt and p53 signaling." In: *Apoptosis* 12.8 (2007), pp. 1419–1432.

Zielecki F, Semmler I, Kalthoff D, Voss D, Mauel S, Gruber AD, Beer M, and Wolff T. "Virulence determinants of avian H5N1 influenza A virus in mammalian and avian hosts: role of the C-terminal ESEV motif in the viral NS1 protein." In: *Journal of Virology* 84.20 (2010), pp. 10708–10718.

Zietara N, Łyszkiewicz M, Gekara N, Puchałka J, Dos Santos VAPM, Hunt CR, Pandita TK, Lienenklaus S, and Weiss S. "Absence of IFN-beta impairs antigen presentation capacity of splenic dendritic cells via down-regulation of heat shock protein 70." In: *Journal of Immunology* 183 (2009), pp. 1099–1109.

Zinzula L and Tramontano E. "Strategies of highly pathogenic RNA viruses to block dsRNA detection by RIG-I-like receptors: Hide, mask, hit". In: *Antiviral Research* 100 (2013), pp. 615–635.

Zohari S, Gyarmati P, Ejdersund A, Berglöf U, Thorén P, Ehrenberg M, Czifra G, Belák S, Waldenström J, Olsen B, and Berg M. "Phylogenetic analysis of the non-structural (NS) gene of influenza A viruses isolated from mallards in Northern Europe in 2005". In: *Virology Journal* 5 (2008), p. 147.