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SANDRA SÖDERHOLM

Phosphoproteomic Characterization of Viral Infection

INSTITUTE OF BIOTECHNOLOGY AND
DEPARTMENT OF BIOSCIENCES
FACULTY OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES
DOCTORAL PROGRAMME IN INTEGRATIVE LIFE SCIENCE
UNIVERSITY OF HELSINKI

Institute of Biotechnology and Department of Biosciences
Faculty of Biological and Environmental Sciences
University of Helsinki
Helsinki, Finland

Finnish Institute of Occupational Health
Unit of Systems Toxicology
Helsinki, Finland

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PHOSPHOPROTEOMIC CHARACTERIZATION OF VIRAL INFECTION

Sandra Söderholm

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 lecture hall 5, Latokartanonkaari 7 (Building B), on May the 20th at 12 o'clock noon.

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Supervisors

Docent Tuula Nyman, Ph.D.
Institute of Biotechnology
University of Helsinki
Helsinki, Finland

Docent Sampsa Matikainen, Ph.D.
Unit of Systems Toxicology
Finnish Institute of Occupational Health
Helsinki, Finland

Expert members of the thesis advisory committee

Docent Kirsi Savijoki, Ph. D.
Dept. of Food and Environmental Sciences
Faculty of Agriculture and Forestry
University of Helsinki, Finland

Docent Tero Ahola, Ph. D.
Dept. of Food and Environmental Sciences
Faculty of Agriculture and Forestry
University of Helsinki, Finland

Docent Denis Kainov, Ph. D.
Institute for Molecular Medicine Finland (FIMM)
University of Helsinki, Finland

Pre-examiners

Docent Tero Ahola , Ph. D.
Dept. of Food and Environmental Sciences
Faculty of Agriculture and Forestry
University of Helsinki, Finland

Docent Maciej Lalowski, Ph. D.
Medicum
Biochemistry/Developmental Biology
Meilahti Clinical Proteomics Core Facility
University of Helsinki, Finland

Opponent

Matthias Trost, Ph. D.
MRC Protein Phosphorylation & Ubiquitylation Unit
University of Dundee
Dundee, United Kingdom

Custos

Professor Kari Keinänen
Department of Biosciences
Faculty of Biological and Environmental Sciences
University of Helsinki, Finland

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"The cure for anything is salt water -- sweat, tears, or the sea."

Karen Blixen (*nom de plume* Isak Dinesen)

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Phosphoproteomics combined with quantitative 14-3-3-affinity capture identifies SIRT1 and RAI as novel regulators of cytosolic double-stranded RNA recognition pathway. *Molecular & Cellular Proteomics*, 13, 10, 2604-2617 (2014)

- SS performed the phosphoproteomics part of the work. The functional studies were conducted together with TÖ.

II **Söderholm S***, Hintsanen P*, Öhman T, Aittokallio T, Nyman TA.
PhosFox: a bioinformatics tool for peptide-level processing of LC-MS/MS-based phosphoproteomic data. *Proteome Science*, 12, 36 (2014)

- SS performed the experimental case study, helped with the development of the analysis tool, and drafted the manuscript.

III Öhman T, **Söderholm S**, Paidikondala M, Lietzén N, Matikainen S, Nyman TA.
Phosphoproteome characterization reveals that Sendai virus infection activates mTOR signaling in human epithelial cells. *Proteomics,* 15, 12, 2087-2097 (2015)

- SS performed the phosphopeptide enrichment together with MP, carried out phosphoprotein identification from LC-MS/MS data, and performed functional studies together with TÖ.

IV **Söderholm S**, Kainov DE, Öhman T, Denisova OV, Schepens B, Kulesskiy E, Imanishi SY, Corthals G, Hintsanen P, Aittokallio T, Saelens X, Matikainen S*, Nyman TA*.
Phosphoproteomics to characterize host response during influenza A virus infection of human macrophages. *Under review*.

- SS performed the phosphoprotemics work, the majority of functional studies, and drafted the manuscript.

* Authors with equal contribution
The original articles are reprinted with the kind permission of the copyright holders. Parts of the supporting information of the original publications are not included in this thesis book. This material is available from the author or via the Internet at http://www.mcponline.org (I), http://proteomesci.biomedcentral.com (II), and http://onlinelibrary.wiley.com (III). Additional unpublished data will also be presented.

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ABBREVIATIONS

AKT	v-akt murine thymoma viral oncogene homolog 1
ALR	AIM2-like receptor
APC	antigen-presenting cell
AP-I	activator protein 1
ASC	apoptosis-associated speck-like protein containing a CARD
ATP	adenosine-5'-triphosphate
Bcl-2	B cell lymphoma 2
CARD	caspase activation and recruitment domain
Caspase	cysteine-aspartic acid protease
Cdc42	cell division control protein 42 homolog
CDK	cyclin-dependent kinase
CDKI	cyclin-dependent kinase inhibitor
cGAS	cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) synthase
CID	collision induced dissociation
CLR	C-type lectin receptor
CTD	C-terminal domain
DAMP	danger-associated molecular pattern
DC	dendritic cell
ECD	electron capture dissociation
eIF2α	α subunit of eukaryotic translation initiation factor 2
EMCV	encephalomyocarditis virus
ESI	electrospray ionization
FDR	false discovery rate
GM-CSF	granulocyte-macrophage colony-stimulating factor
GO	gene ontology
GSK-3β	glycogen synthase kinase 3 β
HCD	higher collision induced dissociation
HCV	hepatitis C virus
HDAC	histone deacetylases
HMCV	human cytomegalovirus
hpi	hours post infection
HPLC	high-pressure liquid chromatography
IAV	influenza A virus
IFIT	interferon-induced protein with tetratricopeptide repeat
IFN	interferon
IKK	IκB kinase
IL	interleukin
IMAC	immobilized metal ion affinity chromatography
IPA	ingenuity pathway analysis
IRF	interferon regulatory factor
ISG	IFN stimulated gene
iTRAQ	isobaric tags for relative and absolute quantitation
JNK	Jun N-terminal kinase
KEA	kinase enrichment analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes
LGP2	Laboratory of Genetics and Physiology gene 2
LPS	lipopolysaccharide
m/z	mass-to-charge ratio

MAPK	mitogen-activated protein kinase
MAVS	mitochondrial antiviral-signaling protein
MDA5	melanoma differentiation factor 5
MHC	major histocompatibility complex
MOAC	metal oxide affinity chromatography
MS	mass spectrometry
MS/MS	tandem mass spectrometry
mTOR	mechanistic target of rapamycin
MyD88	myeloid differentiation primary response gene 88
NAD^+	oxidized nicotinamide adenine dinucleotide
NEMO	NF-κB essential modulator
NF-κB	nuclear factor-κB
NK	natural killer cell
NLR	nucleotide-binding domain leucine-rich repeat containing receptor
NP	nucleoprotein
NS1	non-structural protein 1
PAK1	p21 protein (Cdc42/Rac)-activated kinase 1
PAMP	pathogen-associated molecular pattern
pI:C	polyinosinic:polycytidylic acid
PI3K	phosphatidylinositol-3-kinase
PKR	dsRNA dependent protein kinase
PRR	pattern-recognition receptor
PSM	peptide spectrum match
PTM	post-translational modification
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RAC1	Ras-related C3 botulinum toxin substrate 1
RAI	RelA-associated inhibitor
RelA	nuclear factor NF-κB p65 subunit
RIG-I	retinoic acid-inducible gene I
RIP	receptor-interacting serine/threonine-protein kinase
RLR	RIG-I-like receptor
ROS	reactive oxygen species
SCX	strong cation exchange chromatography
SeV	Sendai virus
siRNA	small interfering RNA
SIRT1	NAD^+-dependent deacetylase sirtuin 1
STAT	signal transducer and activator of transcription
STING	stimulator of interferon genes
TAK1	transforming growth factor β-activated kinase 1
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFR1	TNF receptor 1
TRADD	TNFR1-associated death domain protein
TRAF	TNF receptor-associated factor
TRIF	Toll-interleukin 1 receptor homology-domain-containing adapter-inducing IFN-β
TRIM25	tripartite motif-containing protein 25
UniProtKB	Universal Protein Resource Knowledgebase
vRNP	viral ribonucleoprotein complex
VSV	vesicular stomatitis virus

ABSTRACT

Phosphorylation is one of the most important post-translational modifications of proteins. Phosphorylation is a rapid and reversible way of modifying proteins; it is involved in the regulation of many cellular processes, serving as the main transducer of intracellular signaling cascades. It is possible to identify thousands of protein phosphorylation sites from a single sample with mass spectrometry (MS)-based phosphoproteomics. This is the main reason why MS-based phosphoproteomics is such an excellent method for revealing global changes in phosphoproteomes. Computational analysis of the MS data is vital for identifying the proteins and post-translational modifications. The data analysis steps in the phosphoproteomics workflow are also crucial for biological interpretation, *e.g.* identifying activated kinases and kinase substrates, as well as unravelling signaling pathways and networks.

Cells of the innate immune system are central players in the host defence against pathogens such as viruses. Their pattern-recognition receptors (PRRs) recognize pathogen-associated molecular patterns (PAMPs), which are conserved structures present in pathogens. The most important PAMPs in viral infections are the viral genomes and replication intermediates, and their detection evokes pro-inflammatory and antiviral responses in the infected cell. Host factors and signaling cascades that promote or inhibit virus infections can serve as potential drug targets. Since protein phosphorylation is vital for the progression of nearly all signaling cascades, there is an increasing interest in applying phosphoproteomics and combining it with bioinformatics in studying the regulation of cellular signaling under various conditions, including viral infections.

The main aim of the studies included in this PhD thesis was to characterize the global changes in the cellular phosphoproteomes of virus infected and dsRNA stimulated innate immune cells, and to identify the host proteins and cell signaling pathways involved in the early stages of the host response to Sendai virus (SeV), influenza A virus (IAV), and viral dsRNA. A computational analysis tool, named PhosFox, was developed for processing and comparing MS-based phosphoproteomic data generated by multiple database search algorithms. PhosFox was used for cross-sample comparisons of phosphopeptide identifications. PhosFox also facilitated the identification of those proteins whose phosphorylation was different between samples, and the clarification of phosphorylation sites not described in the literature. The findings from the phosphoproteomic data were explored further by implementing functional studies involving small interfering RNAs (siRNAs) and kinase inhibitors. There were extensive alterations in the phosphorylation of proteins in human epithelial cells and macrophages that were transfected with the synthetic dsRNA-mimic polyinosinic:polycytidylic acid (pI:C) or infected with SeV or IAV. Many of these proteins were determined to be members of pathways with little or no previously known role in the antiviral response to these particular viruses and viral PAMPs. Two novel host factors, RelA-associated inhibitor (RAI) and sirtuin 1 (SIRT1), were identified as negative regulators of dsRNA-induced apoptosis and NF-κB regulated cytokine expression by combining 14-3-3 interactome and phosphoproteome characterizations. The p38 mitogen-activated protein kinase (MAPK) signaling pathway was found to be regulating

cytokine expression and apoptosis in dsRNA-transfected human keratinocytes. MAPK signaling pathways were also regulated in SeV and IAV infected cells. The mTOR signaling pathway was shown to be critical for the interferon response and virus replication in SeV infected human lung epithelial cells. A substantial number of those cellular proteins whose phosphorylation status changed after SeV or IAV infection or viral dsRNA challenge were involved in Rho GTPase signaling. Major changes in protein phosphorylation in IAV infected primary human macrophages were linked to cyclin-dependent kinases (CDKs). CDK activity was shown to be required for efficient viral replication and host response in IAV infection. Administration of one specific CDK inhibitor, SNS-032, also protected mice from IAV-induced death.

The studies included in this PhD thesis are some of the first to apply phosphoproteomics for characterizing host-virus interactions. They emphazise the benefit of applying phosphoproteomics and bioinformatics in innate immunity and viral research, *i.e.* it is possible not only to identify cell signaling pathways, but also the specific host factors that regulate the cellular responses to viral infections. The results of these studies underline the importance and the potential MS-based phosphoproteomics has in the discovery of novel host factors, which can serve as possible antiviral drug targets. In conclusion, the MS-based phosphoproteomics approach led to the discovery of novel host factors and cell signaling circuits in virus infected innate immune cells.

I REVIEW OF THE LITERATURE

1. PHOSPHOPROTEOMICS

1.1 Proteoforms bring diversity to biological systems

Proteins are biological macromolecules that have countless functions in the cell. Natural proteins are made of 20 canonical amino acids. Proteins are important building blocks of life; they play a role in almost every cellular process. In many aspects, they can be considered as the effector molecules of the cell. Proteins can be classified into structural proteins, enzymes, regulators, antibodies, sensors, transporters, pumps, and transducers.

The mapping of a human genome was completed about 15 years ago, and revealed that there were approximately 20,300 genes (Venter et al. 2001). Before the first draft of the human genome was published, the number of human protein-coding genes was thought to be greater than 100,000 (Pennisi 2000). The surprisingly low gene number led to the conclusion that the complexity of our biological systems lies at the level of protein variation rather than due to a large number of genes. Currently, much effort is being expended on deciphering the entire collection of human proteins, the human proteome. The protein research community is dependent on protein databases; these are being constantly updated (**Table 1**). The Universal Protein Resource Knowledgebase (UniProtKB) includes an enormous collection of annotated proteins and is considered to have the largest and best quality collection of protein sequences, and especially the manually curated 'Swiss-Prot' database is widely used.

Table 1. Selected protein databases for human protein entries.

Database	Number of protein entries	References
neXtProt	20,066 41,998 (isoforms*)	http://www.nextprot.org/db/, release *2015-09-01*, (Paik et al. 2015)
UniProtKB/Swiss-Prot	20,193 (reviewed)	http://www.uniprot.org/, release *2015-12-09*
UniProtKB/TrEMBL	129,397 (unreviewed**)	http://www.uniprot.org/, release *2015-12-09*

*The protein isoforms are protein molecules arising from the same gene by alternative splicing or variable usage of promoter. **UniProtKB/TrEMBL contains protein sequence entries with computationally generated annotations and classifications.

Variations in the expression of coding genes are controlled at multiple levels, from transcription through mRNA processing and finally at the translation stage. Alternative transcription and translation initiation as well as alternative splicing are features that make it possible for the cell to produce more than one mature mRNA, and consequently also more than one protein from one single gene (Djebali et al. 2012). Due to the generation of multiple mature mRNAs from each protein-coding gene, the human body most likely produces more than 80,000 transcripts (de Klerk, 't Hoen 2015). Moreover, the proteins translated from these mRNAs can still be further modified by various post-translational modifications (PTMs), giving rise to even more protein variants, or so-called 'proteoforms'. PTMs comprise modifications such as glycosylation, acetylation, methylation, SUMOylation, ubiquitination, lipidation, and phosphorylation. There are more than 200 PTMs described in the literature, which are directed to the side chains of the amino acids or to the N- or C-termini of the protein main chain (Mann, Jensen 2003). PTMs are a key mechanism to increase the diversity of the proteome, and because many of these PTMs are reversible, one particular protein can be present in different proteoforms during its lifetime (**Figure 1**). The translated polypeptide chains can also be cleaved into proteolytic products, which adds another dimension of versatility. Genetic variations and somatic recombination taking place in the human cell further increase the proteoform complexity. As a consequence, the collection of proteoforms is enormous; it has been estimated that as many as one billion proteoforms belong to the cell-based human proteome, and one of the goals of the Human Proteome Organisation (HUPO) and its Human Proteome Project (http://www.thehpp.org, *2016-01-21*) is to detect and analyse all these proteoforms (Kelleher 2012). There are also other projects that are contributing to completing the picture of the human proteome, for instance the Human Protein Atlas, which uses antibody-based proteomics to map the human proteome (Uhlen et al. 2015).

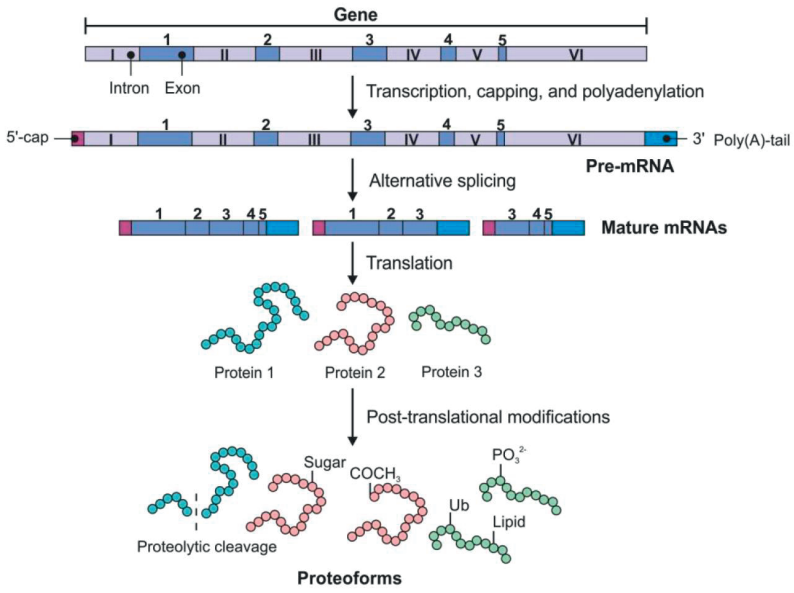

Figure 1. The complexity of the human proteome and an explanation of 'proteoform'. One protein-encoding gene can give rise to several different mRNA transcripts due to alternative splicing; introns are excised by the action of the spliceosome in order to generate mature mRNAs. In eukaryotes, a 5′-cap and a poly(A) tail are added to the mRNA before it is exported out of the nucleus. The 5'-cap structure consists of a 7-methylguanosine residue joined to the RNA transcript's initial nucleoside via a 5'-5' triphosphate bridge. The poly(A) tail is added to the 3' end of the transcript and is usually about 250 nucleotides long. After mRNA translation, the proteins can be irreversibly proteolytically cleaved or reversibly modified, *e.g.* phosphorylated, ubiquitinated, glycosylated, lipidated, or acetylated. These are the different proteoforms in which the protein product of a single gene can be found. The figure is based on descriptions in (de Klerk, 't Hoen 2015, Smith, Kelleher & Consortium for Top Down Proteomics 2013).

1.2 The human phosphoproteome

Protein phosphorylation is a PTM, which involves the addition of a covalently bound phosphate group. Protein phosphorylation is a reversible PTM, regulated by two types of enzymes: kinases and phosphatases. The kinases add phosphoryl groups to proteins, and these phosphoryl groups can be hydrolytically removed by phosphatases. Protein phosphorylation and dephosphorylation are considered to be the main activating and deactivating switches for signal transduction in cells, affecting many cellular processes including cell differentiation, cell cycle progression, apoptosis, energy metabolism, transcription, and cytoskeleton regulation (Hunter 2000, Humphrey, James & Mann 2015). Aberrantly functioning kinases and phosphatases have been linked to many different diseases, especially to several forms of cancer (Pawson, Scott 1997, Mahajan, Mahajan 2015). Therefore, still today, 112 years after the first phosphorylated protein, vitellin, was

identified (Levene PA 1904), protein phosphorylation remains a major topic in biomedical research.

In the 1950s, it was observed that many tissues, especially tumors, possess high concentrations and turnover rates of phosphorylation, but the enzymatic mechanisms behind this process were not completely understood. Phosphatase activity had already been described by this time, but it was still unclear how proteins were enzymatically phosphorylated. Protein kinase activity was discovered by Kennedy and Burnett in 1954 (Burnett, Kennedy 1954). At that time, it was known that an enzyme system situated in the mitochondria of rat liver cells was able to catalyse the oxidative phosphorylation reaction. Burnett and Kennedy observed that this enzyme was able to catalyse the transfer of a radioactive phosphate group from adenosine-5'-triphosphate (ATP) to serine residues in casein, but not to free serine molecules. As a consequence, the term 'protein kinase' was coined. This was followed by an increased interest in the role and regulation of protein phosphorylation, and in 1979, tyrosine phosphorylation was discovered (Eckhart, Hutchinson & Hunter 1979). Before this observation, it was thought that only serine and threonine could be phosphorylated in biological systems.

Up to 30% of the human proteins are thought to be phosphorylated at any given time point (Nagaraj et al. 2011). The extent to which one protein is phosphorylated can vary extensively from one amino acid residue to hundreds of amino acid residues. Protein phosphorylation events are tightly controlled by protein kinases and phosphatases, which recognize unique sequence motifs in their target proteins. Protein kinases transfer the terminal γ-phosphoryl group (PO_3^{2-}) from ATP to the side chains of specific amino acids. More rarely, the phosphoryl group can be transferred from another nucleoside triphosphate such as guanosine-5'-triphosphate (GTP). The addition of the phosphoryl group adds two negative charges to the protein, increasing its electronegativity. This explains why electrostatic interactions with other biomolecules can be disrupted or formed when a protein has been phosphorylated. Hence, phosphorylation can influence the properties of proteins in multiple ways; it can make the protein fold into a specific conformation, make it translocate to a specific site in the cell, change its enzymatic activity, and alter its substrate binding or interaction with adaptor proteins.

The most common phosphorylation in eukaryotes is *O*-phosphorylation; the addition of a phosphoryl group to the hydroxyl group on the side chain of serine (S), threonine (T), or tyrosine (Y). These three amino acids make up approximately 17% of the total average amount of amino acids in the human proteome (Echols et al. 2002). Serine accounts for most of the phosphorylated sites (86.4%), followed by threonine (11.2%), and tyrosine (1.8%) (Olsen et al. 2006). Already in 1980, this S/T/Y ratio was estimated quite correctly by Hunter and Sefton as 90:10:0.05 based on autoradiographic findings (Hunter, Sefton 1980). In addition to *O*-phosphorylation, *N*-phosphorylation of histidine, arginine and lysine, and *S*-phosphorylation of cysteine acid can be detected, particularly in prokaryotes and some eukaryotes such as fungi and plants, but their abundance and biological roles are thought to be less important compared to *O*-phosphorylation. These phosphorylation types are also less studied due to experimental difficulties. In particular, it is challenging to analyse *N*-phosphorylated amino acids, phosphoramidates, because they are rather unstable under acidic conditions. However, this should not be construed that they are unimportant; there

are reports of *N*-phosphorylation of histidine influencing the functions of histone H4 and GTPases (Attwood 2013).

It is estimated that the human genome encodes 518 kinases (Manning et al. 2002) and 228 active phosphatases (Duan, Li & Kohn 2015), representing approximately 3% of the total encoding genes. One protein kinase or phosphatase normally has several substrates, and there can be variations in the spectrum of kinases and phosphatases, for instance depending on the cell type. The overexpression of kinases and phosphatases can also perturb the dynamic regulation of protein phosphorylation, and this has been encountered in many diseases (Brognard, Hunter 2011). In addition, kinases are regarded as key targets for therapeutic agents, since mutations in kinases have been linked to abnormal phosphorylation, leading to the development of tumors and autoimmune diseases (Blume-Jensen, Hunter 2001, Brognard et al. 2011). Depending on the amino acid residue or residues they phosphorylate or dephosphorylate, protein phosphatases and kinases are divided into tyrosine and serine/threonine kinases and phosphatases. There is a large number of tyrosine kinases encoded in the human genome; 90 out of 518 protein kinases are tyrosine kinases. This is puzzling, because the phosphorylated tyrosines account for only about 1% of all phosphorylated residues in the human phosphoproteome. There are several possible explanations for this paradox; one being that tyrosine kinases are strictly negatively regulated and activated only under specific circumstances. Another explanation is that Y-phosphorylation is short-lived, because of the high activity of tyrosine phosphatases. It is not known what type of relationship Y-phosphorylation has with S- and T-phosphorylation, but in the recent report published by Sharma *et al.*, it was speculated that that phospho-Y should be considered as a functionally separate PTM and distinct from phospho-S/T and furthermore that Y-phosphorylation would be more important than S/T-phosphorylation for PTM crosstalk with acetylation, ubiquitination or SUMOylation because of the enrichment of phospho-Y residues located adjacent to lysine residues (Sharma et al. 2014).

The human phosphoproteome is estimated to contain close to one million different phosphorylation sites (Sharma et al. 2014, Boersema et al. 2010). Mapping the human proteome seems a somewhat easier task than mapping the complete human phosphoproteome. Nonetheless, there is an increasing desire to accomplish this task, mainly because it is believed that this knowledge is crucial if one wishes to monitor fluctuations in the cell phosphoproteome. For example, changes in a particular state may clarify system-wide alterations, possibly revealing interconnecting mechanisms lying behind the observed phenotype or cellular state of interest. Recently, major advances have occurred in the analytical capacity to map these mechanistic details of protein phosphorylation in cellular signal transductions, and to identify candidate targets for kinases and phosphatases of interest. These advances mainly can be traced to the ability to analyse protein phosphorylation by mass spectrometry (MS).

1.3 Biological mass spectrometry

Mass spectrometry determines the exact mass-to-charge ratio (*m/z*) of an ionized molecule. The MS instrument consists of three main components: an ion source that ionizes the analytes, a mass analyser that separates the ions, and an ion detector. With MS, it is possible to analyse and determine the abundance of even large biomolecules such as proteins, peptides, and lipids. The requirement is that they can be converted into the gas phase and ionized before they are introduced into the mass analyzer.

Scientists were able to apply MS to protein research in the late 1980s, when soft ionization methods, which are compatible with non-volatile and thermally unstable molecules, were developed. Soft ionization methods include electrospray ionization (ESI) (Whitehouse et al. 1985, Fenn et al. 1989), matrix assisted laser desorption ionization (MALDI) (Karas, Hillenkamp 1988), and soft laser desorption (Tanaka et al. 1988). The importance of these innovations was highlighted in 2002, when John B. Fenn was awarded the Nobel Prize in Chemistry for the invention of ESI, and Koichi Tanaka for the development of the soft laser desorption technique for ionizing proteins. In MALDI, the analytes are crystallized on a metal plate with a matrix that often contains specific organic molecules. When a laser beam irradiates the sample, the organic compound in the matrix absorbs the laser light, providing enough energy for desorption of the analytes, which become volatilized and ionized. Instruments applying ESI are usually connected to a high-pressure liquid chromatography (HPLC) instrument. Simultaneously as the solution containing the analytes is sprayed into an electric field in the proximity of the inlet to the mass analyzer, small charged droplets are formed and the desolvated molecules are ionized. Next, the generated ions are separated in the mass analyzer and their *m/z* ratios are measured. Nowadays, it is possible to analyse thousands of biological macromolecules from complex samples with ESI-MS.

In MS-based proteomics, the ionized molecules are either peptides or proteins. Nowadays, MS-based proteomics is used routinely in biomedical research, due not only to its high sensitivity and the speed of the MS analysis, but also because there is excellent computational software for the rapid identification of peptides and proteins. Additionally, MS-based proteomics provides comprehensive analyses of PTMs, including protein phosphorylation. Peptide-based 'discovery proteomics', also known as 'shotgun proteomics', is a universally used high-throughput proteomics method, enabling the identification of thousands of proteins from a single sample. In shotgun proteomics, the protein sample is digested into peptides and the resulting peptide mixture is fractionated by liquid chromatography before it is ionized and injected to the mass analyzer. There are several different mass analyzers intended for different types of samples, analytes, and purposes in proteomics, *e.g.* time-of-flight (TOF), quadrupole, Orbitrap, and Fourier-transform ion cyclotron resonance (FT-ICR) mass analyzers. Nowadays, hybrid or tribrid instruments, containing two or three different types of mass analyzers, are widely used for proteomics. Hybrid mass analyzers that scan rapidly, have a high resolving power and very high mass accuracy, such as the quadrupole-Orbitrap instruments, are popular in high-throughput proteomics (Lesur, Domon 2015).

Usually, successive rounds of *m/z* measurements with ion fragmentation steps in between are performed; this is called tandem MS (MS/MS) or MS^n (n being the number of fragmentation steps). For tandem MS (**Figure 2**), selected precursor ions are isolated after the initial mass separation, also called the survey scan (MS1). These precursor ions are fragmented into product ions before the second mass separation (MS2). The masses of the fragment ions are analysed and recorded as product ion spectra that allow for the determination of the amino acid sequence.

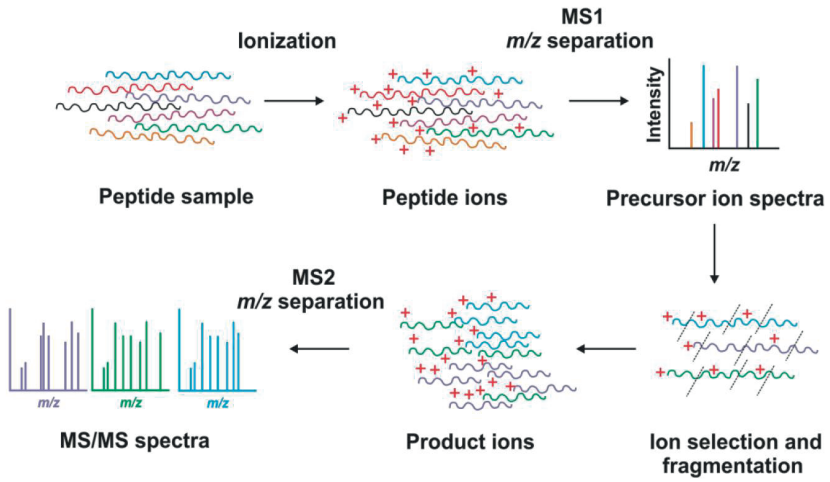

Figure 2. The principle of tandem mass spectrometry. The peptides in the sample are ionized for instance by ESI and injected to the mass analyzer. The ionized peptides are separated based on their *m/z* ratios in the initial mass separation step (MS1). A pre-determined amount of the most abundant precursor ions usually with a minimum of +2 charges are selected for the next mass separation step, the MS/MS analysis. The selected precursor ions are fragmented and the product ions are separated (MS2). The collected mass spectra are used for determing the amino acid composition of the peptide sequences.

The fragmentation step in MS is carried out by applying enough energy to break the bonds between the amino acids in the peptide; this can be performed with different methods. Collision induced dissociation (CID) and higher collision induced dissociation (HCD) generate mostly *b* and *y* ions (**Figure 3**), and are the most widely applied fragmentation methods in proteomics (Jedrychowski et al. 2011). In CID methods, the ion is energized above its dissociation threshold and the weakest bonds are cleaved first. The loss of phosphoryl groups is common, which leads to incomplete fragmentation of the peptide backbone. On the other hand, electron transfer dissociation (ETD) can leave undisturbed certain labile PTMs such as phosphorylations (Syka et al. 2004, Molina et al. 2007). ETD fragmentation relies on the addition of an electron to a multiply charged protein or peptide ion, generating mainly *c*- and *z*-type fragment ions. Electron capture dissociation (ECD) is another extensively used fragmentation technique in proteomics. ECD uses low-energy electrons to fragment the backbone of the ionized peptide (Hakansson, Zubarev & Hakansson 1998). Similarly to ETD, ECD cleaves the backbone bonds almost stochastically,

which provides a high sequence coverage, and preserves PTMs on the amino acid side chains (Kelleher et al. 1999).

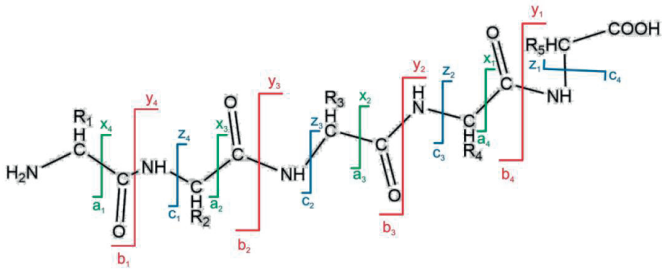

Figure 3. Different fragmentations of a precursor peptide ion. CID and HCD generate mostly *y* and *b* ions, which fragment from the C-terminus (*y* ions) and the N-terminus (*b* ions) of the peptide. Other types of fragment ions (*x*, *a*, *z*, and *c*) can also be generated. In CID and HCD, the fragmentation of the precursor peptide ion occurs through collisions with neutral gas molecules (for instance, nitrogen or helium), contributing vibrational energy that redistributes over the ion and results in the dissociation of amide bonds along the peptide backbone. This fragmentation process requires the ion to be protonated. ETD transfers electrons to a multiply protonated precursor ion, which can lead to the cleavage of the N-Cα bonds, generating *c* and *z* fragment ions. ECD also generates mostly *c* and *z* fragment ions. The generation of *x* and *a* product ions through the cleavage of Cα-C bonds rarely occurs in MS/MS. Figure adapted from (Macek, Mann & Olsen 2009). R= amino acid side chain.

1.4 Mass spectrometry-based phosphoproteomics

Protein phosphorylation can be detected and visualized in many different ways, *i.e.* sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with ^{32}P or ^{33}P labelling, Western blotting, immunoprecipitation, and flow cytometry with specific anti-phosphosite antibodies (Collins, Yu & Choudhary 2007). However, it is very challenging to use these methods for discovering new phosphoproteins, in particular for locating previously unknown phosphorylation sites. Moreover, it is laborious and time-consuming to determine quantitatively the phosphorylation sites or phosphoproteins with these techniques. MS-based phosphoproteomics is an excellent method for resolving these issues. MS-based phosphoproteomics offer a new level of detail compared to the traditional biochemical techniques; with MS-based phosphoproteomics, it is possible to study even the whole repertoire of phosphorylated proteins in a given cell or tissue. Today, MS-based phosphoproteomics is a superior tool for sensitive and high-throughput analysis of protein phosphorylation. With MS-based phosphoproteomics techniques, it is possible to identify and quantify tens of thousands of phosphopeptides from one single cell culture or tissue sample, and to localize the phosphorylation sites in a peptide sequence. For example, Sharma and co-workers demonstrated recently that their label-free workflow enabled a deep quantitative phosphoproteome analysis of epidermal growth factor receptor activated HeLa cells, identifying a total of 38,229 phosphosites on 7,832 proteins (Sharma et al. 2014).

There are several publicly available protein phosphorylation resources with experimentally verified phosphorylation sites, which are especially useful for the identification of already experimentally verified phosphorylation sites (**Table 2**). The largest resource of mammalian PTMs, including phosphorylation, is PhosphoSitePlus (http://www.phosphosite.org, *2016-03-18*) (Hornbeck et al. 2015). Approximately 95% of the data in this database has been gathered from high-throughput MS experiments.

Table 2. Selected protein phosphorylation site databases.

Database	Number of human phosphosite entries (non-redundant)	References
Phosida	24,262	http://www.phosida.de/ *2016-03-20* (Gnad, Gunawardena & Mann 2011)
Phospho.ELM	42,914	http://phospho.elm.eu.org (Diella et al. 2004, Dinkel et al. 2011) release 9.0
PhosphoSitePlus	204,477	http://www.phosphosite.org (Hornbeck et al. 2015, Hornbeck et al. 2012), release *2015-09-18*

1.4.1 Experimental procedures in phosphoproteomics

The workflow of a typical shotgun MS-based phosphoproteomics experiment generally consists of five main steps (**Figure 4**). The workflow starts with the preparation of the sample, solubilizing the sample proteins. Protease and phosphatase inhibitors are typically added to the sample lysis buffer in order to preserve the integrity and the phosphorylation status of the proteins. For example, the sample can be a serum, tissue, cell, or subcellular fraction sample. The sample preparation can be performed in many different ways, but usually it includes a protein digestion step. This step is often done enzymatically with trypsin or lysyl endopeptidase (LysC). Trypsin cleaves on the C-terminal side of arginine and lysine residues, and LysC on the C-terminal side of lysine. These enzymes generate peptides of suitable mass (700-3500 Da) for the MS analysis, and due to the amino side groups of arginine and lysine, the peptides can be readily ionized through protonation. Since the phosphopeptides constitute approximately 5% of the digested sample, the total amount of protein in the original sample typically needs to be in the milligram range, especially if Y-phosphorylated peptides are to be analysed or if both protein and phosphopeptide abundances are to be analysed from the same sample. One major challenge in the phosphoproteomics workflow is the hampering of the proteolytic cleavage of proteins by the phosphoryl groups and other PTMs. Therefore, it is often beneficial to use a combination of different enzymes in order to increase the phosphopeptide coverage, also because the negative phosphoryl groups typically decrease the digestion efficacy of certain digesting enzymes *i.e.* trypsin (Gauci et al. 2009). Giansanti and co-workers recently published a large-scale phosphoproteomics study of prostaglandin E2 stimulated human T cells where they used five different proteases (Asp-N, chymotrypsin, glutamyl endopeptidase, LysC and trypsin) for protein digestion and subsequently were able to

identify 18,430 unique phosphosites and 37,771 unique phosphopeptides originating from 5,326 proteins (Giansanti et al. 2015).

One of the largest challenges in phosphoproteomics is the low relative abundance of phosphorylated peptides compared to their non-phosphorylated counterparts. This is due to sub-stoichiometric site-specific phosphorylation of proteins; after protein digestion, the phosphopeptides represent a small proportion of all the generated peptides. In order to analyse phosphopeptides efficiently with LC-MS/MS, they need to be enriched from the large pool of non-phosphorylated peptides. If the phosphopeptides are not enriched, they become suppressed by the non-phosphorylated peptides, because these are ionized more efficiently compared to the negatively charged phosphopeptides. Successful phosphoproteomic experiments rely on peptide fractionation and phosphorylation-selective enrichment techniques that increase the relative abundance of the phosphopeptides so that they can be detected by MS and maximize the sampling depth in phosphoproteomics (**Table 3**) (Ficarro et al. 2011). The LC-based fractionation can be carried out in one dimension (1D) or in two dimensions (2D) through the combination of two different chromatography methods. In strong cation exchange chromatography (SCX), phosphorylated peptides are separated from their non-phosphorylated counterparts at a low pH. The applied increasing salt gradient in the SCX is optimized for peptides with only one or two positive charges, and since the phosphoryl groups reduce the solution charge, phosphorylated peptides elute early (Beausoleil et al. 2004). The enrichment methods typically favor certain amino acid compositions or degree of phosphorylation. In particular, immobilized metal ion affinity chromatography (IMAC) and metal oxide affinity chromatography (MOAC) are popular methods for phosphopeptide enrichment. Sequential separation of monophosphorylated peptides and multiply phosphorylated peptides can be achieved with sequential elution from IMAC (SIMAC) (Thingholm, Jensen & Larsen 2009). It is also possible to couple two or more enrichment methods in order to decrease the peptide complexity, for instance MOAC, SIMAC and HILIC (Engholm-Keller et al. 2012). MOAC or IMAC, in combination with peptide fractionation with SCX, have been shown to be effective methods in large-scale phosphoproteomics workflows (Olsen et al. 2006, Sharma et al. 2014, Pan et al. 2008). However, the present consensus is that no method is superior and no method alone is sufficient to achieve efficient enrichment. Due to the relatively low proportion of tyrosine phosphorylation, studies on tyrosine phosphorylation usually utilize immunoaffinity enrichment with phospho-Y-specific antibodies (Boersema et al. 2010, Rush et al. 2005, Kettenbach, Gerber 2011).

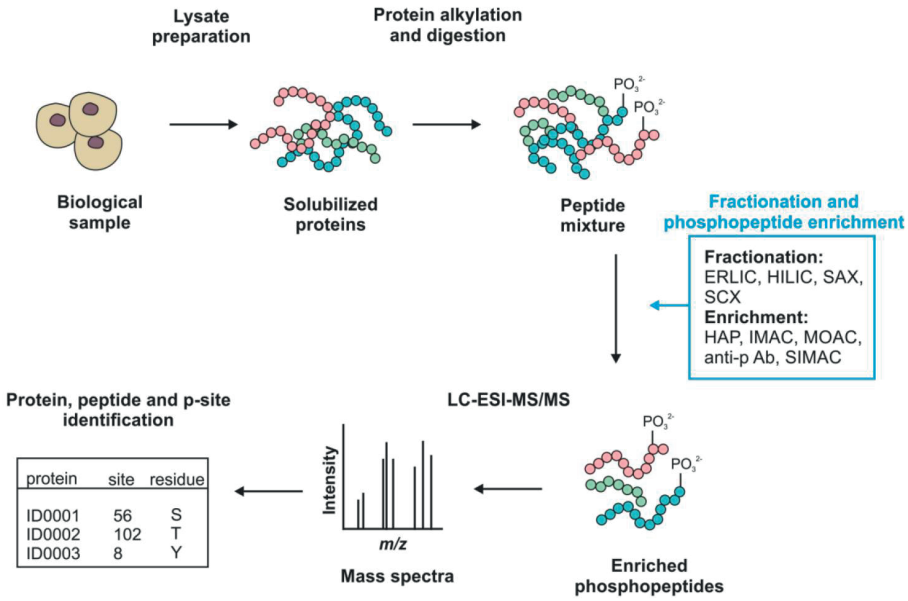

Figure 4. Outline of a typical shotgun MS-based phosphoproteomics workflow. The sample is lysed and the solubilized proteins are alkylated before protein digestion. Next, the peptides are fractionated and the phosphopeptides are enriched. LC fractionation methods are usually performed on-line and combined with one or several phosphopeptide enrichment techniques. The enriched pool of phophopeptides is analysed with LC-ESI-MS/MS. The collected mass spectra are searched against a protein sequence database with a database search algorithm, in order to identify the proteins, peptides, and PTMs. Figure adapted from (Boekhorst et al. 2011).

Table 3. Selected peptide fractionation and phosphopeptide enrichment techniques used in phosphoproteomics.

Method	Specificity and interaction	References
Peptide fractionation		
ERLIC; electrostatic repulsion-hydrophilic interaction chromatography	A combination of HILIC and ion exchange, often performed with a weak anion exchange column and rather unpolar mobile phase; non-phosphorylated peptides elute at solvent front. Singly and multiply phosphorylated species can be separated by increasing the polarity of the mobile phase	(Alpert 2008)
HILIC; hydrophilic interaction chromatography	Hydrophilic interaction between a polar resin and the peptides, eluting peptides by increasing the polarity of the mobile phase	(McNulty, Annan 2008)
SAX; strong anion exchange chromatography	Electrostatic interaction between the negatively charged residues in the peptides and the positively charged SAX resin (usually $-NR_3^+$ groups)	(Han et al. 2008)
SCX; strong cation exchange chromatography	Electrostatic interaction between the positively charged residues in the peptides and the negatively charged SCX resin (usually $-SO_3^-$ groups)	(Villen, Gygi 2008)

Table 3 continued.

Method	Specificity and interaction	References
Phosphopeptide enrichment		
HAP; Hydroxyapatite affinity chromatography	Interaction between phosphoryl groups in the peptides and Ca^{2+} in the resin	(Mamone et al. 2010)
IMAC; immobilized metal ion affinity chromatography	Electrostatic interaction between the negatively charged phosphoryl groups in the peptides and positively charged metal ions, *e.g.* Fe^{3+}, Ti^{4+}, or Ga^{3+} on the beads or resin	(Andersson, Porath 1986, Nuhse, Yu & Salomon 2007)
MOAC; metal oxide affinity chromatography	Lewis-acid interactions/bridging bidentate binding between the phosphoryl groups in the peptides and the metal oxides, such as TiO_2, ZrO_2, Nb_2O_5	(Sano, Nakamura 2007, Pinkse et al. 2004, Kweon, Hakansson 2006, Ficarro et al. 2008)
Phospho-Y immunoaffinity purification	Anti phospho-tyrosine specific antibodies for enrichment of tyrosine phosphorylated peptides	(Boersema et al. 2010, Kettenbach, Gerber 2011)
SIMAC; sequential elution from IMAC	Separation of multiply from singly phosphorylated peptides. The method applies also TiO_2	(Thingholm, Jensen & Larsen 2009)

There are certain aspects that need to be borne in mind about the sample preparation and MS analysis of phosphopeptides. It is important to appreciate that the phosphoester bond is very labile; especially *N*-phosphoryl groups are cleaved easily. Additionally, there is an on-going debate about whether the relocalization of *O*-phosphates takes place inside the MS instrument. There is, however, no clear evidence that the relocation of phosphoryl groups would be significant (Mischerikow et al. 2010). Phosphopeptide identification can be challenging, because the obtained fragmentation spectra might not contain sufficient fragmentation ions to allow the determination of the amino acid sequence and phosphosite localization. In order to obtain more accurate and reliable phosphopeptide sequences and phosphorylation site localizations, different fragmentation techniques can also be combined, such as CID and ETD (Bertsch et al. 2009).

1.4.2 Computational analysis of phosphoproteomic data

Once the MS data of the samples has been collected, the next phase is a very critical part of the phosphoproteomics workflow: the computational pipeline for phosphopeptide identification, quantification, phosphorylation site localization, and biological interpretation of the results. This so-called data mining pipeline consists of multiple steps, and each of these steps can be performed by utilizing various algorithms, analysis tools, and databases.

First, the raw (binary) MS/MS data (including *m/z* values and intensities) is converted into a peak list by a process that is called peak picking. This is generally done by the instrument data system or a specific peak picking software. The peak list contains all of the unprocessed spectra (MS1 and MS2) plus additional spectrum and instrument annotations. Next, the peak list is searched against a protein sequence database with a database search algorithm. In this step, the experimental fragment spectra are matched against theoretical database-derived fragment spectra. This step generates so-called peptide spectrum matches (PSMs). The individual PSMs are typically ranked according to a database search engine score and usually filtered at a controlled false discovery rate (FDR), which allows the user to discriminate the random matches using target decoy searches (Elias, Gygi 2007).

The most commonly used database search engines are summarized in **Table 4**. The difference between the database search algorithms is usually in the spectral comparison method and scoring scheme. The obtained results from database searches are greatly influenced by parameters and thresholds that the user selects. The protein sequence database applied in the searches also has an impact on the final results.

Table 4. Selected database search engines for identifying peptides, proteins, and PTMs from mass spectrometry data.

Database search algorithm	Comments	References
Andromeda	Integrated in the MaxQuant proteomics analysis platform	(Cox, Mann 2008, Cox et al. 2011)
Mascot	Among the most widely used database search algorithms	(Perkins et al. 1999)
MS Amanda	Also available as a plug-in in the Proteome Discoverer interface (Thermo Scientific). Particularly popular for phosphoproteomic data	(Dorfer et al. 2014)
OMSSA	Open source search algorithm	(Geer et al. 2004),
Paragon	Included in the ProteinPilot interface (AB Sciex)	(Shilov et al. 2007)
Sequest	Among the first database search engines	(Yates et al. 1995)
X! Tandem	Open source search algorithm	(Craig, Beavis 2004)

There are several bioinformatic platforms, which have been developed explicitly for managing proteomic and phosphoproteomic data and search engine identification results, for instance MaxQuant (Cox, Mann 2008), Armone (Jiang et al. 2010), ProteoConnections (Courcelles et al. 2011), and FragMixer (Vandenbogaert et al. 2012).

The FDR does not account for errors in the assignment of phosphorylation sites nor any other PTM sites. Instead, the corresponding false localization rates (FLRs) can be estimated with phosphosite localization algorithms (**Table 5**). Some phosphosite localization algorithms are integrated into the database search algorithms, for instance Mascot Delta Score in Mascot and PTM score in MaxQuant. Others can be used separately after the database search has been completed. These algorithms assign the confidence for specific phosphosites in a given peptide; hence they associate the different phosphoisomers

according to a level of statistical significance. The phosphosite localization tools seek out diagnostic fragment ions, which make it possible to distinguish between potential sites of the phosphorylation modification. Kim *et al.* recently argued that as the typical tryptic peptide is about ten amino acids and the estimated number of S, T, and Y residues reaches three million of the totally estimated 20 million amino acids in the complete human proteome, there are 1.5 potential phosphorylation sites per peptide (Kim, Zhong & Pandey 2015). This underlines the importance of estimating the FLR of phosphosites.

After the phosphopeptide identifications and phosphosite localization assignment have been completed, the phosphosites can be quantified and potentially normalized against measured protein expression levels (Wu et al. 2011).

Table 5. Selected phosphosite localization algorithms used for MS-based phosphoproteomic data.

Algorithm	Reference	Link (URL)
Ascore	(Beausoleil et al. 2006)	http://ascore.med.harvard.edu/, *2016-03-22*
Mascot Delta Score	(Savitski et al. 2011)	-
PhosCalc	(MacLean et al. 2008)	https://github.com/danmaclean/PhosCalc, *2016-03-22*
Phosphinator	(Swaney et al. 2009)	-
PhosphoRS	(Taus et al. 2011)	http://ms.imp.ac.at/?goto=phosphors, *2016-03-22*
PhosphoScan	(Wan et al. 2008)	-
PhosphoScore	(Ruttenberg et al. 2008)	http://dir.nhlbi.nih.gov/papers/lkem/phosphoscore/, *2016-03-22*
PhosSa	(Saeed et al. 2013)	https://hpcwebapps.cit.nih.gov/ESBL/PhosSA/, *2016-03-22*
Prophossi	(Martin et al. 2010)	http://www.compbio.dundee.ac.uk/prophossi, *2016-04-11*
PTM score	(Cox et al. 2011)	http://www.coxdocs.org/doku.php?id=maxquant:start, *2016-03-22*
SLoMo	(Bailey et al. 2009)	http://www.sanger.ac.uk/science/tools/turbo-slomo-high-throughput-protein-modification-localisation, *2016-03-22*

1.4.3 Interpretation of phosphoproteomic data

The final task in the phosphoproteomics workflow is to conduct a functional interpretation of the data in order to gain biological understanding and generate testable hypotheses. Biological insights can be gained by utilizing various bioinformatics tools and databases. However, these tools have not been explicitly developed for phosphoproteomic data, but for all types of 'omics' data including genomics, proteomics, and transcriptomics. One step in the computational analysis pipeline is to map and visualize protein-protein interactions, *i.e.* to identify signaling networks and pathways, using various knowledgebases and analysis tools. Usually, the proteins are initially connected to their associated Gene Ontology (GO) terms (http://www.geneontology.org, *2015-11-22*) (Ashburner et al. 2000). This is called a functional annotation. Some proteomics data analysis platforms, such as MaxQuant and

Proteome Discoverer, have an implemented GO term annotation step. There are many commercial and open-access depositories which assist in functional annotation, pathway, and network analysis (**Table 6**).

Table 6. Selected bioinformatics resources for biological interpretation of 'omics' data.

Resource	Freeware/ commercial	Application	References
BioGRID	Freeware	Protein-protein interaction database	(Chatr-Aryamontri et al. 2015)
Cytoscape	Freeware	Visualization of molecular interaction networks and biological pathways	(Shannon et al. 2003)
DAVID	Freeware	Data-mining platform with a biological knowledgebase and various analytic tools for functional annotation	(Huang da, Sherman & Lempicki 2009)
InnateDB	Freeware	Functional annotation tools and knowledgebase focused on innate immunity related pathways, networks, and genes	(Breuer et al. 2013)
Ingenuity Pathway Analysis (IPA)	Commercial	Software application for the analysis, integration, and interpretation of omics data	Qiagen
GeneTrail	Freeware	Analysis of gene sets for statistically significant accumulations of genes that belong to some functional category	(Backes et al. 2007)
Kyoto Encyclopedia of Genes and Genomes (KEGG)	Freeware	Resource for analysis of pathways and biological processes	(Kanehisa et al. 2015)
Reactome	Freeware	Signaling pathway database	(Croft et al. 2014)
PathVisio	Freeware	Pathway analysis and visualization tool	(van Iersel et al. 2008)
PhosphoPath	Freeware	Visualization tool for phospho-proteomics data, integrated in Cytoscape	(Raaijmakers et al. 2015)
PhosSigNet	Freeware	Human phosphorylation-mediated signal transduction networks database and visualization tool	(Zhang et al. 2015)
STRING	Freeware	Protein-protein interaction database	(Franceschini et al. 2013)
TRANSPATH	Freeware	Database of mammalian signal transduction and metabolic pathways	(Krull et al. 2006)

Information on active protein kinases and phosphatases and their substrates in the samples are usually of particular interest. Various analysis tools and algorithms can be utilized for identifying kinase substrates or kinase motifs in phosphorylated proteins. Some tools predict also which kinase or kinases could possibly have phosphorylated a putative phosphosite. These so-called 'phosphorylation site predictors' make the predictions based on information derived from the amino acid residues surrounding the phosphorylated

amino acid. Examples of various kinase, kinase motif, kinase substrate, and site prediction tools are presented in **Table 7**.

Table 7. Selected computational tools for identifying kinase motifs, kinase substrates, and predicting activated kinases.

Resource	Application	References
Kinase Enrichment Analysis (KEA)	Identification tool for kinase substrates	(Lachmann, Ma'ayan 2009)
KinasePhos	Prediction of phosphorylation sites within a given protein sequence	(Huang et al. 2005)
KinomeXplorer	Identification tool for kinase-substrate relationships	(Horn et al. 2014)
MMFPh	Kinase motif finder	(Wang et al. 2012)
Motif-X	Kinase motif finder	(Schwartz, Gygi 2005)
NetPhorest	Atlas of consensus sequence motifs that covers 179 kinases and 104 phosphorylation-dependent binding domains. Integrated in KinomeXplorer	(Miller et al. 2008)
NetworKIN	Prediction tool for kinases associated with specific phosphosites. Integrated in KinomeXplorer	(Horn et al. 2014, Linding et al. 2008)
PhosphoMotif Finder	Database for curated kinase/phosphatase substrates and binding motifs. In HPRD*	(Amanchy et al. 2007)
PhosphoNetworks	Identification tool for kinase substrates	(Hu et al. 2014)
PhosphoSiteAnalyzer	Platform for deciphering phosphoproteomes using kinase predictions retrieved from NetworKIN	(Bennetzen et al. 2012)
PhosphoSitePlus	Phosphosite database, but includes also an application for finding kinase motifs	(Hornbeck et al. 2012)
RegPhos	Knowledge-base and prediction tool for identification of kinase-substrate relationships	(Huang et al. 2014a, Lee et al. 2011)
Scansite	Kinase motif finder	(Obenauer, Cantley & Yaffe 2003)

*HPRD: Human Protein Reference Database, http://www.hprd.org, *2015-12-11.*

2. INNATE IMMUNITY

2.1 The innate and adaptive immune system

The innate immune system is the first line of defence against pathogenic microorganisms; this includes the physical barriers of our body, for instance the skin and the mucosal epithelia of the gastrointestinal and respiratory tract. The innate immunity comprises also chemical defence mechanisms, such as the low pH of our stomach, and humoral defence

mechanisms such as antimicrobial peptides in the mucus of the lungs and gut, as well as the > 20 pro-proteins of the complement system, which are produced by hepatocytes and circulate in the bloodstream (Trouw, Daha 2011). Additionally, the innate immune system possesses specialized phagocytic and killer cells that can eliminate infectious microorganisms. Phagocytes also maintain homeostasis by removing apoptotic cells and cell debris. The innate immune system is activated within hours after an infection. Should it be the case that the innate immune system is unable to clear the infection, the next phase is to transmit a message to the adaptive immune system. The adaptive immune system then comes into play and continues the unresolved battle with the infectious organism. Thus, the adaptive immune system acts as a second line of defence and can also protect against re-exposure to the same pathogen (Medzhitov 2007). The adaptive immune system is antigen-specific and with time, it develops an immunological memory in the individual. The adaptive immune system depends on a process called somatic gene rearrangement of lymphocytic B cell and T cell receptors. This process gives rise to an extensive repertoire of T cells with different antigen-specific cell-bound receptors, and B cells with antigen-specific immunoglobulins.

The main function of the innate immune system is to detect invasive microorganisms, infected cells, and danger signals, and to attract other cells of the immune system to the site of infection. It is important that the innate immunity can distinguish between 'self' and 'non-self', activating responses to invading pathogens when recognizing 'non-self' (Medzhitov, Janeway 2002). However, the 'danger model' is currently more widely accepted for describing its function (Pradeu, Cooper 2012). It was coined by Dr. Polly Matzinger, who proposed that our innate immune system is designed to combat danger, and that "endogenous cellular alarm signals from distressed or injured cells" are the triggers of innate immune responses (Matzinger 1994).

The innate immunity relies on a collection of distinct pattern-recognition receptors (PRRs) that can detect pathogen-associated molecular patterns (PAMPs) such as fungal β-glucan, viral nucleic acids, and bacterial lipopolysaccharide (LPS). PAMPs are unique and conserved structures of pathogens that usually have crucial roles in microbial physiology. The PRRs are encoded in the germline and are susceptible to change only on the evolutionary scale, meaning that they cannot be influenced during the lifespan of an individual. In addition to PAMPs, the innate immune system can react to so-called danger-associated molecular patterns (DAMPs), such as those released upon tissue damage or secreted by activated inflammatory cells. An example of a DAMP released due to cellular damage is extracellular ATP (Mariathasan et al. 2006). Some DAMPs are also directly associated with certain dietary disorders, as in the case of gout-associated monosodium urate (MSU) crystals (Martinon et al. 2006).

2.2 Cells of the immune system

Many of the innate immune cells circulate in the bloodstream, but when they are activated, they leave it and migrate to the sites of infection or danger, for instance to peripheral tissues such as the epidermis or the lung tissue. Some cells, such as dendritic cells (DCs)

and macrophages, reside in these tissues. Therefore, they are among the first cells to encounter pathogens that have succeeded in penetrating through the epithelial layers of the body. The innate immune cells react rapidly to the danger signals and pathogens that they detect. In particular, phagocytic cells start secreting large amounts of cytokines that initiate inflammatory responses (Iwasaki, Medzhitov 2015).

The most important bridge between innate and adaptive immunity is the interaction between the receptors of lymphocytes, most importantly T cells, and the major histocompatibility complex (MHC) molecules of antigen-presenting cells (APCs). The APC is often an activated DC that has migrated from a site of infection to the lymph nodes. This APC will present the MHC in complex with a small antigen (in the form of a peptide), which is originating from the pathogen that is to be eliminated. The interaction between the APC and the naïve T cell together with CD4 or CD8 co-receptors and secreted co-stimulatory cytokines, activates the T cell and initiates hereby the adaptive immune system (Iwasaki, Medzhitov 2015). This is the starting point for the proliferation of antigen-specific T cells and B cells. These cells are intended to eliminate any persistant pathogens, and protect the host from re-infections by that particular pathogen.

The cells of the immune system are derived from pluripotent hematopoietic stem cells of the bone marrow (**Figure 5**). The mast cells, DCs, monocytes, macrophages, and granulocytes (basophils, eosinophils and neutrophils) are derived from a common myeloid progenitor and belong to the innate immune system. The neutrophils, macrophages, and DCs are the main inflammatory and phagocytic cells of the innate immune system. DCs and macrophages are also important APCs, presenting peptides derived from engulfed pathogens on their MHC class II proteins. Macrophages and DCs reside primarily in tissues, while the neutrophils circulate in the blood. Basophils and eosinophils release toxic granules upon activation and are also important players in the innate immunity. The natural killer cells (NKs) are derived from a common lymphoid progenitor, but classified as innate immune cells, because they lack antigen-specific receptors. The NKs are especially important for detecting and killing virus infected cells. A subset of T cells, γδ T cells, which have limited antigen receptor diversity, also participate in the innate immunity (Bendelac, Bonneville & Kearney 2001).

The lymphoid lineage comprises mainly B and T cells. B cells mature in the bone marrow, whereas the precursors of T cells migrate from the bone marrow to the thymus to undergo development. The maturation process of B and T cells includes somatic gene rearrangements and multiple negative and positive selection steps in order to generate antigen-specific and self-tolerant cells. The B and T cells that survive the development and maturation process in the central lymphoid tissues migrate to the peripheral lymphoid organs, such as the spleen and lymph nodes. The pro-B cell matures and differentiates into plasma producing B cells or long-lived memory B cells. The plasma B cells produce large amounts of antibodies that bind to their specific epitopes, opsonizing infected cells or pathogens thereby marking them for uptake by phagocytic cells. T cells develop along three main lineages and become either γδ T cells, iNKT cells, or αβ T cells. The αβ T cells are either CD4 or CD8 positive. CD4+ T cells recognize peptides bound to MHC II molecules, and can further mature into T regulatory cells and T helper cells (T_H cells). The CD8+ T cells, also

known as cytotoxic T cells, recognize the peptides bound to MHC I molecules and recognize and kill mainly virus infected cells.

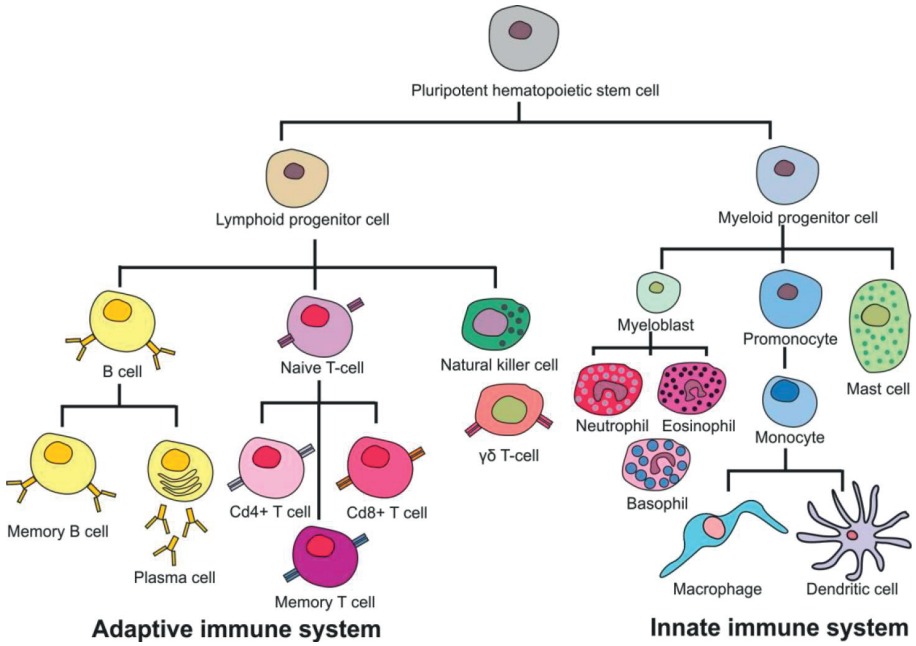

Figure 5. Cells of the immune system. The cells of innate and adaptive immune systems are derived from pluripotent hematopoietic stem cells residing in bone marrow. These cells divide into two different stem cells: lymphoid and myeloid stem cells, which differentiate along two separate pathways. The lymphoid progenitor stem cells give rise to B and T cells, which form the adaptive immune system. The myeloid progenitor stem cells give rise to granulocytes (neutrophils, basophils, and eosinophils), monocytes, macrophages, DCs, and mast cells, which shape the innate immune system. NKs and γδ T cells are considered part of the innate immune system, although they belong to the lymphoid lineage. Figure modified from (Murphy 2011).

2.2.1 Macrophages

Monocytes, which make up approximately 10% of the total leukocytes in the blood, can mature into macrophages *e.g.* during their migration to the site of infection (Robbins, Swirski 2010). The monocyte to macrophage development processes are regulated by growth factors, such as macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), along with cytokines such as interleukins (ILs) IL-4 and IL-34 (Italiani, Boraschi 2014). Macrophages are very abundant throughout the body and they are often resident in tissues and concentrated at locations of exposure to pathogenic infiltration such as the epithelial linings and microvasculature. Macrophages are found especially in the epidermis, gut and lung tissue. There are several different types of macrophages in the body, but their roles are essentially the same regardless of where they reside: osteoclasts in the bone, alveolar macrophages in the lung, microglial cells in

the central nervous system, histiocytes in the connective tissue, Kupffer cells in the liver, and Langerhan cells in the skin (Gordon, Pluddemann & Martinez Estrada 2014). The main role of macrophages is to scavenge and engulf invading microorgansims, infected cells, dead cells, and other possible debris inside our bodies. Especially the Kupffer cells and the macrophages in the spleen remove daily large amounts of dying and dead cells from the blood. In addition to being important phagocytic cells, macrophages play a very crucial role in the induction of the immune response. Macrophages express a large repertoire of PRRs, and upon activation, they secrete large amounts of soluble cytokines and chemokines that induce the innate immune response. In some special cases, macrophages can also function as APCs and expose pathogen-derived peptides to lymphocytes.

In response to the changes in the surrounding infected or damaged tissues, macrophages polarize into two principal phenotypes: M1 and M2. The M1 macrophages are pro-inflammatory, while the M2 macrophages are anti-inflammatory (Mills, Lenz & Ley 2015). The M1 macrophages differentiate from monocytes by GM-CSF or interferon-γ (IFN-γ) exposure, and they promote T_H1- or T_H17-mediated immune responses. The M2 macrophages are activated by IL-4, IL-10, or M-CSF and they suppress T cell and host defence responses (Martinez et al. 2008). The M2 macrophages are able to differentiate into M1 type, but the reverse route is not considered possible, mainly due to the production of toxic nitric oxide in activated M1 macrophages, which is thought to be the main reason for the determined cellular state and death of the M1 cells (Italiani, Boraschi 2014).

2.2.2 Cells of the body linings

The epithelial lining of the inside and outside of our body is not only a physical barrier against pathogens: the epithelial cells of the skin, lungs, gastrointestinal tract, and genital area express PRRs and participate actively in the recognition of pathogens and danger signals, producing inflammatory cytokines and chemokines. Although epithelial cells are not part of the so-called professional cellular part of the innate immunity, they play a major role in the immune response.

The epidermis is the outermost layer of the skin. Keratinocytes are the most abundant cells in epidermis, representing over 90% of the cell types (Nestle et al. 2009). Keratinocytes are not only needed for the physical defence, but are believed to be some of the first cells to encounter invading pathogens and actively participating in the immune response against these microorganisms. In the presence of an infection, keratinocytes start to express large amounts of pro-inflammatory chemokines and cytokines, especially IL-1α and tumor necrosis factor (TNF), but also antimicrobial peptides such as β-defensins (Yang, Chertov & Oppenheim 2001, Albanesi et al. 2005). Other cell types of the epidermis are mast cells, DCs, Langerhan cells and macrophages. They secrete antimicrobial peptides, inflammatory cytokines, and chemokines upon ligand binding or danger signal detection. The layer under the epidermis, the dermis, is also important in immune surveillance. The dermis contains numerous dermal fibroblasts, which produce connective tissue, but also DCs, and memory T cells. The most abundant cells in the lining of the respiratory tract are

epithelial cells, but macrophages and DCs also reside in the tissue lining the airway, especially in the lung tissue (Kopf, Schneider & Nobs 2015).

2.3 Activation of the innate immune response

In order for the immune responses to be initiated, the cellular part of the innate immunity has to recognize the pathogen or 'danger signal'. This recognition is dependent on the PRRs, which initiate intracellular signaling pathways that induce changes in gene expression, particularly in the expression of inflammatory cytokines and chemokines. These cytokines are secreted and they recruit, activate, or inhibit other cells of the immune system. They subsequently increase the expression of PRRs, antigen-receptors, and co-stimulatory receptors, and recruit leukocytes to the site of infection or damage. The cytokines also affect the proliferation of immune cells, and the phagocytic activity of macrophages, neutrophils and DCs.

2.3.1 Pathogen- and damage-associated molecular patterns (PAMPs and DAMPs)

There are numerous PAMPs and DAMPs, which are able to activate the innate immune system. For instance, LPS is a major structural component of the outer wall of Gram negative bacteria and a very well-studied PAMP. Other well-characterized PAMPs are viral nucleic acids (Wilkins, Gale 2010), Gram negative bacterial heptose-1,7-biphosphate (Gaudet et al. 2015), and β-glucan (McIntosh, Stone & Stanisich 2005). There is not always the need to detect a specific antigen in order to activate the innate immune system; sometimes a DAMP can be sufficient, in accordance with the 'danger model'. Cellular K^+ efflux (Munoz-Planillo et al. 2013), the generation of reactive oxygen species (ROS) (Sorbara, Girardin 2011), extracellular ATP (Mariathasan et al. 2006), MSU (Martinon et al. 2006), and cholesterol crystals (Rajamaki et al. 2010) are considered as DAMPs which are very potent inducers of innate immune responses.

2.3.2 Pattern recognition receptors (PRRs)

The innate immune system is strictly dependent on the germline-encoded PRRs, which detect and initiate the response to microbial components, danger signals, and environmental irritants. The family of PRRs includes Toll-like receptors (TLRs), Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), AIM2-like receptors (ALRs), NOD-like receptors (NLRs; nucleotide-binding domain leucine-rich repeat containing receptors), and C-type lectin receptors (CLRs). The TLRs and CLRs are transmembrane receptors, while the RLRs, ALRs, and NLRs are cytoplasmic receptors (Takeuchi, Akira 2010). The PRRs initiate and transduce signaling pathways that activate transcription factors such as nuclear factor-κB (NF-κB), interferon regulatory factors (IRFs), and activator protein 1 (AP-1) family proteins (such as c-Jun, and c-Fos) (Takeuchi, Akira 2010). These transcription regulators initiate or increase the expression of inflammatory cytokines and antiviral interferons

(IFNs). This ultimately culminates, at best, in restored homeostasis or in the elimination of the invading microbe.

Detecting foreign or self DNA in the cytoplasm is a sign of danger to the host (O'Neill 2013). Quite recently, a cytoplasmic sensor known as cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) synthase (cGAS) was identified to be an innate immune sensor, especially of viral and bacterial DNA, but also of endogenous DNA (Ishikawa, Barber 2011, Wu et al. 2013). When cGAS binds to cytoplasmic DNA, and is activated, it starts to catalyse the synthesis of a specifc cGAMP isomer (Ablasser et al. 2013). The endoplasmic reticulum-resident adaptor protein, stimulator of interferon genes (STING), binds cGAMP, activating inhibitory κB kinases (IKKs) and TANK-binding kinase 1 (TBK1), which in turn activate both NF-κB and IRF3 (Ishikawa, Barber 2011).

2.3.3 Toll-like receptors

TLRs are membrane-bound glycoproteins consisting of a cytoplasmic Toll-interleukin 1 receptor (TIR) domain and a horse-shoe-shaped N-terminal leucine-rich repeat domain, which is the ligand-binding domain and located either on the extracellular side of the cell membrane or inside endosomes. The TLRs detect exogenous and endocytosed PAMPs, but can also sense endogenous DAMPs such as β-defensins (Piccinini, Midwood 2010). Activated TLRs mediate signaling via their adaptor proteins myeloid differentiation primary response gene 88 (MyD88) and TIR domain containing adaptor protein (TIRAP) (MyD88-dependent pathway) or TIR-domain-containing adapter-inducing interferon-β (TRIF) (MyD88-independent pathway) (Kenny, O'Neill 2008).

There are ten *TLR* genes in the human genome (Takeuchi, Akira 2010). The TLRs are located either in the plasma membrane or in the membranes of endosomes. The TLR1-TLR2 and TLR2-TLR6 heterodimers detect bacterial peptidoglycans and lipoproteins. TLR3 recognizes extracellular dsRNA, which is internalized into the endosomes (Alexopoulou et al. 2001). TLR3 activates IRF7 through TRIF, and NF-κB and AP-1 through the TNF receptor 1 (TNFR1)-associated death domain protein (TRADD)/receptor-interacting serine/threonine-protein kinase 1 (RIP1) complex. The TLR3 pathway can also induce TBK1-mediated phosphorylation of IRF3. TLR4 is the most extensively studied TLR, and it recognizes LPS. TLR4-induced signaling is mediated through MyD88/TIRAP, activating MAPKs and NF-κB. The TLR4 downstream cascade can also be activated via the MyD88-independent way, through TRIF, if the TLR4-LPS complex is taken up through endocytosis. Thus, the same kinases and transcription factors, MAPKs, NF-κB, but also IRF3 are activated. TLR5 is expressed on specialized DCs and recognizes flagellin, leading to pro-inflammatory cytokine expression, in particular *TNF* expression (Choi et al. 2010). TLR7 (which is expressed only in mice) and TLR8 detect endosomal ssRNA of viral origin, and TLR9 detects endosomal unmethylated CpG DNA of bacterial origin, activating signaling cascades via MyD88 (Heil et al. 2004). TLR10 was recently identified and its function and ligands are not completely characterized, but TLR10 has been postulated to interact with TLR2 (Oosting et al. 2014).

2.3.4 Inflammasomes

The best-established function of NLRs and ALRs is their assembly of inflammasomes in response to infection or tissue damage. Inflammasomes are cytosolic multiprotein sensory complexes that are able to detect and respond to different kinds of stress signals, not only those derived from pathogens but also from metabolic dysregulation and tissue damage. The ALRs are, in addition to cGAS, important sensors of cytosolic DNA; the ALR members AIM2 and IFI16 inflammasomes sense both bacterial and viral DNA, which they bind directly via their HIN-200 domain (Burckstummer et al. 2009, Hornung et al. 2009, Unterholzner et al. 2010). Inflammasomes are responsible for the activation of many inflammatory caspases, including caspase-1, caspase-4/5 (caspase-11 in mice), and caspase-12. The canonical inflammasome complex consists of a sensor, an adaptor and an effector; it regulates the activation of caspase-1 and the secretion of IL-1β and IL-18 (Martinon, Mayor & Tschopp 2009). The non-canonical inflammasomes, on the other hand, activate caspases other than caspase-1.

The best-characterized canonical inflammasome is the NLRP3 inflammasome; this consists of the sensor protein NLRP3, the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD), and the effector molecule caspase-1 (Mariathasan, Monack 2007, Franchi et al. 2009). Caspase-1 is synthesized as an inactive zymogen (pro-caspase-1), and is activated by autocatalytic cleavage upon inflammasome assembly and activation, forming an enzymatically active 10 kDa (p10)/20 kDa (p20) heterodimer (Martinon, Burns & Tschopp 2002). ASC functions as a bridge between NLRP3 and pro-caspase-1, binding to the N-terminal pyrin domain of NLRP3. When NLRP3 detects an agonist, ASC can oligomerize and form large macromolecular ASC oligomers, or so-called ASC specks, to which pro-caspase-1 is recruited (Stutz et al. 2013, Lin et al. 2015).

The assembly and activation of the NLRP3 inflammasome requires two signals; 'signal 1' and 'signal 2'. Signal 1, also called the priming step, represents the induction of pro-IL-1β and NLRP3 expression through NF-κB-dependent gene transcription. Signal 1 can be mediated in several ways *e.g.* by TLR ligands such as LPS (Embry et al. 2011). Signal 2 is the activation step of the NLRP3 inflammasome, initiating the proteolytic processing of pro-caspase-1. Active caspase-1 cleaves pro-IL-1β and pro-IL-18, leading to their unconventional secretion (ER/Golgi-independent secretion). The secreted IL-1β upregulates adhesion factors and chemokines, resulting in an influx of neutrophils at the site of inflammation (Dinarello 2005). Moreover, IL-1β promotes the differentiation of T cells into the T_H17 lineage, which further promotes the neutrophil response (Coccia et al. 2012). Activated caspase-1 induces also a lytic form of programmed cell death, called pyroptosis (Martinon, Burns & Tschopp 2002). Pyroptosis has important roles in the innate immune response, but it is primarily induced by bacterial pathogens (Latz, Xiao & Stutz 2013). Signal 2 can be delivered in the form of PAMPs or DAMPs and it was described more than 25 years ago, when Hogquist and colleagues discovered that mouse macrophages required stimulation by ATP in order to cleave pro-IL-1β (Hogquist et al. 1991).

In contrast to the other inflammasomes, the NLRP3 inflammasome is known to be activated by a broad array of agonists. There are reports that extracellular ATP first binds to and then opens the P_2X_7 receptor (Mariathasan et al. 2006), leading to cellular K^+ efflux,

consequently activating the NLRP3 inflammasome (Munoz-Planillo et al. 2013). Endogenous stimuli such as crystalline MSU or cholesterol, which are known to damage phagolysosomes and impair mitochondrial function, can also activate the NLRP3 inflammasome (Dostert et al. 2008). In addition, the NLRP3 inflammasome can be activated by microbial components such as viral and bacterial RNA, and the dsRNA analog pI:C (Mariathasan et al. 2006, Kanneganti et al. 2007, Kanneganti 2010, Sutterwala et al. 2006). Furthermore, the NLRP3 inflammasome is activated by RNA viruses such as IAV (Kanneganti et al. 2006, Allen et al. 2009, Thomas et al. 2009), SeV (Kanneganti et al. 2006), encephalomyocarditis virus (EMCV) (Ito, Yanagi & Ichinohe 2012), and vesicular stomatitis virus (VSV) (Rajan et al. 2011).

The non-canonical inflammasomes do not activate caspase-1. Instead, they activate principally caspase-4 (caspase-11 in mice) and caspase-8 (Broz, Monack 2013). One cytoplasmic protein, gasdermin D, is crucial in non-canonical inflammasome signaling (Kayagaki et al. 2015). The caspase-8 non-canonical inflammasome comprises mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1), ASC, and caspase-8. Upon detection of extracellular fungal or mycobacterial PAMPs by the CLR Dectin-1, caspase-8 processes pro-IL-1β to its mature form (Gringhuis et al. 2012). After stimulation of the Fas receptor, which is a TNFR receptor known to induce apoptosis, there is also a non-canonical caspase-8 mediated cleavage of pro-IL-1β and pro-IL-18 (Bossaller et al. 2012). Some PAMPs such as LPS, do not need to act via TLR4, instead they can directly activate the canonical NLRP3 inflammasome through the non-canonical inflammatory pathway involving caspase-11 activation, leading to caspase-1 mediated processing of pro-IL-1β, pro-IL-18 and pyroptosis (Broz et al. 2012, Kayagaki et al. 2011). Moreover, caspase-4 and caspase-5 have been shown to bind intracellular LPS, directly inducing pyroptosis and IL-1β cleavage (Shi et al. 2014).

2.3.5 RIG-I-related receptors

The RLRs sense RNA in the cytoplasm and they are the main detectors of viral RNAs (Pichlmair, Reis e Sousa 2007). There are three members of the RLR family: RIG-I, melanoma differentiation factor 5 (MDA5), and Laboratory of Genetics and Physiology gene 2 (LGP2). The RLRs have a central ATP hydrolyzing DExD/H box helicase domain and a RNA binding C-terminal domain (CTD) (Bruns, Horvath 2014). RIG-I and MDA5, but not LGP2, have two caspase activation and recruitment domains (CARDs) in tandem at their N-termini, and the CARDs are essential for mediating signaling (Gorbalenya et al. 1988). LGP2 detects a wide range of dsRNA species, but due to the lack of CARDs, LGP2 is thought to be a positive regulator of responses by MDA5 and RIG-I to viral RNA (Venkataraman et al. 2007, Bruns, Horvath 2015). Upon RNA binding to the CTD, the CARDs of MDA5 and RIG-I interact with their common adaptor protein, a scaffold molecule called mitochondrial antiviral-signaling protein (MAVS), which is localized on the outer membrane of the mitochondria and serves as a platform for coordinating downstream innate immune and antiviral signaling. This interaction initiates MAVS oligomerization (Bruns, Horvath 2014). MAVS filaments recruit several downstream signaling molecules, such as TNF receptor-

associated factor 2 (TRAF2), 3 (TRAF3), and 6 (TRAF6). This leads to activation of the non-canonical IKK-related kinases TBK1 and IKKε, which are important players in NF-κB and IRF3/7 activation (Fitzgerald et al. 2003, Hou et al. 2011). Tripartite motif-containing protein 25 (TRIM25) was shown to be critical for RIG-I activation (Gack et al. 2007). TRIM25 interacts with RIG-I and delivers the K63-linked ubiquitin moiety to the second CARD domain on the N-terminus of RIG-I, resulting in its binding to MAVS and a marked increase in RIG-I downstream signaling and type I IFN induction.

MDA5 binds primarily long segments of dsRNA (Kato et al. 2008), while RIG-I recognizes viral RNAs containing 5'-triphosphates (5'-ppp), which are features of many negative-sense single-stranded RNA, (-)ssRNA, viruses such as IAV (Pichlmair et al. 2006, Hornung et al. 2006). This 5'-ppp modification is recognized as a PAMP, because it is absent in the cytoplasmic spliced and capped host mRNAs. In particular, short viral RNAs containing 5'-ppp are recognized by RIG-I (Baum, Sachidanandam & Garcia-Sastre 2010). The 5'-ppp RNA binds to the CTD of RIG-I, leading to the dimerization of RIG-I with the subsequent conformational change, which stimulates its ATP activity (Cui et al. 2008). RIG-I binds also short segments (300-2000 bp) of dsRNA and synthetic dsRNA molecules such as pI:C, however, pI:C has also been found to be a very strong activator of MDA5 (Pichlmair et al. 2006, Hornung et al. 2006, Rehwinkel et al. 2010, Kato et al. 2006, Gitlin et al. 2006). The recognition of pI:C by RIG-I is facilitated by the dsRNA endonuclease Dicer, which cleaves pI:C so that it becomes a suitable substrate for RIG-I (Kato et al. 2008).

3. VIRAL INFECTIONS

Viruses are very small, usually nanoscale-sized, pathogens. They lack metabolism and are classified as obligate parasites, meaning that they are dependent on a host cell for their reproduction. There are many ways to classify viruses, but the most common approach is based on the genome they contain. According to this classification, viruses can be divided into ssRNA, dsRNA, ssDNA and dsDNA viruses.

Viruses try to hijack the host cell, while the infected cell tries to defend itself against the viral infection by triggering various molecular mechanisms. Viral infections usually induce a strong immune response, which is vital for the clearance of the infection. The extent of the response is dependent on the cellular interactions between the innate immune cells and the cells of the adaptive immune system, in particular the T cells. Another important factor in the clearance of viral infections is the type and magnitude of cytokines that are produced by the innate immune cells, because they determine the extent and quality of the specific adaptive immune responses. **Figure 6** shows a schematic model of one of the most common routes of viral infection and the early steps of infection progression.

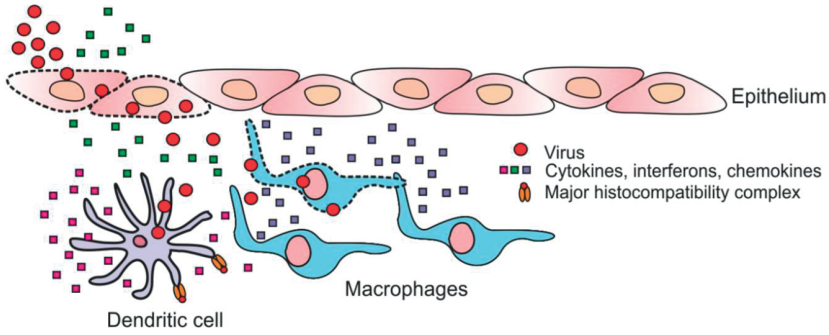

Figure 6. Schematic model of progression and effect of a typical viral infection. First, the incoming virus penetrates the epithelial lining of the body, *e.g.* of the respiratory tract or skin, infecting the epithelial cells. The virus replicates inside the cells and the progeny viruses are released into the extracellular space. These virus particles, in turn, can infect nearby uninfected cells. The viral infection induces pro-inflammatory and antiviral cytokine reponses; the infected cells secrete IFNs, chemokines and interleukins. These cytokines act locally around the infected tissue and prime nearby cells; enhancing their expression of PRRs and other proteins involved in antiviral signaling and defence. The cytokines also have systemic effects, attracting other cell types of the innate immune system to the site of infection, *e.g.* macrophages and DCs. Activated DCs, which present peptides derived from the virus with their MHC molecules, migrate from the infected tissue to the nearby lymph nodes where they activate naïve T and B cells. This awakens the adaptive immune system to respond to the ongoing infection. Other innate immune cells, including macrophages, can also be infected by viruses, triggering mainly the release of large amounts of antiviral IFNs, pro-inflammatory cytokines, and DAMPs. Some infected cells also sacrifice themselves through programmed cell death in order to limit the infection.

3.1 Antiviral cytokine expression in virus infected innate immune cells

In virus infected innate immune cells, the induction of an antiviral innate immune response is dependent on the presence of PRRs, in particular the RLRs and TLRs. These initiate signaling pathways that lead to the production of cytokines with pro-inflammatory and antiviral effects, especially IFNs. The expression and secretion of chemokines *e.g.* CXCL10, CXCL8, CCL3, and acute-phase cytokines such as IL-6 and TNF, IL-1α/β, and IL-18 are often enhanced upon viral infections, especially upon IAV infection (Scheller et al. 2011, Geiler et al. 2011). IL-6, and in particular IL-1β are potent pro-inflammatory factors during viral infections, and their expression is tightly controlled by *e.g.* NF-κB, and the processing of IL-1β into its mature and secreted form is dependent on the NLRP3 inflammasome. In summary, antiviral cytokines and chemokines are critical for controlling virus replication and for activating the adaptive immune response.

3.1.1 Interferons

It has long been known that IFNs play critical roles in antiviral defence (Muller et al. 1994). IFNs restrict the replication of viruses and regulate the development and activity of most effector cells of the immune system (Hertzog 2012). The production of IFNs by

infected cells or recruited innate immune cells is an important protective initial immune response to viral infections. IFNs are also used in therapeutic settings, for instance as treatments of chronic viral infections and autoimmune disorders such as multiple sclerosis (Lamers et al. 2012, Croze et al. 2013). The IFNs are divided into three different classes: type I (IFN-α, IFN-β, IFN-κ, IFN-ε, IFN-ω), type II (IFN-γ), and type III IFNs (IFN-λ1 or IL-29, IFN-λ2/3 or IL-28A/B, and IFN-λ4). Each of these IFN classes has its own specific roles in the regulation of the immune response; type I IFNs have antiviral, antiproliferative and immunomodulative functions, type II and III IFNs modulate immune responses, and type III IFNs are also very important in the fight against viral infections (Odendall, Kagan 2015). IFN-α and IFN-β are the two most important IFNs in viral infections since they have a broad spectrum of biological functions; they activate NKs and T cells, increase T cell life span, induce IFN-γ and chemokine production, enhance antigen presentation, and increase the expression of TLRs, T_H1 cytokine receptors, and adhesion molecules (Katze, He & Gale 2002). In particular, type I IFNs are crucial in limiting viral replication and for inducing a robust immune response. The type I IFNs are able to promote apoposis, inhibit protein synthesis and thus also inhibit viral replication and translation of viral proteins (Gonzalez-Navajas et al. 2012, Sadler, Williams 2008).

Viral infections activate IRF3, IRF7, and NF-kB signaling pathways, leading to the expression of inflammatory genes including type I (*IFN-α/β*) and III IFNs (*IL-28* and *IL-29*), pro-inflammatory cytokines and chemokines (Weber, Wasiliew & Kracht 2010). In addition to IRF3 and NF-kB transcription factors, MAPKs, in particular Jun N-terminal kinase (JNK) and p38, also induce *IFN-α/β* expression through the activation of the transcription factor AP-1 heterodimer c-Jun:ATF-2 (Bonjardim, Ferreira & Kroon 2009).

When the IFNs bind to their cognate receptors, a cascade of signaling events is triggered, which include the phosphorylation of a pair of Janus kinases (JAKs) and the intracellular domains of the IFN receptor (de Weerd, Samarajiwa & Hertzog 2007). These activated signaling pathways lead to the phosphorylation of transcription factors called STATs (Signal Transducer and Activator of Transcription), which induce the expression of IFN stimulated genes (ISGs) such as interferon-induced protein with tetratricopeptide repeats (IFITs) (Vladimer, Gorna & Superti-Furga 2014). The IFNs can also induce phosphatidylinositol-3-kinase (PI3K) and MAPK signaling pathways, which are known to play roles in the host response to viral infections (Platanias 2005). In addition to antiviral effects, type I IFNs have also been shown to excert anti-inflammatory effects through STATs in viral infections; they induce the expression of IL-10 via STAT1, and IL-10 consequently suppresses pro-IL-1α and pro-IL-β expression through STAT3 (Guarda et al. 2011). Moreover, activated STAT1 has been shown to inhibit the NLRP3 inflammasome-mediated caspase-1 activation (Guarda et al. 2011). These mechanisms could explain the effectiveness of IFN treatment in some autoimmune disordes such as multiple sclerosis, but also the susceptibility for an invidual to suffer bacterial infections following certain viral infections, including IAV infections.

3.2 Programmed cell death in virus infected cells

Apoptosis is a type of programmed cell death that is regulated by proteins of the B cell lymphoma 2 (Bcl-2) and caspase families. Apoptosis is an important homeostatic mechanism to maintain the cell populations in tissues, but also an important self defence mechanism of virus infected cells, because it can limit the spread of the virus. Apoptosis involves morphological changes in the cell; the cytoplasm shrinks, the plasma membrane starts to bleb, chromosomal DNA is degraded, and finally the apoptotic cells form cell fragments called apoptotic bodies (Kerr, Wyllie & Currie 1972).

Viruses use different strategies to control the cell death machinery by encoding anti- or pro-apoptotic virulence factors. Viruses have evolved to exploit various immune responses including IFN, NF-κB, and autophagy pathways to fine-tune or disrupt programmed cell death (Liang, Oh & Jung 2015). Some viruses, mainly non-enveloped ones, actively manipulate the cell to succumb; some picornaviruses encode viroporins in the late phase of infection that break the host cell membrane, releasing the newly produced virions (Nieva et al. 2003). IAV has been shown to promote apoptosis by activating caspase-3; caspase-3 activation is needed for efficient export of its progeny ribonucleoprotein complexes (vRNPs) (Wurzer et al. 2003). The accumulation of dsRNA in the cytoplasm is one event that principally occurs in virus infected eukaryotic cells. This leads to the activation of dsRNA dependent protein kinase (PKR), which phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF2α), attenuating the overall protein translation, thus triggering apoptosis (Gil, Alcami & Esteban 1999).

Generally, apoptosis proceeds via a caspase-dependent cascade that is triggered either extrinsically (the death receptor-mediated pathway) or intrinsically (the mitochondria-dependent pathway). The essential mediator of the death receptor pathway is caspase-8, whereas caspase-9 is the mediator of the mitochondrial pathway (Green 2000). Both caspase-8 and caspase-9, which are called initiator caspases, proteolytically activate pro-caspase-3, which ultimately leads to the caspase-mediated destruction of the cell (Hengartner 2000). Activated caspase-3 fragments the cytoskeleton and activates lysosomal proteases, cathepsins, in the cytoplasm. In particular, cathepsin D has been shown to be activated by caspase-3 in viral dsRNA-induced apoptosis (Rintahaka et al. 2008, Rintahaka et al. 2011).

The death receptor-mediated pathway is activated by death receptors, *e.g.* TNFR1, Fas, TNF-related apoptosis-inducing ligand (TRAILR) 1 and 2. Upon ligand binding, these death receptors oligomerize and the adaptor proteins Fas-associated death receptor domain (FADD) or TRADD directly or indirectly binds to the cytoplasmic part of these receptors, consequently activating caspase-8 (Bossaller et al. 2012).

In the mitochondria-dependent pathway, the delicate balance between activities of anti- and pro-apoptotic Bcl-2 family proteins dictates whether or not a cell will succumb to an apoptotic stimulus (Autret, Martin 2009). If pro-apoptotic signals are received, pro-apoptotic Bak and Bax channels that are localized on the outer side of the mitochondria, are opened and cytochrome c is released from the intermembrane space, leading to disruption of the mitochondrial membrane potential. The released cytochrome c is sensed by apoptotic protease-activating factor 1 (Apaf-1), which initiates the formation of the

apoptosome, followed by the recruitment and activation of pro-caspase-9 (Adams, Cory 2002, Yuan, Akey 2013). Caspase-9 then activates the effector caspases, caspase-3 and caspase-7.

The RIG-I/MDA5-MAVS signaling pathway and IRF3 can also induce the activation of apoptosis during viral infections (Rintahaka et al. 2008, Heylbroeck et al. 2000). After its phosphorylation, IRF3 has been shown either to relocalize to the nucleus (where it induces gene expression) or to the mitochondrion, where it induces the intrinsic apoptotic pathway via an interaction with the cytosolic pro-apoptotic protein Bax (Chattopadhyay et al. 2010).

3.3 Antiviral signaling induced by RNA viruses

The viral nucleic acids are the most important PAMPs for the activation of antiviral signaling in the infected cell. Viral replication intermediates, such as dsRNA and 5'-ppp RNA, and the viral genome of ssRNA viruses, are recognized by specific cytoplasmic and endosomal PRRs, activating cell signaling pathways, which induce the expression of antiviral and pro-inflammatory genes and regulate programmed cell death.

The TLRs and the RLRs are the most important PRRs in RNA virus infections, and in particular, MDA5, RIG-I, and TLR3 play important roles in the recognition of RNA viruses (**Table 8**), activating primarily transcription factors IRF3, IRF7, and NF-κB (Bruns, Horvath 2012). **Figure 7** represents a summary of the main signaling molecules involved in the recognition of RNA viruses. Many PRRs achieve the same outcome but via slightly different signaling routes. There are also interconnections between different signaling pathways, especially in the final steps of the pathways.

Long segments of dsRNA, which are produced by many RNA viruses as replication intermediates, are mainly sensed by TLR3 and MDA5. TLR3 recognizes dsRNA in the extracellular milieu or inside endosomes (Kawai, Akira 2011). The TLR3-TRIF pathway initiates *IFN-α/β* expression (through the activation of IRF3 and IRF7) and pro-inflammatory cytokine expression (through the activation of NF-κB) (Hiscott 2007). RIG-I is the main sensor for (-)ssRNA viruses SeV, IAV, VSV, rabies virus, and (+)ssRNA viruses including Japanese encephalitis virus and hepatitis C virus (HCV), while MDA5 senses mainly (+)ssRNA viruses such as poliovirus, rhinoviruses, and EMCV (Pichlmair, Reis e Sousa 2007, Kato et al. 2006).

Table 8. The main PRRs involved in viral RNA recognition and innate immune response initiation.

PRR	Ligand	References
MDA5	dsRNA, high molecular weight RNAs, intracellular pI:C	(Kato et al. 2006, Gitlin et al. 2006, Pichlmair et al. 2009)
RIG-I	ssRNA, 5'ppp RNA, short dsRNA	(Pichlmair et al. 2006, Hornung et al. 2006, Rehwinkel et al. 2010)
TLR3	dsRNA, pI:C	(Alexopoulou et al. 2001, Heinz et al. 2003)
TLR7	ssRNA	(Diebold et al. 2004, Lund et al. 2004)
TLR8	ssRNA	(Heil et al. 2004, Triantafilou et al. 2011)

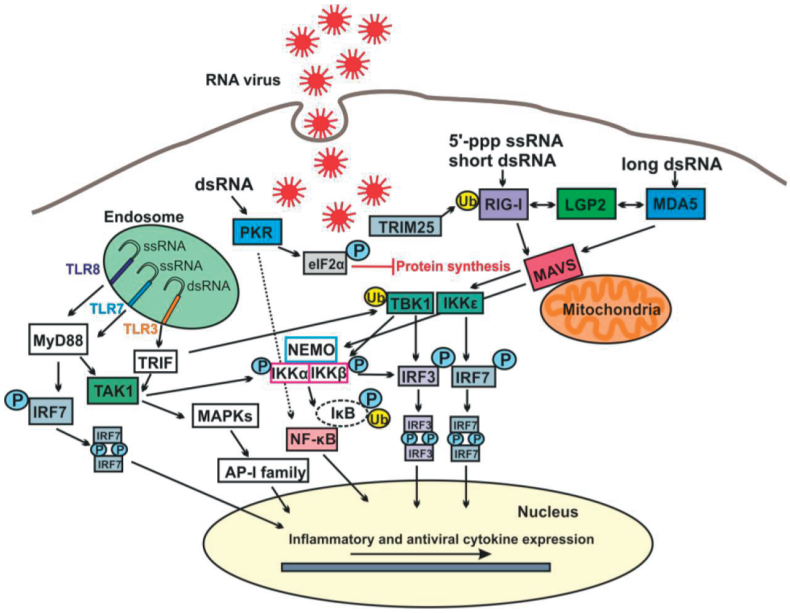

Figure 7. Pattern recognition receptors and signaling pathways involved in the recognition of RNA viruses. Upon recognition of viral nucleic acids, specific PRRs activate signaling cascades, which induce the expression of antiviral and pro-inflammatory cytokines, in particular type I and III IFNs, TNF, and IL-1β. RLR-mediated signaling is dependent on MAVS, which serves as a signalosome, activating TBK1 and IKKε. These kinases phosphorylate and activate IRF3 and IRF7, which are required for type I IFN expression. TLR3 signaling is mediated through the adaptor protein TRIF, leading to the activation of IRF3 (through the activation of TBK1), IRF7 (through the activation of IKKε), AP-1 family transcription factors (through the activation of TAK1 and MAPKs) and NF-κB (through the activation of TAK1) (Karin, Ben-Neriah 2000). TLR8/7 signal through MyD88 and activates IRF7. TLR8/7 can also activate NF-κB through the IKK complex, which consists of two canonical IKKs (IKKα and IKKβ) and the regulatory subunit NEMO (Israel 2010). PKR binds dsRNA and phosphorylates eIF2α, inhibiting protein synthesis. PKR also activates indirectly NF-κB. Figure modified from (Wilkins, Gale 2010, Liu et al. 2015, Yu, Levine 2011, Barral et al. 2009, Amaya et al. 2014).

3.4 Interferon regulatory factors (IRFs) in antiviral signaling

Specific members of the interferon regulatory factor (IRF) family are major regulators of the immune responses against viral infections. In particular, IRF3 and IRF7 are known to regulate the expression of type I IFNs (Honda, Taniguchi 2006). In particular, RIG-I and MDA5 together with MAVS coordinate downstream antiviral signaling through TBK1 and IKKε, which phosphorylate IRF3 and IRF7. The E3 ubiquitin ligases Mind Bomb 1 and 2 catalyze the K63-polyubiquitination of TBK1, which enables TBK1 to bind to NF-κB essential modulator (NEMO) and be recruited to MAVS. This facilitates the TBK1 regulated phosphorylation of IRF3 (Wang, Li & Dorf 2012). The phosphorylation of IRF3 leads to its dimerization followed by its nuclear relocalization. IRF3 induces the expression of type I

IFNs, which induce ISGs such as IFIT1, IFIT2, IFIT3, IFIT5 and viperin (Zhou et al. 2013). IFIT proteins and viperin have also been shown to recognize and bind viral nucleic acids, inhibiting the action of viruses (Abbas et al. 2013, Pichlmair et al. 2011).

In addition to phosphorylation, the stability and activity of IRFs can be modulated by other PTMs. Virus-triggered SUMOylation of IRF3 and IRF7 was shown to negatively regulate IFN expression (Kubota et al. 2008). In response to viral infection, Forkhead box O protein 1 (FoxO1) was also shown to promote IRF3 degradation, independently of E3 ubiquitin ligases RBCK1 and RAUL, which induce the degradation of IRF3 through K48-linked polyubiquitination (Lei et al. 2013). Another Forkhead box O protein, FoxO3, was demonstrated earlier to negatively regulate IRF7 (Litvak et al. 2012).

3.5 NF-κB in antiviral signaling

In addition to the IRFs, NF-κB is also a central transcription factor in the response to viral infections. The NF-κB transcription factor is activated through the RIG-I/MDA5-MAVS pathway and TLR pathways when there are viral infections, and this activation leads to the expression of NF-κB target genes such as *IL-6* and *IL-1β* (Johnson, Gale 2006). There are five members of the mammalian NF-κB family: RelA (nuclear factor NF-κB p65 subunit), RelB and c-Rel, and the precursor proteins NF-κB1 (p105) and NF-κB2 (p100), which are processed into p50 and p52, respectively (Oeckinghaus, Hayden & Ghosh 2011). The canonical and most abundant form of NF-κB is composed of NF-κB1 and RelA subunits, which can form homodimers or heterodimers with other NF-κB family members. The NF-κB transcription factor complex is normally kept inactive through binding to the IκB family proteins, principally IκBα (Hayden, Ghosh 2004). The IKK complex (NEMO-IKKα-IKKβ) is critical for the release of IκBα and the activation of the NF-κB transcription factor. IKKα and IKKβ are activated when they are phosphorylated on S177/S181 and S176/S180, respectively (Mercurio et al. 1997). The transforming growth factor β-activated kinase 1 (TAK1) is essential for this activation of the IKKα and IKKβ kinases (Ninomiya-Tsuji et al. 1999). Subsequently, IκBα becomes phosphorylated by IKKα and IKKβ on specific serine residues, allowing IκBα to be polyubiquitinated and targeted for proteasomal degradation (Hayden, Ghosh 2004). This releases active NF-κB, which translocates to the nucleus and initiates the expression of its target genes.

3.6 Viral dsRNA

In addition to dsRNA viruses, many viruses generate dsRNA during their replication cycle, in particularly (+)ssRNA and DNA viruses mainly due to convergent bidirectional transcription (Weber et al. 2006). The dsRNA activates TLR3, MDA5, RIG-I, but also the NLRP3 inflammasome (Rintahaka et al. 2011, Yu, Levine 2011, Jiang et al. 2011). Synthetic pI:C is often used in studying the immune response to viral infections. RIG-I and MDA5 are strongly activated by transfected pI:C. In addition to the expression of NF-κB stimulated genes and IFN genes, intracellularly delivered pI:C induces the mitochondrial apoptosis

pathway and this apoptotic signaling is mediated mainly by MDA5 and RIG-I and their adaptor protein MAVS (Besch et al. 2009). This apoptotic induction can be inhibited in certain primary cells by upregulation of anti-apoptotic proteins such as Bcl-xL, which counteracts the effects of pro-apoptotic Bcl-2 proteins such as Bik, Bid, Bim, and Bad.

Intracellular pI:C has been shown to activate the NLRP3 inflammasome in dendritic cells and macrophages, independently of MDA5 (Rajan et al. 2010). Lu *et al.* showed that PKR, which is phosphorylated and activated upon dsRNA recognition, physically interacts with the NLRP3 inflammasome and is important for caspase-1 activation as well as for IL-1β cleavage and secretion of the inflammatory mediator high mobility group box 1 (HMGB1) protein (Lu et al. 2012).

3.7 Sendai virus

SeV has a non-segmented (-)ssRNA genome and belongs to the *Paramyxoviridae* family of viruses. SeV is a respiratory virus and its natural hosts are rodents including rats, mice, guinea pigs, and hamsters. SeV is an enveloped virus with a lipid bilayer, which is derived from the plasma membrane of the host cell where the virus was produced. The SeV genome (the Ohita strain) encodes nine different proteins: protein C, fusion glycoprotein F0, hemagglutinin-neuraminidase (HN), RNA-directed RNA polymerase L, matrix protein, nucleoprotein, phosphoprotein, protein V, and protein W (reviewed proteins retrieved from UniProtKB, *2015-12-10*). The other SeV strains encode similar proteins, but the number of proteins varies from seven to nine (Lamb RA 2007). HN and fusion protein are transmembrane glycoproteins required for the binding to sialic acids on the target cell and for the uptake of the virus particle. HN is also needed for the detachment of the newly synthetized virions. The matrix protein has a structural function, forming a layer of molecules on the inner side of the lipid bilayer (Mottet-Osman et al. 2014). The RNA segment is wrapped by numerous nucleoprotein molecules. The nucleoproteins are also associated with the RNA-directed RNA polymerase L and the phosphoprotein, forming the viral transcriptase complex. SeV replicates in the cytoplasm of the host cell. SeV replication and virion production induce actin remodelling involving major changes in the cellular β- and γ-actin patterns (Miazza et al. 2011). The accessory V protein is able to suppress the innate immunity by binding to MDA5, consequently inhibiting IFN-β expression (Irie et al. 2012, Sakaguchi et al. 2011). Protein C has also been seen to suppress the IFN response upon infection (Komatsu et al. 2004). Protein C inhibits also IFN-α/β/γ-regulated signaling by binding to STAT1 (Oda et al. 2015).

The genome of SeV is principally recognized by RIG-I (Kato et al. 2006). Previously, SeV was demonstrated to infect human A549 cells and to induce a strong cytokine and chemokine response (Matikainen et al. 2000, Veckman et al. 2006). SeV infection also initiates apoptosis of infected human cells. SeV infection has been linked particularly to IRF3-mediated apoptosis that involves the interaction of IRF3 with the pro-apoptotic protein Bax on the mitochondrial membrane, leading to cytochrome c release (Heylbroeck et al. 2000, Chattopadhyay et al. 2011, Chattopadhyay et al. 2013). Recently, Hare and co-workers demonstrated that an incoming viral genome consisting of as few as 13 SeV

particles would be sufficient to trigger virus detection by cytosolic PRRs and consequent IRF3 activation (Hare et al. 2015).

3.8 Influenza A virus

The influenza viruses belong to the *Orthomyxoviridae* family. Similar to SeV, they also have (-)ssRNA genomes. However, the genome of influenza viruses is segmented. There are three genera of influenza viruses: A, B, and C. Influenza A virus is the most common type; it can be further divided into different subtypes depending on the two surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). To date, there are 18 HA and 11 NA known variants (reviewed in (Neumann, Kawaoka 2015)). Of these, IAV strains with combinations of H1, H2, H3, N1, and N2 are established in humans.

The IAVs target the upper respiratory tract of humans, in particular the epithelial cells (Le Goffic et al. 2007, Pothlichet, Chignard & Si-Tahar 2008, Sanders, Doherty & Thomas 2011). Phagocytic cells such as macrophages and DCs residing in the respiratory tissue are also subjected to infection (Hashimoto et al. 2007). IAVs spread very easily through airborne droplets in sneezes and coughs. The most common symptoms of influenza infections are high fever, cough, muscle and joint pain, headache, and occasionally, a sore throat and a runny nose.

3.8.1 The seasonal and pandemic influenza A viruses

The seasonal influenza viruses circulate throughout the year across the entire globe. The IAVs cause the major part of all seasonal influenza infections and every year, they are estimated to infect up to 10% of the adult population. During the 2014-2015 seasonal influenza period in Europe, 51% of the cases were IAV subtype A (H3), 30% influenza B virus, 14% IAV subtype A (H1) and 5% IAV but unsubtyped (www.ecdc.europa.eu, *2015-09-24*).

The IAVs change their genome quickly through two main evolutionary pathways - *antigenic drift* and *antigenic shift*. *Antigenic drift* includes the gradual accumulation of point mutations in the viral genes, contributing particularly to structural changes in the HA. This alters the antigenicity of the virus, enabling the virus to infect an individual even though that particular individual had previously encountered the virus. The *antigenic shift* includes the reassortment of gene segments, which can take place when two different influenza virus subtypes infect the same cell simultaneously. The progeny virus may contain segments from both viruses. A third way for the IAV to introduce novel viruses into the human population is by animal-human transmission. The introduction of a novel virus, against which the human population completely lacks immunity, can result in a very serious pandemic, causing hundreds of thousands, even millions of deaths. The most recent pandemic took place in 2009, and was caused by a H1N1 strain, which is currently circulating as a seasonal influenza.

The IAV has a broad species distribution, and this large animal reservoir and constant inter-species transmission of IAV makes it impossible to eradicate influenza. The most important hosts of IAV are water fowl, pigs, and humans. So far, the H5 and H7 subtypes have developed into highly pathogenic avian influenza strains. In particular, H5N1 has been causing sporadic mortality in South Asia (Korteweg, Gu 2008). Other avian IAVs have been detected to infect people, for instance H5N2, H7N9, H7N7, H9N2, and recently an H10N8 virus (Chen et al. 2014). Patients infected with avian IAVs suffer often from acute respiratory distress syndrome, mainly because of the stronger cytokine response in the lung than that evoked by human-to-human transmissible IAVs. This pro-inflammatory cytokine 'storm' is often thought to be the major reason for the higher lethality attributable to the avian influenzas (de Jong et al. 2006).

Annual vaccination is the principal approach taken to prevent infections by seasonal influenzas. The viruses in the influenza vaccines are re-evaluated twice a year. The annual vaccine protects against two IAVs (one H1N1 and one H3N2) virus, and one or two influenza B viruses. The present treatments of severe IAV infections are antiviral drugs, which target the viral proteins. There are two main classes of anti-IAV drugs that are available and licensed world-wide: adamantanes and NA inhibitors. The adamantanes impair the function of the ion channel protein matrix protein 2 (M2), but they are not recommended for treating currently circulating H3N2 and 2009 pandemic H1N1 virus infections, because these strains are resistant to adamantanes (http://www.cdc.gov/flu/professionals/antivirals/antiviral-drug-resistance.htm, *2015-10-12*). The NA inhibitors *e.g.* oseltamivir (Tamiflu®), zanamivir (Relenza®), and peramivir (Rapivab®) are used as treatment of seasonal IAV infections, because few of the circulating 2009 pandemic H1N1 strains are resistant to oseltamivir, and resistance to zanamivir is rare.

3.8.2 The structure and life-cycle of influenza A virus

A total of eleven proteins have been identified in the IAV virion. These proteins are structural proteins (nine proteins) and non-structural proteins (two proteins) that are required for infecting the target cell and completing the first round of genome replication (**Figure 8**). IAV is an enveloped virus with a lipid membrane inside which the genome, consisting of eight segments of ssRNA, is packed as vRNPs. The eight vRNP segments are made of ssRNA, nucleoprotein (NP), and trimeric polymerase complexes comprising basic polymerase 1 (PB1) and 2 (PB2) and polymerase acidic protein (PA). The transmembrane glycoproteins HA and NA and matrix protein M2 are embedded in the lipid bilayer. HA functions as a receptor, attaching to sialic acids on the surface of the target cell, and is therefore important for virus entry. NA cleaves the α-ketosidic linkage between the sialic acid and the adjacent sugar residue, usually galactose (Varghese, Colman 1991), and is also required during the budding process of newly formed viruses, preventing the aggregation of viral particles. NA also cleaves neuraminic acid residues of mucins in the respiratory tract, facilitating the access of the virus to the target cell. M2 facilitates the fusion of viral membrane and endosome membrane, and the assembly and release of new virions. The

oligomerized matrix protein 1 (M1) molecules are located beneath the lipid bilayer. M1 is the most abundant protein in the virion, with up to 3000 copies. The nuclear export protein (NEP), also known as the NS2 protein, is crucial for the export of vRNPs out of the nucleus. Non-structural protein 1 (NS1) was also recently found to be a component of the virion, however it is present at very low abundances (Hutchinson et al. 2014). In addition to the viral proteins, Hutchinson *et al.* identified >100 host proteins with MS both inside the virion and in the envelope (Hutchinson et al. 2014). The function of these host proteins is still unclear, but it has been suggested that these cellular proteins might play active roles in the virus life cycle.

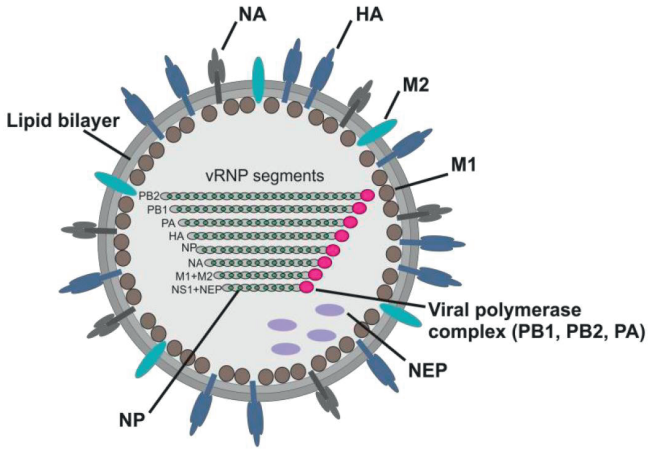

Figure 8. A simplified illustration of the IAV structure. Transmembrane proteins are embedded in the lipid bilayer: the surface proteins HA and NA, as well as the channel protein M2. The inner surface of the lipid bilayer is coated with M1 molecules. The vRNPs include the (-)ssRNA segments, which are wrapped around NP molecules, and the heterotrimeric viral polymerase complexes located on the termini of the RNA segments (Moeller et al. 2012). In the resting vRNP, the RNA-NP complex forms two antiparallel strands twisting around each other, and the polymerase binds both the 3' and 5' ends of the RNA (Resa-Infante et al. 2011). The NEP proteins are also located inside the virion.

An illustration of the IAV life cycle is shown in **Figure 9**. The IAV enters the target cell by binding with its HAs to sialic acids on the extracellular side. Avian influenza binds to α-2,3-linked sialic acids and human influenza binds to α-2,6-linked sialic acids. The primary targets of human-adapted IAV are the epithelial cells of the respiratory tract where α-2,6-linked sialic acids are abundant, however, deep in the alveoli there are mainly α-2,3-linked sialic acids (van Riel et al. 2006), which is one reason why it is believed that the avian influenza strains encounter greater difficulties in infecting humans.

Once the virus has become attached to the target cell, the virus is taken up through clathrin-mediated endocytosis. The uncoating process of the virion is initiated in the early endosome, when the early endosome is mildly acidified. This acidification triggers the M2 ion channel to open, leading to the influx of H^+ and K^+ (Martin, Helenius 1991, Pinto, Holsinger & Lamb 1992, Stauffer et al. 2014). As the pH decreases further in the late endosome, conformational changes in HA takes place and it fuses with the endosomal

membrane through its fusion peptide (White, Matlin & Helenius 1981, Matlin et al. 1981). Next, the M1 envelope disperses and it then terminates its interaction with the vRNPs in a process called 'uncoating' and the vRNPs are released into the cytosol. The vRNPs escape into the nucleus through the nuclear pore complexes. Once the vRNPs have been imported into the nucleus, the replication and transcription processes start.

The first step in the viral replication is transcription of (-)ssRNA by PB1 and PB2 to produce positive sense RNA called complementary RNA (cRNA), which serves as a template for the synthesis of more vRNA. cRNA and vRNA are assembled with newly expressed PB1, PB2, and NP to form vRNPs and cRNPs, respectively. The vRNPs are exported to the cytoplasm and packed into the progeny viruses. The viral polymerase complex transcribes vRNA segments into mRNAs, which are 5′ capped and 3′ polyadenylated. The transcription is initiated with 10- to 13-nucleotide-long 5′ methylated capped primers that are endonucleolytically cleaved from host mRNAs by PA in a process called 'cap-snatching' (Reich et al. 2014). The heterotrimeric viral polymerase complex is dependent on host RNA polymerase II for the generation of mature viral mRNAs, which are exported to the cytoplasm where they are translated into viral proteins. Finally, the intracellular levels of virus proteins and vRNPs reach a critical concentration and this triggers the assembly process.

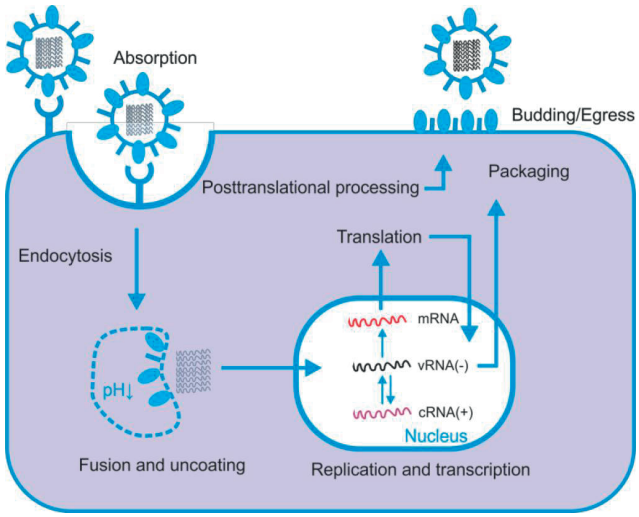

Figure 9. Influenza A virus life cycle. The figure is modified from (Paterson, Fodor 2012, Das et al. 2010). The HA binds to sialic acids on the surface of the cell and the virion is taken up by the cell through clathrin-mediated endocytosis. In the late endosome, acidification enables the virus to fuse with the endosomal membrane and release its viral genome into the cytoplasm. This fusion is mediated through extrusion of the fusion peptide of HA. After fusion, the viral RNAs move into the nucleus through nucleopores. The IAV is an unusual ssRNA virus, because it replicates in the nucleus and not in the cytoplasm of the host cell. The newly synthesized vRNPs are exported to the cytoplasm for envelopment and budding during the late stage of infection. Finally, the assembled virus leaves the cell through a budding process.

3.8.3 The innate immune responses to influenza A virus infection

At least five PRRs are particularly important in IAV recognition and host activation: TLR3, TLR7 (in mice), TLR8 (in humans), RIG-I, MDA5, and the NLRP3 inflammasome. TLR3 recognizes endosomal dsRNA that is produced as a replication intermediate, TLR7/TLR8 recognize the (-)ssRNA genome inside endosomes, RIG-I bind to the cytoplasmic (-)ssRNA and MDA5 to the viral dsRNAs (Rehwinkel et al. 2010, Baharom et al. 2015). The NLRP3 inflammasome is one of the key intracellular sensors of IAV infection as well as being the main mediator of the innate immune response to the infection, especially in IAV infected APCs (Thomas et al. 2009, Pothlichet et al. 2013). The 'signal 1' is triggered by the viral RNA recognition by TLR7/8 and RIG-I, leading to enhanced transcription of pro-IL-1β, pro-IL-18, and NLRP3. IAV promotes the expression of pro-IL-1β in primary monocytes and GM-CSF-differentiated macrophages, and induces the caspase-1 activity, which is required for the secretion of IL-1β and IL-18 (Pirhonen et al. 1999). The caspase-1-activating 'signal 2' is thought to be largely mediated by the activity of M2 and its generated ion flux (mainly K^+ efflux) and cellular ROS production (Ichinohe, Pang & Iwasaki 2010). The mechanisms through which NLRP3 detects RNA viruses such as IAV and SeV are still not completely understood, but recent findings suggest that the RIP1-RIP3 complex plays an important role. The RIP1-RIP3 complex has been shown to assemble upon IAV infection, activating the GTPase dynamin-related protein 1 (DRP1), which promotes mitochondrial damage and ROS production, consequently activating the NLRP3 inflammasome (Wang et al. 2014). IAVs also trigger apoptosis of the infected host cell (Fesq et al. 1994). In primary human macrophages, the induction of apoptosis was shown to be mediated by both the death receptor-mediated pathway and the mitochondria-dependent pathway, independently of the IAV strain (Geiler et al. 2011).

3.8.4 Cell signaling pathways and host factors regulating influenza A virus infection

The host response to IAV comprises cell signaling pathways that initiate the antiviral and pro-inflammatory cytokine expression, but also pathways that induce programmed cell death. In order to facilitate its own life cycle and replication events, the IAV can counteract, avoid or in some cases even promote certain responses through a wide variety of mechanisms, in particular through the actions of its own proteins (**Table 9**). This is a strategy that is used by many viruses, which encode proteins that suppress or control the activation of host signaling and IFN production. For instance, the VP35 protein of the (-)ssRNA Ebola virus can inhibit the phosphorylation of IRF3 and thus antagonize the activation of type I IFN expression (Basler et al. 2003), and the NS3/4A protein of the (+)ssRNA HCV can inhibit IFN production by binding to TBK1 (Otsuka et al. 2005). HCV can also activate PI3K signaling through the action of its NS5A protein (Street et al. 2004) and its NS3/4A proteins can inhibit MAVS by proteolytically cleaving it (Li et al. 2005). Recently, a serotype of the (+)ssRNA Dengue virus was shown to inhibit the RIG-I and MDA5/MAVS signaling pathway by blocking the phosphorylation of TBK1/IRF3 with its NS2A and NS4B proteins (Dalrymple, Cimica & Mackow 2015).

Post-translational modifications of viral proteins have been shown to regulate different stages of infection. The phosphorylation of M1 is necessary for its interaction with importin 1α and its nuclear import (Wang et al. 2013). The cellular protein kinase C (PKC) and JAKs have been shown to phosphorylate M1 and their inhibition decreases viral replication (Halder et al. 2013). On the other hand, phosphorylation of NP inhibited its nuclear export (Zheng et al. 2015).

Table 9. IAV proteins with described effects on host cell functions and antiviral responses.

IAV protein	Effect	References
NA	Activation of Akt signaling pathway	(Gaur et al. 2012)
NEP	Slow accumulation of NEP in the nucleus due to alternative splicing of segment 8 (coding for NS1 and NEP), is critical for the timed export of vRNPs	(Chua et al. 2013)
NP	Inhibits PKR by dissociating $P58^{IPK}$ from Hsp40	(Sharma et al. 2011)
NS1	Suppresses cytokine production, especially type I IFNs by inhibiting IRF3. Counteracts the TRIM25-mediated CARD ubiquitination of RIG-I. Activates NF-κB in later stages of infection through activation of PI3K/Akt pathway and promotes cell survival. Inhibits PKR activation by binding to dsRNA. Inhibits JNK activation. Interferes with nuclear export and stability of cellular mRNAs	(Talon et al. 2000, Gack et al. 2009 Wang et al. 2000, Noah, Twu & Krug 2003, Fortes, Beloso & Ortin 1994, Ehrhardt et al. 2007, Hale et al. 2006, Lu et al. 1995)
PA	Manipulates RNA polymerase II and thus suppresses protein synthesis by the binding of the viral trimeric polymerase complex to the phosphorylated C-terminal domain of RNA polymerase II during viral mRNA transcription	(Engelhardt, Smith & Fodor 2005, Desmet et al. 2013)
PB2	Performs 'cap-snatching' for generation of viral mRNAs: PB2 binds to the 5'-cap of pre-mRNAs and cleaves it at nucleotide 10–13 downstream of the cap with its endonucleolytic activity	(Plotch et al. 1981)

In addition to inhibiting and promoting host signaling pathways with their own proteins, IAVs exploit the host's proteins in different stages of the IAV life cycle (**Table 10**). This host factor-specific targeting has various outcomes for the progression of the infection. Many of the identified host proteins are involved in MAPK signaling, PI3K signaling, and actin organization and function. In particular, genome-wide RNA interference screenings have identified host factors crucial for early phases of IAV replication (Konig et al. 2010, Karlas et al. 2010).

Table 10. Selected cellular host factors involved in the IAV life cycle.

Host factor	Function	References
Serum- and corticoid-regulated kinase (SGK1)	Viral replication and nuclear export of vRNPs	(Alamares-Sapuay et al. 2013)
Chromosome region maintenance protein 1 (CRM1)	Nuclear export of vRNPs, interacts with M1 and NEP	(O'Neill, Talon & Palese 1998, Cao et al. 2012)
Nup153	In the later stages of infection, Nup153 (an integral subunit of nuclear core complex) is caspase-dependently degraded, facilitating the nuclear vRNP export	(Muhlbauer et al. 2015)
Importin 1α	Nuclear import of PB2, NP, and M1	(Gabriel, Herwig & Klenk 2008)
Ran binding protein 5 (RanPB5)	Nuclear import of the PB1-PA dimer	(Fodor, Smith 2004, Deng et al. 2006)

4. PHOSPHOPROTEOMICS IN INNATE IMMUNITY AND HOST-VIRUS INTERACTION STUDIES

Within the last few years, MS-based proteomics methods have been applied in studies on the immune system and host responses to pathogens. Primary immune cells have also been used, *e.g.* the phosphoproteome of primary human neutrophils was recently characterized (Muschter et al. 2015). The exploitation of MS-based phosphoproteomics is also increasing in mammalian cell signaling-based experiments, however, there are rather few publications where phosphoproteomics has been used in studying signaling related to innate immune responses.

The host response to various bacteria (Glowinski et al. 2014, Nakayasu et al. 2013, Schmutz et al. 2013, Chen et al. 2012, Holland et al. 2011) and bacterial components such as LPS (Matsumura et al. 2010), as well as yeast (Reales-Calderon et al. 2013) has been studied with MS-based phosphoproteomics. Phagocytosis of infected host cells and bacteria plays a central role in innate immunity, and *e.g.* the phosphoproteome of phagosomes in IFN-γ activated macrophages has been characterized (Trost et al. 2009). The application of phosphoproteomics for clarifying the response to different TLR ligands (Sjoelund, Smelkinson & Nita-Lazar 2014, Weintz et al. 2010) and chemokines (Wojcechowskyj et al. 2011) revealed novel information on cellular immune signaling. Moreover, TLR signaling components that define viral-sensing circuits have also been characterized with phosphoproteomics (Chevrier et al. 2011).

Viral infections alter the human proteome, as has been demonstrated with Japanese encephalitis virus (Zhang et al. 2013), measles virus (Billing et al. 2014), IAV (Liu et al. 2008, Vester et al. 2009, Vester et al. 2010, Dove et al. 2012, Emmott et al. 2010, Coombs et al. 2010), SeV (Zhou et al. 2015), and human respiratory syncytial virus (Munday et al. 2010).

The proteome of human cytomegalovirus (HMCV) infected cells was comprehensively studied with quantitative proteomics, revealing a changed expression of > 8,000 cellular proteins and alterations of cell signaling involved in innate and adapted immune defences (Weekes et al. 2014). Some proteomics studies have been conducted in primary human cells instead of cell lines. For example, the global protein expression has been shown to change in IAV-infected primary human macrophages (Ohman et al. 2009, Lietzen et al. 2011, Liu et al. 2012) and epithelial cells (Kroeker et al. 2013, Kroeker et al. 2012).

There is only a limited number of phosphoproteomics publications about virus-host responses in mammalian cells (**Table 11**). Since viruses rely on cell signaling for their life cycle stages including their cellular uptake, replication, and egress, they have been shown to alter the protein phosphorylation in infected cells. The application of phosphoproteomics in cell signaling studies of virus infected host cells can aid in revealing the functional annotations of identified proteins and their role in cellular pathways and networks. Moreover, bioinformatics analyses of the phosphoproteomics data may make it possible to identify kinases and kinase substrates. Based on these results, potential players with unknown or poorly understood roles in the identified pathways and networks can be selected for further functional studies with pharmacological inhibitors or RNA interference.

Table 11. Selected studies that utilized MS-based phosphoproteomics for studying the virus-host interactions *in vitro*.

Virus	Cell/tissue	Reference
HMCV	MRC5 human lung fibroblasts	(Oberstein et al. 2015)
HCV	Human hepatocarcinoma 7.5.1 cell line (Huh7.5.1)	(Chong et al. 2016)
HIV-1	Jurkat E6-1 and MAGI cells	(Wojcechowskyj et al. 2013)
Influenza A virus	Human A549 lung epithelial cells	(Dapat et al. 2014).
Lytic gammaherpes virus	Swiss-albino 3T3 fibroblasts (mouse)	(Stahl et al. 2013)
Porcine reproductive and respiratory syndrome virus (PRRSV)	Primary swine pulmonary alveolar macrophages	(Luo et al. 2014)
West Nile virus	Human glial cell line (U251)	(Zhang et al. 2015)

II AIMS OF THE STUDY

The main aim of this PhD project was to study the early innate immune response to viral infections, by applying large-scale phosphoproteomics combined with bioinformatics. Functional studies were subsequently performed based on the findings from the phosphoproteomic characterizations, revealing information about the regulation and roles of cell signaling pathways and host factors in virus infected human innate immune cells.

The specific aims were:

1. To characterize phosphoproteomes of innate immune cells infected by a virus or viral replication intermediates (publications I, III, IV).

2. To develop an open-access data analysis tool for processing and comparing high-throughput LC-MS/MS phosphopeptide identifications from multiple search engines and samples (publication II).

3. To utilize small molecules to study the cellular regulation of viral responses and viral replication progress in innate immune cells (publications I, III, IV).

III MATERIALS AND METHODS

This chapter summarizes the materials and methods used in this work. More detailed descriptions are found in the original publications (I-IV).

1. CELL CULTURES

The cell types used in this study are listed in **Table 12**.

Cell lines (I-IV): HaCaT (I, II), A549 (III), and MDCK (IV) cells were propagated in tissue culture flasks at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% heat inactivated fetal bovine or calf serum (Gibco), 2 mM L-glutamine (Lonza), and 50 U/mL penicillin–streptomycin (Lonza).

Primary human macrophages (IV): Human peripheral blood mononuclear cells (PBMC) were isolated from leukocyte-rich buffy coats prepared from healthy blood donors, as previously described (Pirhonen et al. 1999, Sareneva et al. 1998). The PBMCs were isolated using Ficoll-Paque PLUS™ (GE Healthcare) density gradient centrifugation and the mononuclear cell layer containing the monocytes was collected. A total of 1.4 x 10^6 monocytes were seeded per well on 6-well plates, 7 x 10^5 monocytes per well on 12-well plates, and 6 x 10^4 per well on 96-well plates. The PBMCs were left to adhere onto the plates for 45 min at 37°C and 5% CO_2 in RPMI medium 1640 (Gibco) supplemented with 50 U/mL penicillin–streptomycin (Lonza) and 2 mM L-glutamine (Lonza). The unadhered cells were washed away and the attached cells were cultured in serum free macrophage media (Gibco) supplemented with 10 ng/ml GM-CSF (Biosource International) and 50 U/mL penicillin–streptomycin (Lonza) at 37°C and 5% CO_2 for six days, polarizing the monocytes into macrophages of the acute pro-inflammatory M1-phenotype. The M1 macrophages are capable of producing pro-inflammatory cytokines such as IL-1 and IL-18, and as such, represent suitable primary human cells for studies on innate immune response to various PAMPs and DAMPs (Mosser, Edwards 2008).

Table 12. Cell types used in this study.

Name	Description	Reference	Study
A549	Adenocarcinomic human alveolar basal epithelial cell line	American Type Culture Collection, CCL-185	III
HaCaT	Human immortalized keratinocyte cell line	(Boukamp et al. 1988)	I, II
Primary human macrophages	PBMC-derived macrophages from healthy blood donors	Finnish Red Cross Blood Transfusion Service	IV
MDCK	Madin-Darby canine kidney cell line		IV

2. VIRAL INFECTIONS AND CELL STIMULATIONS

Human macrophages, keratinocytes, and lung epithelial cells were stimulated with various viruses, virus- or bacteria-derived and other immunostimulatory molecules. The details of viruses and virus-derived molecules used in the study are presented in **Table 13**. The cells were stimulated in their culture media, except for the primary human macrophages, whose media was changed to GM-CSF free media before virus stimulations.

DsRNA molecules (I, II): For studying the response to viral RNA, HaCaT cells were transfected for 4 h (I) or 1 h (II) with a mimetic of dsRNA, pI:C (Sigma-Aldrich), using Lipofectamine™ 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions, for delivery into the cell cytoplasm. The final concentration of pI:C was 10 μg/ml. The commercial synthetic RNA polymer pI:C is roughly 125-250 base pairs long and contains 5'-mono- or diphosphate ends (Rajan et al. 2010).

Encephalomyocarditis virus (I): EMCV was propagated in L929 cells grown in DMEM with 2% fetal calf serum and antibiotics as previously described (Ohman et al. 2010). HaCaT cells were infected at a multiplicity of infection (MOI) of 1 for 15 h (I). EMCV is a (+)ssRNA virus that causes myocarditis and encephalitis in certain mammalian species; it is widely used to study the dsRNA response in immune cells (Carocci, Bakkali-Kassimi 2012).

Sendai virus (III): A549 cells were infected with SeV for 4 h or 1 h, MOI = 2 (III). The virus was grown in 11-day-old embryonated hen eggs and the hemagglutinin titer for the virus stock was 4096 (Veckman et al. 2006).

Influenza A viruses (IV): Human pathogenic influenza A/Udorn/307/1972 (H3N2) was cultured in 11-day-old embryonated hen eggs and the virus stock, with a titer of 256 hemagglutination U/ml, was stored at -80°C (Pirhonen et al. 1999). A virus dose of 2.56 hemagglutination U/ml was used in the infection experiments unless stated otherwise (IV). The virus experiments were carried out under BSL-2 conditions and in compliance with the regulations of the University of Helsinki (permit No 21/M/09). A mouse-adapted H1N1 A/Puerto Rico/8/34 (PR8), was used in the mice studies (IV).

Table 13. Viruses and virus-derived molecules used in the study.

Virus/viral mimetic	Description	Study
EMCV	Encephalomyocarditis virus, a (+)ssRNA picornavirus	I
Influenza A/Puerto Rico/8/34	Mouse-adapted H1N1 influenza A virus	IV
Influenza A/Udorn/307/1972	Human H3N2 influenza A virus	IV
pI:C	Synthetic dsRNA of 125-250 base pairs	I, II
SeV	Sendai virus, strain Cantell	III

3. REAGENTS AND INHIBITORS

Specific signaling pathways and antiviral effects were assessed using pharmacological inhibitors (I, III-IV) (summarized in **Table 14**) and small interfering RNAs (siRNAs) (I, III). The inhibitors were added to the cells one hour before stimulation unless otherwise mentioned. Pre-designed siRNAs (FlexiTube siRNAs from Qiagen), 50 nM of each, were used for silecing SIRT1 (Hs_SIRT1_1/Hs_SIRT_2) (I), PPP1R13L (Hs_PPP1R13L_8/Hs_PPP1R13L_10) (I), rictor (Hs_RICTOR_3/Hs_RICTOR_6) (III), and raptor (Hs_raptor_3/Hs_KIAA1303_4) (III). Non-targeting, AllStars negative control siRNA was transfected as a control at 100 nM. The HiPerfect transfection reagent (Qiagen) was used for transfecting the siRNAs into the cells. The cells were stimulated 24 h after transfection.

Table 14. Compounds used in this work. More information can be found in the indicated references.

Name	Manufacturer/ Reference	Study
AZD8055 (mTOR pathway inhibitor)	Selleck Chemicals	III
Dinaciclib (CDK inhibitor)	Selleck Chemicals	IV
Flavopiridol (CDK inhibitor)	Selleck Chemicals	IV
Pablociclib (CDK inhibitor)	Selleck Chemicals	IV
Roscovitine (CDK inhibitor)	Selleck Chemicals	IV
SB203580 (p38 inhibitor)	Sigma-Aldrich	I
Sirtinol (SIRT1 inhibitor)	Sigma-Aldrich	I
SNS-032 (CDK inhibitor)	Selleck Chemicals	IV
SP600125 (JNK inhibitor)	Sigma-Aldrich	I
SRT1720 (SIRT1 activator)	Sigma-Aldrich	I
UO126 (MEK1/2 inhibitor, inhibits ERK1/2)	Sigma-Aldrich	I

3.1 Compound efficacy testing *in vitro* (IV)

The compounds were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich), to obtain 10 mM stock solutions. The stock solutions were stored at -80°C. DMSO was used as a control in the experiments. The testing of efficacy was performed in 96-well plates with macrophages at 95% confluence. The compounds were added to the medium and one hour later, the cells were infected with virus or non-infected (mock). Cell viability was analysed with the Cell Titer Glo assay (CTG; Promega) at 24 hours post infection (hpi). The luminescence was read with a PHERAstar FS plate reader (BMG Labtech).

The 50% cytotoxic concentration (CC_{50}) of the compounds was determined with the CTG assay in macrophages treated with different concentrations of the compounds. The half-maximal effective concentration (EC_{50}), *i.e.* the ability of the compounds to reduce virus production to 50%, was calculated by measuring the titers in MDCK cells of viruses grown in macrophages (initial MOI 0.01) for 24 h in the presence of different concentrations of

the compounds in question as described (Denisova et al. 2014). The selectivity index (SI) was defined as the CC_{50}/EC_{50} ratio.

4. WESTERN BLOT ANALYSIS (I, III, IV)

Whole cell lysates were prepared in HEPES lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, pH 7.4) (I, IV-V) or a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1% Tx-100, 150 mM NaCl, 50 mM NaF (III). Both lysis buffers were supplemented with Protease inhibitor cocktail (Sigma-Aldrich) and Phosphatase inhibitor cocktail 2 and 3 (Sigma-Aldrich). The lysates were centrifuged and the protein concentration in the collected supernatants was measured with the DC™ Protein Assay (Bio-Rad). SDS-PAGE samples were prepared with 5 x Laemmli buffer (in-house made) and loaded on Mini-Protean TGX precast gels (Bio-Rad). After SDS-PAGE separation, the proteins were electrophoretically transferred to Immobilon-P membranes (Millipore). The blots were stained with Ponceau S to confirm equal transfer of proteins. The membranes were blocked in 5% milk or 5% BSA in TBS-0.05% Tween-20 or PBS or PBS-0.05% Tween-20, depending on the conditions for the primary antibody used. The incubation with the primary antibody of interest was carried out at +4°C over-night. After this step, the membranes were washed and incubated for 1 h at RT with the appropriate horseradish peroxidase-conjugated secondary antibody (DakoCytomation). Proteins were visualized with enhanced chemiluminescence (ECL) reagents (Elmer Perkin) and images were either captured on HyperMax films (GE Healthcare) (I, III, IV) or with the ImageQuant LAS 4000 mini CCD camera (GE Healthcare) (I, IV, V). The antibodies used to characterize innate immune responses and to verify proteomic data are listed in **Table 15**.

Table 15. Antibodies used in this study. More information can be found in the indicated references and original publications (I-IV).

Antigen	Antibody	Source or Reference	Used in
Akt 1/2/3 (H-138)	Rabbit polyclonal	Santa Cruz Biotechnology	III
Bcl-xL (7B2.5)	Mouse monoclonal	Santa Cruz Biotechnology	IV
Bid	Rabbit monoclonal	Cell Signaling Technology	I
Caspase 3	Rabbit polyclonal	Santa Cruz Biotechnology	I
Cleaved caspase 3 (Asp175)	Rabbit polyclonal	Cell Signaling Technology	I, IV
GAPDH (0411)	Mouse monoclonal	Santa Cruz Biotechnology	I, III, IV
IFIT3 (RIG-G)	Mouse monoclonal	BD Transduction Laboratories	III
IκBα (44D4)	Rabbit monoclonal	Cell Signaling Technology	I, IV
IRF3	Rabbit polyclonal	Santa Cruz Biotechnology	III, IV
ISG15	Rabbit monoclonal	Cell Signaling Technology	I
MAVS*	Rabbit polyclonal	Produced in-house	I
MDA5	Rabbit polyclonal	(Lin et al. 2006), ProSci Inc	I
NP (influenza A virus)*	Rabbit polyclonal	(Pirhonen et al. 1999)	IV
NS1 (influenza A virus)*	Rabbit polyclonal	(Pirhonen et al. 1999)	IV
p-Akt (pSer473)	Rabbit monoclonal	Cell Signaling Technology	III
p-Erk1/2 (pThr202/Tyr204)	Rabbit monoclonal	Cell Signaling Technology	I

Table 15 continued.

Antigen	Antibody	Source or Reference	Used in
p-IRF3 (pSer396)	Rabbit monoclonal	Cell Signaling Technology	III, IV
p-p38 MAPK (pThr180/Tyr182)	Rabbit monoclonal	Cell Signaling Technology	I, III
p38 MAPK	Rabbit monoclonal	Cell Signaling Technology	I, III
p44/42 MAPK	Rabbit monoclonal	Cell Signaling Technology	I
p70 S6 Kinase (p70S6K)	Rabbit monoclonal	Cell Signaling Technology	III
p-p70S6K (pThr389)	Rabbit monoclonal	Cell Signaling Technology	III
RAI (iASPP, 2808C5a)	Mouse monoclonal	Santa Cruz Biotechnology	I
Raptor (regulatory-associated protein of mTOR)	Goat polyclonal	Santa Cruz Biotechnology	III
Rictor (rapamycin-insensitive companion of mTOR)	Mouse monoclonal	Santa Cruz Biotechnology	III
RIG-I*	Guinea pig polyclonal	Produced in-house	I
Sendai virus (total)*	Rabbit polyclonal	(Julkunen, Hautanen & Keski-Oja 1983)	III
SIRT1 (H-300)	Rabbit polyclonal	Santa Cruz Biotechnology	I

*Kindly provided by Professor Ilkka Julkunen.

5. QUANTITATIVE REAL-TIME PCR (I, III, IV)

For the real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis, total cellular RNA was first isolated with the RNeasy Plus Mini Kit (Qiagen). Next, RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions. The qRT-PCR was performed with ABI PRISM 7500 Sequence Detection System controlled by the ABI Prism 7500 Fast System SDS Software v1.4 (Applied Biosciences), applying TaqMan chemistry including pre-developed TaqMan assay probes and TaqMan® Fast Advanced Master Mix (Applied Biosystems). The SYBR Green Master Mix (Applied Biosystems) was applied for the SYBRGreen runs. 18S rRNA and GAPDH were used as endogenous controls for each sample in the PCR amplification to normalize the amount of cDNA between the samples. The relative quantification of the expression of the genes of interest was calculated as described before (Ohman et al. 2010).

6. PHOSPHOPROTEOMICS (I-IV)

The fractionation and phosphopeptide enrichment steps were modified versions of the protocol published by Villén and co-workers (Villen, Gygi 2008). The cells were lysed in HEPES lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, pH 7.4) supplemented with Protease inhibitor cocktail (Sigma-Aldrich) and Phosphatase inhibitor

cocktail 2 and 3 (Sigma-Aldrich). The cell lysates were centrifuged 11,686 x g for 15 min at 4°C to remove cell debris and the protein content was measured with Bio-Rad DC™ protein assay (Bio-Rad). After the cell lysis step (study II), the proteins were precipitated with 10% trichloroacetic acid (TCA) in acetone and resuspended in urea buffer (8 M urea, 400 mM NH_4CO_3, 20 mM DL-dithiotheitol, 1 mM EDTA, pH 8.5). Proteins were reduced, alkylated, and digested in-solution with rLys-C Mass Spec Grade lysyl endopeptidase (Promega) for 2 h (II, III) and sequencing grade modified trypsin (Promega) for 16 h (I, II, III, IV). Afterwards, the samples were centrifuged to remove undigested proteins and other debris, desalted with Sep-Pak Vac RP 1cc RP C18 cartridges (Waters), and acidified with formic acid (FA) to a final concentration of 1% and H_3PO_4 to reach a pH below 2.

6.1 Phosphopeptide enrichment

The desalted peptide solution was fractionated with SCX on an ÄKTApurifier™ HPLC instrument (Amersham Biosciences). The peptides were separated on a 200 x 4.6 mm, 5 µm, 200 Å PolySULFOETHYL A™ column (PolyLC) by applying a gradient run. Buffer A contained 10 mM KH_2PO_4, 20% ACN, with a pH < 3. Buffer B was buffer A and 0.4 M KCl. The gradient was set to 0-50% buffer B in 25 min, followed by 50-100% buffer B in 15 min. The flow rate was 1 ml/min and 1 ml fractions were collected by an autosampler. The early eluting fractions, containing enriched phosphopeptides, were collected and pooled. The samples were acidified with FA, desalted, vacuum centrifuged to decrease the acetonitrile content before the phosphopeptide enrichment step with Fe(III)- nitrilotriacetic acid (NTA) IMAC. IMAC was performed with PHOS-Select Iron Affinity Gel (Sigma-Aldrich) in SigmaPrep™ (Sigma-Aldrich) spin columns according to the manufacturer's instruction. The enriched phosphopeptides were vacuum-dried and dissolved in 0.1% trifluoroacetic acid before LC-MS/MS analysis.

6.2 Mass spectrometry analysis

The enriched phosphopeptide fractions were analysed with nano-LC-ESI-MS/MS. The analyses were performed with an Ultimate 3000 nano-LC system (Dionex) coupled to a QStar Elite hybrid quadropole time-of-flight mass spectrometer (TOF-MS) (Applied Biosystems/MDS Sciex) (I, II, III) or with a Q Exactive ESI-quadrupole-Orbitrap mass spectrometer coupled to an EASY-nLC 1000 nanoflow LC (Thermo Fisher Scientific) (IV).

QStar Elite conditions (I, II, III): The samples were loaded onto a ProteCol C18-Trap column (SGE) and separated on a PepMap C18 analytical column (15 cm x 75 µm, 5 µm, 100 Å) (LC Packings/Dionex) at 200 nl/min with a linear gradient of 0-40% acetonitrile in 120 min. The MS data was acquired with Analyst QS 2.0 software. The information-dependent acquisition (IDA) method consisted of a 0.5 s TOF-MS survey scan of *m/z* 400-1400. The two most abundant ions with charge states +2 to +4 were selected for product ion scans from each of the survey scans, and each selected target ion was dynamically excluded for 60 s once it was selected for fragmentation. Smart IDA was activated with automatic collision energy and automatic MS/MS accumulation.

Q Exactive conditions (IV): The analysis was performed with a Q Exactive hybrid quadrupole-orbitrap tandem mass spectrometer coupled to an EASY-nLC 1000 nanoflow LC (Thermo Fisher Scientific). The peptides were trapped on a 100 µm x 3 cm trap column and separated on a 75 µm x 15 cm analytical column (both were in-house packed with Magic C18AQ resin; 200 Å, 5 µm; Michrom Bioresources). The mobile phases consisted of 2% acetonitrile, 0.2% FA (A buffer) and 95% acetonitrile, 0.2% FA (B buffer). LC gradient elution conditions were 2% B buffer (0 min), 20% B buffer (70 min), 40% B buffer (100 min), followed by 100% B buffer (105-110 min), with a flow rate of 300 nl/min. Data dependent acquisition was performed in the positive ion mode. The MS spectra were acquired from *m/z* 300 to *m/z* 2000 at a resolution of 70,000 at *m/z* 200 with a target value of 1,000,000 and maximum injection time of 120 ms. The 10 most abundant precursor ions with charge states 2+ or higher were selected for HCD with an isolation window of 2 and a normalized collision energy of 30. MS/MS spectra were acquired at a resolution of 17,500 at *m/z* 200 with a target value of 50,000, maximum injection time of 250 ms, and the lowest mass fixed at *m/z* 100. Dynamic exclusion duration was 30 s.

6.3 Database searches

Database searches for protein identifications were performed with the Mascot algorithm (Matrix Science) (Perkins et al. 1999) version 2.4.0 through the Proteome Discoverer interface version 1.3 (Thermo Fisher Scientific) (IV). For studies I, II, and III, the database searches were performed through the Protein Pilot 4.0 interface (Applied Biosystems/MDS Sciex) with the Paragon (Shilov et al. 2007) and Mascot algorithm. The false discovery rates (FDRs) were calculated using the target-decoy strategy with concatenated normal and reversed amino acid sequence databases (Elias, Gygi 2007) (I, II, III, IV), and were < 1% in all studies.

Study I, II, III: The LC-MS/MS data were searched against the human canonical sequences in the Swiss-Prot database: version from 2013-03-01 with 539,616 sequences in total (including all taxonomies) for Mascot searches and version 2013-04-01 with 539,829 sequences for Paragon searches) (I) or version 2013-04-18 for Mascot searches and version 2012-08-13 for Paragon searches (II). Alternatively, human canonical sequences in the UniProt database (version 12/2013 with 20,274 human sequences for Mascot search and version 2014-02-03 with 20,272 human sequences for Paragon search) were used (III). The search criteria for the two distinct search engines are provided in **Table 16.**

Table 16. Search criteria for Mascot and Paragon searches in studies I, II, and III.

Database search engine	Search criteria	Value
Mascot		
	Taxonomy	Homo sapiens
	Digestion	Trypsin
	Fixed modification	Carbamidomethyl (C)
	Variable modifications	Acetyl (K), Oxidation (M), Phospho (S, T, Y)
	Ion score cut-off	0.01
	Significance threshold	$p < 0.05$
	Missed cleavage allowed	1
	Peptide charge	+1, +2, +3
	Mass tolerance for precursor ions	50 ppm
	Mass tolerance for fragment ions	0.2 Da
Paragon		
	Modifications	Cysteine alkylation with iodoacetamide
	Digestion	Trypsin
	ID focus	Biological modifications and phosphorylation emphasis
	Thorough search	Yes
	Detected protein confidence threshold, Unused ProtScore (Conf)	> 1.3, confidence 95%

Study IV: The LC-MS/MS data were searched against a concatenated forward-reverse Swiss-Prot database (release date April 2012, *Homo sapiens*, total 40,678 sequences). Trypsin was chosen as the enzyme and a maximum of two missed cleavage sites was allowed. The peptide charge was specified as 2+ and 3+, the precursor mass tolerance as 5 ppm, the peptide mass tolerance as 50 ppm, and the MS/MS ion tolerance as 0.02 Da. Carbamidomethyl (C) was specified as a fixed modification, and oxidation (M), phosphorylation (S, T, and Y) and acetylation (protein N-terminal) as variable modifications. In order to identify IAV proteins, the LC-MS/MS data were searched against a Swiss-Prot database including both IAV and human protein sequences (version 02/2012 including 21,545 sequences).

6.4 Bioinformatics

As the first step, the identified peptide and proteins from the database search engines were processed with PhosFox in order to compare the control (unstimulated) to the infected (or stimulated) samples, and to retrieve the uniquely phosphorylated proteins and peptides in the respective samples. The final lists of phosphopeptide identification were generated with PhosFox, applying a cut-off score value ≥ 30 for the Mascot peptide ion score (I, II, III, IV) and ≥ 80% (I, III) or ≥ 99% (II) for Paragon peptide confidence.

PhosFox is an in-house developed analysis tool that is suitable for peptide-level processing phosphoproteomic data generated by multiple database search algorithms such as Mascot, Paragon, and Sequest (I). PhosFox supports both qualitative and quantitative

phosphoproteomic data and multiple between-group comparisons. Phosfox is an open source software. PhosFox has been implemented in the Pearl programming language and can be run on most common operating systems.

The uniquely phosphorylated proteins were analysed further with various bioinformatics tools. Pathway analyses and classification of proteins based on Gene Ontology annotations were carried out with the Ingenuity Pathway Analysis (IPA) (Qiagen) (I, II, III, IV) and the InnateDB database (Breuer et al. 2013) (IV). The NetworKIN algorithm (Horn et al. 2014) (IV) in KinomeXplorer was used for predicting the kinases associated with specified phosphosites, and Kinase Enrichment Analysis (KEA) (Lachmann, Ma'ayan 2009) (II, III, IV) was applied for identifying kinase substrates in the phosphoproteomic data. The PhosphoNetworks database (Hu et al. 2014) was also used for identifying kinase substrates (IV).

7. OTHER METHODS

A number of other methods was used in this study; they are listed in **Table 17** and described in more detail in the original publications.

Table 17. Other techniques used in this work.

Method	Used in publication
14-3-3 protein affinity capture and isobaric tags for relative and absolute quantitation (iTRAQ) labelling	I
Caspase activity assay	IV
Cytokine array	IV
Enzyme-linked immunosorbent assay (ELISA)	IV
GeneTrail bioinformatics analysis	I
Lactate dehydrogenase (LDH) cytotoxicity assay	I, III
Luminex	IV
Phospho-kinase array	III
Silver staining of SDS-PAGE gel	I
Virus titration (colony forming unit assay)	IV
In vivo mice experiments	IV

8. STATISTICAL ANALYSIS

Data are given as the mean ±SD. Statistical analysis of results was performed with Student´s *t* test. *P* values of ≤ 0.05 were considered statistically significant.

IV RESULTS AND DISCUSSION

Phosphoproteomics is an excellent approach for studying global changes in protein phosphorylation in cells. In this PhD thesis project, phosphoproteomics was applied to study the host response to viral infections in HaCaT (I, II) and A549 (III) epithelial cells, as well as in primary human macrophages (IV) (**Figure 10**). The phosphoproteomics samples were prepared in similar ways in all four studies and the PhosFox software was developed and used for processing the phosphoproteomic data (II). A variety of bioinformatics analyses were performed following the PhosFox processing of the data. The dsRNA- or virus-host interactions were further elucidated with specific protein-targeting pharmacological inhibitors and activators (I), kinase inhibitors (I, III, IV), and by silencing host regulators of interest with siRNAs (I, III).

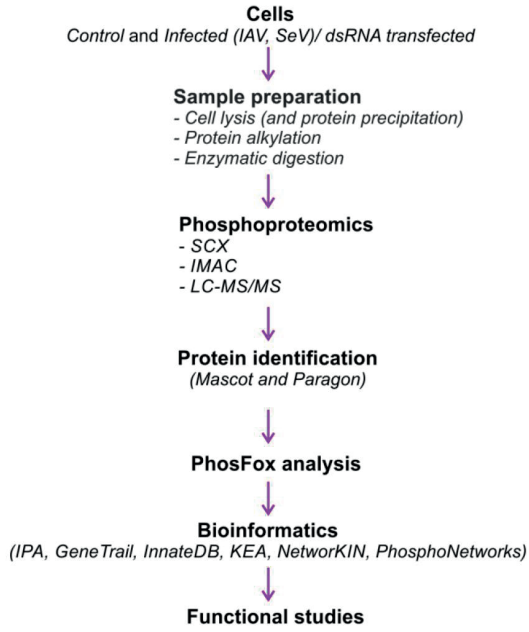

Figure 10. The phosphoproteomics methods used in publications I-IV. The cells were infected with IAV for 6 h (IV) or SeV for 4 h (III), or transfected with the dsRNA mimic pI:C for 1 h (II) or 4 h (I). The cell samples were collected and lysed, followed by either protein alkylation (I, III, IV) or protein precipitation and protein alkylation (II). The proteins were digested and fractionated with SCX before phosphopeptide enrichment with IMAC. The samples enriched in phosphopeptides were analysed with LC-MS/MS, and the data was searched against a protein sequence database, applying either Mascot or/and Paragon database search algorithms. The results were processed with PhosFox, following bioinformatics analysis with the battery of analysis tools indicated. Finally, functional studies were performed.

In such complex experiments as these phosphoproteomic analyses, the processing and interpretation of the data, and the follow-up functional studies, required a close collaboration between various areas of scientific expertise; cell biology, virology, protein chemistry, analytical chemistry, computer science, and bioinformatics. This type of multifaceted collaboration proved to be fruitful, each area contributing to the discovery of novel cellular factors and cell signaling circuits involved in viral infections.

1. PHOSFOX ANALYSIS ENABLED THE DISCOVERY OF CHANGES IN PROTEIN PHOSPHORYLATION (II)

The analysis of LC-MS/MS phosphoproteomic data is a very laborious and time-consuming process. It was decided to develop an open-access tool, PhosFox, for comparing qualitative and quantitative large-scale phosphoproteomic data regardless of the applied LC-MS/MS instrument and database search algorithm (II). PhosFox enables peptide-level processing of phosphoproteomics data generated by multiple database search engines, including Mascot, Paragon, and Sequest. **Figure 11** shows the relevant part of the phosphoproteomics workflow in respect to the PhosFox analysis.

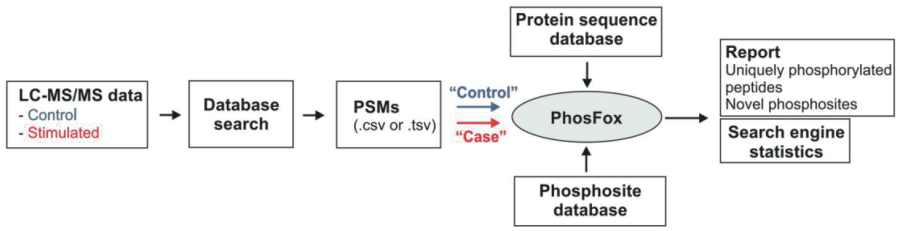

Figure 11. PhosFox processing of phosphoproteomic data generates an output report including uniquely phosphorylated peptides and proteins. The LC-MS/MS data from an experiment, comprising a stimulated sample and its control, are analysed with a database search algorithm in order to identify the peptides, proteins, and PTMs. The raw data (the PSMs) of these searches are submitted to PhosFox in comma-separated values (CSV) or plain text format (TSV). An arbitrary number of input files can be submitted. At the stage of file import, the user defines each data file as either "control" (non-stimulated samples) or "case" (stimulated samples). These data files are then automatically grouped into "control" and "case" and processed as two distinct batches of data. Additionally, the user can apply cut-off values for quality scores such as the Mascot ion score and the Paragon peptide score. It is also possible to set a threshold for the phosphosite confidence *e.g.* if the data includes Mascot Delta or phosphoRS values. A protein sequence database (in FASTA format) is required for mapping the peptide sequences, and data from phosphosite databases such as PhosphoSitePlus, enables PhosFox to pinpoint phosphorylation sites that are described in the literature. PhosFox generates two types of output files: a report of the phosphorylated peptides (in HTML format) and a plain text file of the search engine statistics, which is advantageous if the user wants to compare the results from two or more search engines. Additionally, a log file including the submission details for the analysis and possible error and warning messages are generated (in plain text format).

PhosFox is able to group database search results from multiple algorithms and runs, and perform cross-sample comparisons. For example, PhosFox can compare data from a stimulated sample to a non-stimulated sample and identify the phosphopeptides and phosphoproteins that are common and unique to the samples. The output report of the PhosFox analysis also distinguishes 'novel' phosphosites; sites that are not described in the input phosphosite databases. The advantage of PhosFox is that PhosFox-processed data includes detailed information on changes in the phosphorylation of proteins in one sample compared to some other sample, also for proteins that are phosphorylated in both samples. This information is difficult to obtain by manual compilation, for instance by sorting and combining data in spreadsheet softwares. In addition to phosphorylation, PhosFox is also able to identify and compare other PTMs, for instance those attributable to acetylation. Due to the open source code, PhosFox can be freely supplemented with more features (https://bitbucket.org/phintsan/phosfox, *2016-03-21*).

It was observed that the phosphoproteins generated by PhosFox analysis were different from those obtained by manual compilation, despite the fact that they were generated from the same database search engine identifications. The main reason for this discrepancy is that with manual processing, for instance with Microsoft Excel, it is difficult and time-consuming to even locate the phosphosite(s) on the identified peptide sequences due to the way the database search engines export the PSMs. Moreover, it is even more difficult to compare or integrate PSMs from two or more database search engines. The only way of manually processing the data is to select the phosphorylated proteins without taking into account the different phosphosite locations on the total protein sequence (which is automatically generated with PhosFox). More importantly, it was noted that the two different ways of selecting uniquely phosphorylated proteins in the dsRNA-stimulated in comparison to the non-stimulated sample had a profound impact on the bioinformatics results; the canonical pathways and networks were different when the manually compiled datasets were compared with the PhosFox-processed datasets. The two ways of processing phosphoproteomic data lead to different biological interpretations, assumptions, and hypotheses, which create the basis for follow-up functional studies. In summary, PhosFox processing increases the amount of biological information that can be extracted from phosphoproteomic data.

2. VIRAL dsRNA AND ssRNA VIRUSES MODIFY THE CELLULAR PHOSPHOPROTEOME OF INNATE IMMUNE CELLS (I-IV)

The cells of the innate immune system respond to viral infections by detecting the viral nucleic acids with their PRRs, activating signaling pathways that induce the expression of antiviral and inflammatory cytokines. However, the dynamic changes in cell signaling after the initial triggering of these receptors are still not completely understood. In this thesis, phosphoproteomics was used to characterize the changes in the phosphoproteome and signaling cascades of SeV and IAV infected and dsRNA transfected cells. The success in

characterizing the phosphoproteome by MS-based proteomics is closely related to the methods that selectively enrich the phosphopeptides. Several phosphopeptide enrichment techniques have been developed. Unfortunately, there is no gold standard method that is superior to the others. It is often up to the investigators to implement, combine, and improve the enrichment techniques so that they are appropriate for the sample and the LC-MS/MS analysis. In this PhD project, SCX was applied to separate the peptides based on their solution charge, and this step was followed by phosphopeptide enrichment with an IMAC Fe^{3+}-nitrilotriacetic acid matrix (Villen, Gygi 2008). After performing the database searches and applying the peptide score thresholds (Mascot peptide ion score ≥30 and Paragon peptide confidence ≥80% or 99%) for the phosphoproteomic data, the peptides identified as being phosphorylated accounted for approximately 65% of the total amount of identified peptides (I-IV).

A summary of the uniquely identified phosphorylated peptides and proteins in studies I-IV is shown in **Table 18**. Our studies showed that pI:C transfection, SeV, and IAV infection triggered comprehensive changes in the phosphoproteomes of HaCaT keratinocytes (I, II), A549 lung epithelial cells (III) and primary human macrophages (IV). For example, the phosphorylation of 1113 proteins qualitatively changed after IAV infection, which was 66% of the total number of identified phosphoproteins (IV).

Table 18. Summary of the phosphoproteomics results from publications I-IV. The total numbers of phosphopeptides, phosphoproteins, phosphosites, and uniquely phosphorylated proteins identified in the studies are listed. Case= dsRNA transfected (I, II) or virus infected (III and IV) sample, control= untransfected or non-infected sample.

Study	Phospho-peptides	Phospho-proteins	Phospho-sites (S, T, Y)	Uniquely phosphorylated proteins (control)	Uniquely phosphorylated proteins (case)	Proteins common to control and case (but differently phosphorylated)
I	5380	1614	5214	850	758	339
II	2605	1083	2965	420	428	151
III	3701	1347	3947	364	984	190
IV	4004	1675	4146	771	654	312

In addition to the identification of host phosphoproteins, the IAV protein NS1 was observed to be phosphorylated on T215 (IV). Moreover, the SeV nucleoprotein was also identified by LC-MS/MS to be phosphorylated (III). The role of phosphorylation of IAV NS1 or SeV nucleoprotein is not known, but it could be important for the viral life cycle. The phosphorylation of NS1 T215 was considered to be mediated by the host extracellular signal-regulated kinase (ERK)/cyclin-dependent kinase (CDK) pathway, and the mutation of this site as well as S42 attenuated viral replication (Hale et al. 2009, Hsiang, Zhou & Krug 2012). Additionally, the phosphoproteome of IAV has been mapped, and viral proteins were predicted to be phosphorylated by several human kinases, for instance casein kinase 2 (CK2), Akt, CDKs, and MAPKs (Bretana et al. 2012, Hutchinson et al. 2012).

2.1 Bioinformatic analysis of phosphoproteomic data facilitates biological interpretation (I-IV)

The results obtained from the PhosFox analyses facilitated the downstream bioinformatics analyses and the biological interpretation of the phosphoproteomic data, especially the identification of kinases, kinase substrates, and cell signaling pathways (I-IV).

The canonical pathways (I-IV), and molecular types and cellular locations (**III**, IV) of the identified phosphoproteins were determined by Ingenuity Pathway Analysis (IPA). Most of the proteins, whose phosphorylation changed after IAV or SeV infection, were transcription regulators, kinases, enzymes, and transporters (III, IV Fig. 1D). IPA also linked the phosphoproteomic data to biological functions; about 15% of the proteins, whose phosphorylation changed after IAV infection, were associated with 'viral infection' (IV, Fig. 2). Similarily, about 20% of the uniquely phosphorylated proteins in the SeV infected sample were linked to 'viral infection' (III). In all, 56 of these viral infection-linked proteins were identified in both IAV and SeV datasets, and twelve of these could be classified as transcription regulators (**Table 19**) and nine as kinases (**Table 20**). Some of these transcription regulators were also identified, and their up- or down-regulation in different cellular fractions (cytoplasmic, mitochondrial, and nuclear fractions) was determined with MS by Lietzén and co-workers (Lietzen et al. 2011) (**Table 19**). In that study, the subcellular proteomes of primary human macrophages infected with IAV for 6 h, 12 h, and 18 h were quantified using iTRAQ.

Table 19. Transcription regulators identified as uniquely phosphorylated after SeV and IAV infection and annotated by IPA to be involved in viral infection (studies III, IV). The proteins that were identified in the cellular fraction and whose expression changed in IAV infected primary human macrophages in the publication of Lietzén *et al.* are indicated. The brief descriptions of the functions are retrieved from the UniProtKB database.

Protein symbol	**Protein name**	**Function**	**Found in** (Lietzen et al. 2011)
BCLAF1	Bcl-2 associated transcription factor 1	Death-promoting transcriptional repressor	Upregulated 6 and 12 hpi (nuclear fraction)
DEK	DEK oncogene	Involved in chromatin organization	Upregulated 6 and 12 hpi (nuclear fraction)
GATAD2A	GATA zinc finger domain containing 2A	Transcriptional repressor. Enhances methyl-CpG binding domain (MBD2)-mediated repression	-
GTF2I	General transcription factor IIi	Basal transcription machinery, coordinating also formation of a multiprotein complex at the c-Fos promoter	Upregulated 6 hpi and downregulated 12 hpi (nuclear fraction)
HDAC1	Histone deacetylase 1	Responsible for the deacetylation of lysine residues, *e.g.* on histones. Deacetylates K310 in RelA, inhibiting the transcriptional activity of NF-κB	Identified in the nuclear fraction, but the quantitative data was not statistically significant
HMGA1	High mobility group AT-hook 1	Transcription regulation of genes containing, or close to, A+T-rich regions	-
NCOR2	Nuclear receptor corepressor 2	Transcriptional repression of nuclear receptors	-
PML	Promyelocytic leukemia	Associates with PML-nuclear bodies important in several cellular processes. Antiviral activity against both DNA and RNA viruses	-
RREB1	Ras responsive element binding protein 1	Binds specifically to Ras-responsive elements of gene promoters	-
SPEN	Spen family transcriptional repressor	Important corepressor protein, regulating key pathways, *e.g.* Notch signaling	-
SRSF2	Serine/arginine-rich splicing factor 2	RNA-binding phosphoprotein, controls pre-mRNA splicing	Upregulated 6 and 12 hpi (cytoplasmic fraction) and 6 hpi (nuclear fraction)
YBX1	Y box binding protein 1	Regulates pre-mRNA splicing, endonucleolytically cleaves dsDNA, also involved in translation	Upregulated 6, 12, and 18 hpi (mitochondrial fraction)

With respect to the 'viral infection'-associated transcriptional regulators (**Table 19**), five were uniquely phosphorylated after IAV and SeV infection (III, IV) and upregulated 6 h after IAV infection either in the cytoplasmic or nuclear or both fractions (Lietzen et al. 2011). The specific roles of these transcription regulators in SeV and IAV infections, and the potential role of their regulation though phosphorylation, have not been clarified. The overexpression of BCLAF1 is known to promote apoptosis (Kasof, Goyal & White 1999). DEK has been claimed to inhibit RelA-mediated transcriptional activation (Ko et al. 2006). HDAC1, which was identified at 6 hpi, has been described to inhibit NF-κB activity by deacetylating RelA. HDACs, in particular HDAC6, which is part of the aggresome processing machinery, was demonstrated to be important for IAV capsid disassembly (Banerjee et al. 2014). GTF2I (also known as TFII-I) is involved in many cellular processes, including the immune system by regulating B and T cell signaling (Roy 2012). GTF2I is also crucial in HIV-1 transcription (Taylor et al. 2011). The splicing activity of SRSF2 has been shown to play roles in the expression of proteins of various viruses, including human papilloma virus (McFarlane et al. 2015), HIV-1 (Erkelenz et al. 2015), and IAV (Fortes, Lamond & Ortin 1995). The function of YBX1 in viral infection has primarily been associated with HCV and Dengue virus. YBX1 interacts with the HCV NS5A protein, protecting it from degradation and possibly regulating its phosphorylation (Wang et al. 2015). YBX1 was also identified as an interaction partner of the HCV NS3/4A protein and the viral genome, and the silencing of YBX1 was demonstrated to cause decreased viral replication (Chatel-Chaix et al. 2011). In Dengue virus infections, on the other hand, YBX1 has been reported to suppress viral mRNA translation (Paranjape, Harris 2007).

Table 20. Kinases identified as uniquely phosphorylated after SeV and IAV infection and annotated by IPA to be involved in viral infection (studies III and IV). The brief descriptions of the functions are retrieved from the UniProtKB database.

Protein	Protein name	Function
CCNK	Cyclin K	Transcriptional regulation; phosphorylates the CTD of the large subunit of RNA polymerase II
CDC42BPB	CDC42 binding protein kinase beta (DMPK-like) or MRCKβ	Important downstream effector of CDC42. Plays a role in the regulation of cytoskeleton reorganization and cell migration
MARK2	MAP/microtubule affinity-regulating kinase 2	Involved in cell polarity and microtubule dynamics regulation
MINK1	Misshapen-like kinase 1	Negative regulator of Ras-related signal transduction. Can activate the JNK and p38 pathways and mediates stimulation of p38 MAPK downstream of the Raf/ERK pathway
PAK1	p21 protein (Cdc42/Rac)-activated kinase 1	Involved in small GTPase signaling, links the Rho-related GTPases CDC42 and RAC1 to the JNK pathway. Can directly phosphorylate Bad and protects cells against apoptosis. Phosphorylates also RAF1
PKN2	Protein kinase N2	Rho/Rac effector protein. Role in the regulation of cell cycle progression, actin cytoskeleton assembly, and cell migration
PRKAA1	Protein kinase AMP-activated catalytic subunit alpha 1	Catalytic subunit of AMP-activated protein kinase, which regulates cell energy metabolism. Negatively regulates the mTORC1 complex by phosphorylating Raptor
RAF1	v-raf-1 murine leukemia viral oncogene homolog 1	Mediator of Ras GTPase and MAPK/ERK cascades. Is phosphorylated and activated by PAK1
WNK1	WNK lysine deficient protein kinase 1	Involved in electrolyte homeostasis, activates Na^+-coupled and inhibits K^+-coupled chloride cotransporters

Cyclin K is the regulatory subunit of cyclin-dependent kinase 9 (CDK9), which regulates transcriptional activity through the phosphorylation of the CTD of RNA polymerase II (Fu et al. 1999). MINK1, PAK1, and RAF1 are involved in MAPK signaling. They are also involved in small GTPase signaling together with CDC42BPB and PKN2. PRKAA1 negatively regulates mTOR signaling by phosphorylating Raptor. The effect on electrolyte homeostasis by WNK1 has been described in the context of hypertension (Kahle et al. 2004) and neuron function (Friedel et al. 2015), but the potential role of WNK1 in the antiviral response has not been elucidated. Furthermore, there is no published information on the role of MARK2 in the regulation of the innate immune response.

In addition to IPA, InnateDB was also utilized for identifying the KEGG and REACTOME canonical pathways (IV). Since phosphorylation is regulated by kinases, it was not surprising that a great many of the proteins identified in the phosphoproteomic data were either kinases themselves or kinase substrates. NetworKIN predicted kinases for the identified phosphosites in the phosphoproteomic data and PhosphoNetworks also identified kinase-substrate relationships (IV). NetworKIN predicted kinases for 842 phosphosites in the proteins unique to the IAV dataset and for 1181 phosphosites in the proteins unique to the non-infected dataset (IV). Kinase Enrichment Analysis (KEA) identified kinase substrates in the phosphoproteomic data (II, III, IV). The kinases that were linked to most of the uniquely phosphorylated proteins in SeV and IAV datasets were glycogen synthase kinase 3 β (GSK-3β), CDK1, CDK2, MAPK p38, and PKC (III, Supporting Information Fig. 2B and IV, Table 1).

2.2 SIRT1 and RAI are regulators of antiviral innate immune responses (I)

In addition to changes in the phosphoproteome, the dsRNA stimulation also altered the binding of cellular proteins to 14-3-3 proteins (I). Previously, it was revealed that intracellular dsRNA induces phosphorylation of 14-3-3 adaptor proteins in human keratinocytes, suggesting that 14-3-3 proteins could play important roles in the antiviral innate immune response (Ohman et al. 2010). There are seven human isoforms of 14-3-3 proteins. The 14-3-3 proteins bind primarily to specific phosphorylated motifs on their target proteins, and are known to regulate various cellular processes such as those highlighted in a metadata analysis of 14-3-3 interactome studies (Johnson et al. 2011).

In study I, proteins were identified whose phosphorylation and interaction with 14-3-3s were modulated by dsRNA. Overall, 646 proteins were identified to bind to 14-3-3s, 272 of these were identified as novel 14-3-3 targets and 209 quantitatively changed their binding upon dsRNA stimulation and of these 209 proteins, the binding of 147 increased and the binding of 62 decreased in response to dsRNA. The biological functions of the 14-3-3 target proteins with altered affinity to 14-3-3s after dsRNA stimulation were determined with GeneTrail. The relevant functions with links to innate immunity were intracellular transport, immune system, cytoskeleton organization, apoptosis, and oxidation-reduction (I, Fig. 4A, left panel). Moreover, the proteins, which binding to 14-3-3s increased the most after dsRNA stimulations, were associated with the actin cytoskeleton (I, Fig. 4B).

By combining the phosphoproteomic dataset and the 14-3-3 affinity capture dataset, signaling pathways were identified and examined, leading to the discovery of new targets of 14-3-3 signaling during dsRNA stimulation. Two host proteins, RelA-associated inhibitor (RAI, also known as iASPP, and by its gene name *PPP1R13L*) and the NAD^+-dependent deacetylase sirtuin 1 (SIRT1), were found to regulate dsRNA-induced host responses. RAI was also identified as a novel 14-3-3 target protein and had a four-fold increase in binding to 14-3-3 proteins upon dsRNA transfection. The total expression of RAI, however, was reduced upon dsRNA stimulation (I, Fig. 4C). RAI was phosphorylated on S102 after dsRNA transfection, and this phosphosite was identified as being part of a 14-3-3-recognition motif. RAI was found to regulate dsRNA-induced apoptosis and TNF production in human keratinocytes; depletion of RAI increased tBid formation (causes cytochrome c release from

mitochondria) and caspase-3 activation (I, Fig. 4D), and decreased *TNF* expression independently of NF-κB signaling (I, Fig. 4E). NF-κB signaling was upregulated in cells depleted of RAI, but dsRNA stimulation had no increasing or decreasing effect on this NF-κB signaling (I, Fig. 4D). RAI is known to play a role in the regulation of NF-κB induced transcription via its interaction with the RelA subunit of NF-κB (Yang et al. 1999). Moreover, 14-3-3 proteins are known to bind RelA and IκBα (Aguilera et al. 2006). The p53 family proteins have various functions in the cell such as stimulating cell cycle arrest and apoptosis. p53 is one of the most commonly silenced or mutated genes and therefore it has been extensively studied, and its role in non-infectious diseases such as cancer is well established (Wang, Simpson & Brown 2015). The phosphorylation of RAI, which was postulated to be carried out by cyclin B1/CDK1 (Lu et al. 2013), inhibits its dimerization and promotes nuclear relocalization, where it interacts with p53, preventing p53 from transcribing predominantly apoptosis-related genes (Bergamaschi et al. 2003). Recently, RAI was identified as a caspase substrate, and the cleaved fragment was shown to be an even more potent inhibitor of p53 and RelA (Hu et al. 2015). Thus, if the RAI-mediated inhibition of p53 is abrogated, for instance by depleting cells of RAI as in study I, increased apoptosis independently of NF-κB signaling could be one possible outcome.

The phosphoproteomic data identified phosphorylated SIRT1 only after dsRNA stimulation (I). SIRT1 has been linked to various disorders, including cancer and neurodegenerative ones, but also immune functions, such as T cell proliferation and differentiation (Kong, McBurney & Fang 2012). Furthermore, specific human sirtuins, among them also SIRT1, have been shown to display antiviral activity against various DNA and RNA viruses (Koyuncu et al. 2014). As an example, the specific SIRT1 activator resveratrol has been demonstrated to reduce IAV titers (Palamara et al. 2005). Our results demonstrated that SIRT1 suppresses cytokine production and protects cells from apoptosis in dsRNA transfected keratinocytes (I, Fig. 6A and C-D). SIRT1 activity was also found to decrease apoptosis in EMCV infected keratinocytes (I, Fig. 6E). Previously, the role of SIRT1 in cell death has been elucidated, *e.g.* small-molecule SIRT1 inhibitors induced cell death of T cell leukemia cells (Kozako et al. 2015). SIRT1 deacetylates p53, which suppresses the activity of p53, and the ubiquitin-mediated destabilization of SIRT1 has been shown to increase p53-mediated apoptosis (Lin et al. 2012). The present results demonstrated that the depletion of SIRT1 enhanced the degradation of IκBα and increased the expression of *NFKB1A* and *CCL5*, whose expression is known to be initiated by NF-κB, after dsRNA transfection (I, Fig. 5C-E). An increase in IFN expression was also observed in SIRT1 silenced cells after dsRNA stimulation or EMCV infection (I, Fig. 6A-B). Similar effects on IFN expression, without any effect on viral titers, were seen after treatment with the SIRT1 inhibitor, EX-527, in respiratory syncytical virus infected DCs from mice (Owczarczyk et al. 2015). SIRT1 has not been shown before to play any role in RLR-mediated signaling induced by viral infections. However, SIRT1 has been described to regulate TLR4-mediated signaling and NF-κB transcriptional activity; SIRT1 accumulated at the promotors of *TNF* and *IL-1β* and deacetylated K310 in RelA, consequently deactivating RelA and suppressing the expression of the acute pro-inflammatory genes (Liu et al. 2011).

In summary, RAI and SIRT1 were found to be novel host regulators of the antiviral cytokine response and virus-induced apoptosis in dsRNA transfected human keratinocytes

(**Figure 12**). SIRT1 is a suppressor of pro-inflammatory and antiviral cytokine production and apoptosis, while RAI is mainly a suppressor of apoptosis in dsRNA transfected human keratinocytes. The potential roles of the specific phosphorylation sites in SIRT1 and RAI remain to be clarified. The RAI phosphorylation site might be important for its binding to 14-3-3s, as the site is in a 14-3-3 recognition motif, but it could also be important for its binding to NF-kB. The SIRT1 phosphorylation might also impact on the regulation of NF-kB and p53. SIRT1 seems to negatively regulate multiple pro-inflammatory pathways, since impairing SIRT1 activity increases inflammation, also in the response to viral dsRNA in human epithelial cells. Therefore, SIRT1 activators have been suggested as potential compounds for treating inflammation, in particular due to the downregulatory effects on NF-kB signaling (Yang et al. 2012, Zhu et al. 2011).

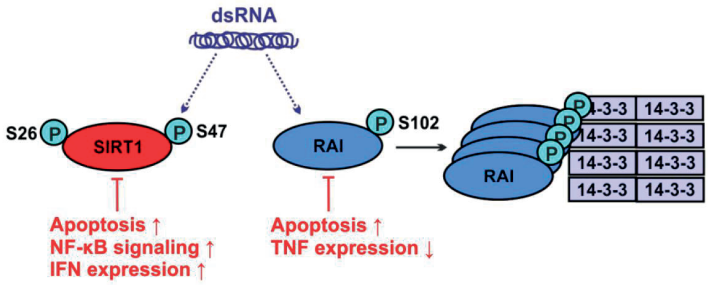

Figure 12. SIRT1 and RAI regulate the host response to intracellular dsRNA. Double-stranded RNA induced the phosphorylation of SIRT1 and RAI. The binding of RAI to 14-3-3s was increased four fold after dsRNA transfection. The silencing of SIRT1 and RAI increased dsRNA-induced apoptosis. SIRT1 depletion also increased NF-κB signaling and mRNA levels of NFKB1A, CCL5, TNF, IFN-β, and IL-29 after dsRNA transfection. Similar results were also obtained after EMCV infection. Depleting keratinocytes of RAI and transfecting with dsRNA decreased the mRNA expression of TNF.

2.3 IAV, SeV, and dsRNA partly activate similar cell signaling pathways (I-IV)

There were major changes in the phosphorylation of proteins in intracellularly dsRNA stimulated and SeV as well as in IAV infected cells, and the majority of these proteins belonged to signaling pathways as summarized in **Table 21**.

Table 21. The main cell signaling pathways that were regulated in IAV/SeV infected and dsRNA transfected cells (studies I-IV).

Signaling pathway	dsRNA	Sendai virus	Influenza A virus
CDK signaling	x	x	x
MAPK signaling	x	x	x
RLR signaling	x	x	x
Small GTPase signaling	x	x	x
GSK-3β signaling	-	x	x
mTOR signaling	-	x	x

2.3.1 CDK signaling (I, III, IV)

The CDKs are a family of serine/threonine kinases whose function and activity is dependent on their regulatory subunits, the cyclins, which are responsible for recruiting the substrates (Meyerson et al. 1992). The CDKs are involved in crucial cellular processes such as cell cycle regulation and transcription. There are a total of 13 CDKs and 25 cyclins known to be expressed in human cells. CDK1, CDK2, CDK3, CDK4, and CDK6 regulate the cell cycle and CDK8, CDK9, CDK10, and CDK11 are involved in the regulation of transcription. CDK7 regulates both the cell cycle and transcription.

CDKs and phosphoproteins involved in CDK signaling were overrepresented in SeV and IAV infected cells, and their phosphorylation profiles were altered upon infection (III, IV). Signaling related to cell cycle regulation was also represented after dsRNA stimulation (I). CDK1, 12, 13, 14, and 17 as well as cyclin K, L1, L2, T2, and Y were identified as phosphorylated in the IAV datasets (IV). Many proteins in both the IAV and SeV phosphoproteomic datasets were also identified as CDK substrates by KEA (III, IV). Cyclin K was uniquely phosphorylated after SeV and IAV infections (III, IV). This cyclin is known to associate with CDK9; it plays a role in transcriptional regulation via its phosphorylation of the CTD of the large subunit of RNA polymerase II. The T215 in the IAV NS1 protein has been suggested to be phosphorylated by cellular CDKs (Hale et al. 2009), and this phosphorylation site was also identified in the phosphoproteomic data (IV). The role and regulation of CDKs and CDK signaling were evaluated in study IV, and the results revealed that CDK activity is required for IAV infection and replication in primary human macrophages (IV).

Several viruses have been shown to regulate the activity of CDKs (Nascimento, Costa & Parkhouse 2012). CDKs have been predicted to phosphorylate IAV proteins, but the role of these phosphorylations is not known (Bretana et al. 2012, Hutchinson et al. 2012). In response to (-)ssRNA RSV infection in A549 cells, it was shown that the cytokine expression was mediated by the phosphorylation (on S276) and acetylation (on K320) of RelA (Brasier et al. 2011). These PTMs were needed for RelA to bind to positive transcription elongation factor (PTEF-b), which contains CDK9 and Brd4. PTEF-b regulates the phosphorylation of the CTD of RNA polymerase II and thus the transcription process. CDK inhibitors and in particular the inhibition of CDK9, blocked the RSV-induced gene expression of *e.g. IL-6*, *CCL5*, and *TNF*. CDK activity could also play a role in dsRNA-induced apoptosis, as cyclin B1/CDK1 was suggested to phosphorylate RAI, promoting its nuclear relocalization and inhibition of p53, consequently suppressing apoptosis (Lu et al. 2013).

2.3.2 MAPK signaling (I-IV)

Mammalian cells express 14 different MAPKs, and MAPK signaling is known to regulate countless functions in the cell, *e.g.* cell survival, apoptosis, proliferation, and differentiation (Kyriakis, Avruch 2012). In particular, extracellular signal-regulated kinase 1 and 2 (ERK1/2), Jun N-terminal kinase 1 and 2 (JNK1, JNK2), and p38α/β have been extensively studied in the context of innate immunity (Arthur, Ley 2013).

Many of the identified kinases in the IAV infected macrophages belong to the MAPK family, and especially the phosphorylation of p38 kinase substrates changed upon infection (IV, Table 1). Bioinformatic analyses detected an overrepresentation of phosphoproteins involved in ERK1/2, p38, and JNK signaling pathways upon SeV infection (III, Fig. 3B). The ERK/MAPK signaling pathway was the pathway with the lowest p-value in SeV infected cells. ERK5 signaling was also overrepresented in SeV infected cells compared to control. Keratinocytes stimulated with dsRNA for 1 h had significantly more enriched substrates for ERK1 and JNK1 compared to the unstimulated keratinocytes (II, Fig. 5). ERK/MAPK canonical signaling was also the most relevant signaling pathway induced after 4 h of dsRNA stimulation (I, Fig. 2D).

The present studies confirmed that MAPK signaling is located downstream of dsRNA-induced RLR activation, as MAPK inhibitors did not significantly affect the protein expression of RIG-I, MDA5, or MAVS (I, Supporting Information Fig. 2A). In particular, p38 signaling played a role in dsRNA-induced cytokine response, whereas both p38 and JNK signaling played a role in dsRNA-induced apoptosis (I, Fig. 3). The inhibitor of p38 reduced the mRNA expression of *TNF*, *IFN-β*, and *IL-29* at 5 h after pI:C transfection (I, Fig. 3A). *IL-29* expression was also significantly reduced when JNK was inhibited (I, Fig. 3A). Both the JNK and the p38 inhibitor reduced the expression of ISG15 protein 17 h after dsRNA stimulation (I, Fig. 3B). After dsRNA stimulation, caspase-3 activation and cell death were downregulated when p38 and JNK were inhibited (I, Fig. 3C-D).

JNK, p38, and ERK1/2 signaling pathways have been shown to be activated in IAV infected cells, in particular in the early stages of infection (Marjuki et al. 2011, Nacken et al. 2014). p38 is considered the most important MAPK in the IAV infection-related immune response (Borgeling et al. 2014). Crosstalk between JNK1/2 and ERK1/2 pathways and NF-κB has been described in IAV infected porcine macrophages, and these MAPKs were needed for RIG-I and MDA5 induction, as JNK1/2 and ERK1/2 inhibition suppressed RIG-I and MDA5 (Gao et al. 2012). PI3K is also involved in TLR-mediated signaling and consequently it can also influence MAPK signaling (Troutman, Bazan & Pasare 2012). TLR signaling is known to affect MAPK signaling, since TAK1 activates both p38 and JNK pathways (Cheung et al. 2003, Whitmarsh et al. 1997). The TAK1-JNK pathway was activated through lysosome rupture, and this activation was required for ASC oligomerization and NLRP3 inflammasome activation, also after RNA virus infection (Okada et al. 2014). MAVS has been shown to recruit and activate MAPK kinase 7 (MKK7), which phosphorylates JNK2, and consequently induces apoptosis (Huang et al. 2014b). The MAPK activated kinases (MAPKAPKs) M2 and M3 are located downstream of p38, and they interact with the PKR repressor $p88^{RIPK}$, resulting in the inhibition of PKR (Luig et al. 2010). The MAPK phosphatases act as negative regulators of the innate immune response to viral infections. In particularly, MAPK phosphatase 5 (MKP5) dephosphorylates IRF3 on S396 and S386, and reduces the type I IFN expression in IAV, SeV, and VSV infected cells (James et al. 2015). However, this interaction of MKP5 with IRF3 was independent of its interaction with ERK, JNK, and p38, as reported by Nomura *et al.* (Nomura et al. 2012).

2.3.3 RLR signaling (I, III, IV)

The RLRs are important PRRs for detecting viral nucleic acids and for activating transcription factors regulating cytokine expression, including IRFs and NF-κB. Keratinocytes express all of the known dsRNA sensing receptors, including PKR, RIG-I, MDA5, and TLR3 (Kalali et al. 2008). RIG-I and MDA5 signaling was activated upon pI:C transfection in human keratinocytes (I, Fig. 1B). This confirms the results of previous studies showing that MDA5 and RIG-I detects pI:C (Kato et al. 2008, Rehwinkel et al. 2010, Gitlin et al. 2006, Pichlmair et al. 2009). The RLR signaling induced the expression of *TNF*, *IFN-β*, and *IL-29* already after 5 h of dsRNA stimulation (I, Fig. 3A). Additionally, both SeV and IAV activated IRF3 signaling at early timepoints of infection, as shown by Western blots of phosphorylated IRF3 (III, Fig. 1A and IV, Fig. 1A). The NF-κB p105 subunit was phosphorylated on S907 only after IAV infection (IV). The NF-κB p105 subunit is known to be processed by the proteasome into the p50 subunit that forms the active NF-κB transcription heterodimer together with RelA. The S907 phosphorylation has been described to act as a cleavage signal (Demarchi et al. 2003).

The ubiquitin E3 ligases TRIM22 and TRIM25 were found to be phosphorylated only after IAV infection (IV). TRIM22 was phosphorylated on S383 and TRIM25 on S97, and both of these sites have been identified by MS before and are included in the PhosphositePlus and PhosphoNetworks databases. The TRIM22 S383 was postulated to be phosphorylated by PAK1 and the TRIM25 S97 by Akt. The roles of these phosphorylations in viral infection have, however, not been characterized. TRIM22 was shown to restrict the replication of viruses including EMCV, hepatitis B virus, and HIV (Hattlmann, Kelly & Barr 2012). TRIM22 inhibits IAV infection by targeting the viral NP for degradation (Di Pietro et al. 2013). TRIM25 has been shown to be essential for RIG-I mediated IFN-α and IFN-β production (Gack et al. 2007); TRIM25 ligates a K63-linked ubiquitin moiety to the second CARD domain on RIG-I, enabling the binding of RIG-I to MAVS. Influenza A virus NS1 was shown to specifically counteract this TRIM25-mediated CARD ubiquitination (Gack et al. 2009).

Mitochondria are critical platforms for the assembly of signaling complexes, in particular for the MAVS adaptor protein and its interacting proteins that regulate RLR signaling (Jacobs, Coyne 2013). The phosphorylation profile of MAVS changed upon IAV infection; MAVS was phosphorylated on S165 and S222 in response to infection (IV). The S165 amino acid residue is part of a target sequence motif (pS/pTXR/K) for protein kinase A (PKA) and PKC. The S222 amino acid residue, on the other hand, is situated in the kinase substrate motifs of ERK1/2, CDK5, GSK-3β, and casein kinase I. The possible role of S165 and S222 phosphorylation in IAV infection, however, remains to be elucidated. It was recently shown that MAVS is phosphorylated by TBK1 at a consensus motif, *p*Lx**I**S (*p*, hydrophilic amino acid, *x*, nonaromatic amino acid) (Liu et al. 2015). MAVS was also phosphorylated on two different C-terminal serine clusters, including S426/S430/S433 and S442/S444/S445/S446 residues (Liu et al. 2015). Phosphorylation of S442 was essential for the interaction between MAVS and IRF3, and enabled the recruitment of IRF3 to MAVS and consequently the phosphorylation and activation of IRF3 by TBK1. In addition to MAVS, other adaptor proteins of the innate immunity including TRIF, STING, and ASC, are regulated by phosphorylation. TRIF was reported to be phosphorylated on the pLxIS consensus motif

upon TLR4 stimulation with LPS, leading to the recruitment and activation of IRF3 (Liu et al. 2015). The phosphorylation of S210/S212/T214 on TRIF was mediated by TBK1. A similar phosphorylation of the TRIF adaptor in TLR3-mediated signaling could be predicted, but has not been characterized. STING is an essential adaptor protein for the DNA sensing pathway, activating TBK1 and IKKs (Ishikawa, Barber 2011). The phosphorylation of STING at S366 was shown to be important in the activation of IRF3 (Tanaka, Chen 2012). However, another report indicated that the ULK1-mediated phosphorylation of S366 impaired the function of STING (Konno, Konno & Barber 2013). Liu and co-workers reported that also the phosphorylation of consensus motif pLxIS in STING was essential for IRF3 recruitment and phosphorylation by TBK1 (Liu et al. 2015). ASC is the adaptor protein in the NLRP3, NLRP4, and AIM2 inflammasomes, and the main activator of pro-capase-1 (Hara et al. 2013). Spleen tyrosine kinase (Syk) was observed to phosphorylate ASC at Y146 and Y187 residues, and these phosphorylations were critical for ASC oligomerization and the recruitment of pro-caspase-1, but not for its interaction with NLRP3 (Lin et al. 2015).

2.3.4 Small GTPase signaling (I, III, IV)

Rho family of GTPases are small guanine nucleotide-binding proteins, involved in *e.g.* the reorganization of actin cytoskeleton, cell movement, cell cycle progression, and endocytosis (Murali, Rajalingam 2014). Many of the proteins whose phosphorylation changed upon IAV and SeV infection and which were associated with the annotation term 'viral infection' were linked to Rho family GTPase signaling (III, IV). Additionally, the top pathway of the InnateDB analysis of REACTOME pathways was 'Signaling by Rho GTPases'; 26 of in total 120 proteins associated with the pathway were differently phosphorylated upon IAV infection (IV). RhoA signaling was statistically enriched in SeV-infected A549 cells (III). Additionally, the Rho family GTPase signaling, including cell division control protein 42 homolog (Cdc42) signaling, was modulated after 4 h of cytoplasmic dsRNA stimulation in keratinocytes (I). The kinases involved in small GTPase signaling and whose substrates were enriched in IAV infected macrophages, included PAK1 and Rho-associated coiled-coil containing protein kinase 2 (ROCK2), also associated with cytoskeleton regulation. SeV infection induced phosphorylation of proteins involved in cytoskeleton dynamics, especially substrates of PAK1 (III). PAK1 functions as a GTPase effector protein and links Cdc42 and Ras-related C3 botulinum toxin substrate 1 (RAC1) to the JNK and ERK pathways. Moreover, PAK1 is located upstream of IKKε and TBK1 (Ehrhardt et al. 2004). A signaling axis consisting of PI3K-RAC1-TIPE2 (TNF induced protein 8 like-2) plays a role in the reponse to dsRNA and pI:C (Sun et al. 2012). This also links the PI3K pathway to the small GTPase signaling pathway. Taken together, small GTPase signaling seems to be involved in the regulation of MAPK, PI3K/mTOR, and NF-κB signaling in response to dsRNA, SeV, and IAV.

2.3.5 GSK-3β signaling (III, IV)

Glycogen synthase kinase 3 α and β (GSK-3α/β) regulate multiple signaling pathways involved in *e.g.* glycogen metabolism and gene expression. The phosphoproteomics data

included an overrepresentation of substrates for GSK-3β (IV). A significant portion of the uniquely phosphorylated proteins (124 out of 749) in SeV infected A549 cells was also identified as GSK-3β substrates (III). GSK-3β is an important player in IAV entry (Konig et al. 2010). Recently, GSK-3β was shown to phosphorylate β–catenin in response to SeV infection; phosphorylated β-catenin in turn was recruited to the IRF3 holocomplex, which then induced the activation of IRF3-regulated ISGs (Khan et al. 2015). GSK-3β has also been shown to positively regulate TLR3 signaling; the E3 ubiquitin ligase TRAF6 was observed to polyubiquitinate GSK-3β, promoting the formation of the TRIF-GSK-3β-TRAF6-TAK1 complex, which in turn activates IKKs and MAPKs (Ko et al. 2015).

2.3.6 mTOR signaling (III, IV)

The mTOR signaling pathway is a central regulator of cell metabolism, proliferation and survival (Albert, Hall 2015). The bioinformatics analysis of the phosphoproteomic data indicated that mTOR signaling is activated in SeV infected lung epithelial cells (III). In addition to the upregulation of mTOR signaling, there were also many differently phosphorylated substrates of PKC upon infection, and PKC is involved in the PI3K/mTOR signaling pathways (III). The activation of mTOR signaling in SeV infected cells was confirmed by a phospho-kinase array and immunoblotting, detecting the phosphorylation of the mTOR complex 1 (mTORC1) target p70S6K upon infection (III, Fig. 4B-C). Akt is a substrate of mTOR complex 2 (mTORC2), and mTORC2 activation was confirmed after infection by an increase in Akt phosphorylation (III, Fig. 4B-C). The inhibition of mTOR signaling with a compound specific for both mTORC1 and mTORC2, AZD8055, completely prevented the phosphorylation of p70S6K and Akt and exerted an inhibitory effect on the IFN-response in SeV infected cells (III, Fig. 5C-D and Supporting Information Fig. 4). Additionally, the treatment with AZD8055 decreased the expression of SeV HN protein (Fig. 5A). Additionally, silencing Raptor (mTORC1) decreased the expression of viral HN protein, emphasizing that mTORC1 is a crucial player in SeV protein synthesis (Fig. 5B).

The InnateDB analysis of KEGG pathways also exhibited that the cellular proteins, whose phosphorylation changed after IAV infection, were associated with mTOR signaling (IV). Many uniquely phosphorylated proteins in both the SeV and IAV phosphoproteomic datasets were identified as substrates for PKC. Additionally, PRKAA1, which negatively regulates mTOR signaling by phosphorylating Raptor, was identified as being uniquely phosphorylated after SeV and IAV infections (III, IV).

The role of mTOR signaling in viral infections has been studied, in particular in DNA virus infections (Buchkovich et al. 2008). The mTOR signaling pathway is activated by several viruses, for instance West Nile virus (Shives et al. 2014). There are no previous studies about the effects of SeV on mTOR signaling, but the present results demonstrated that SeV requires activated mTOR signaling for its infection and replication.

PI3K is activated in response to growth factors, cytokines, and other stimuli, and regulates various cellular functions, including proliferation, survival, cell metabolism, and protein translation (Cantley 2002). PI3K affects mTOR signaling via its downstream kinases 3-phosphoinositide dependent protein kinase-1 (PDK1) and Akt (Katso et al. 2001). TRAF6

K63-polyubiquitinates Akt, and this is critical for its membrane recruitment and phosphorylation by PDK1 and mTORC2 (Yang et al. 2010). Viruses can activate PI3K in order to prevent cell death and promote replication. For instance, IAV replication is inhibited by antagonising the PI3K-Akt-mTOR pathway (Murray et al. 2012). PI3K/Akt signaling is involved in dsRNA and IAV induced antiviral responses, by enhancing the phosphorylation of IRF3 and *IFN-β* expression (Ehrhardt et al. 2006). The inhibition and silencing of Akt decreased the viral titers in pandemic H1N1 IAV infected human cells (Denisova et al. 2014). PI3K is activated and promotes the entry of IAV in the early phase of infection through the action of epidermal growth factor receptor (EGFR) (Eierhoff et al. 2010), while PI3K activation precedes the endosomal transport of IAV particles (Ehrhardt et al. 2006). In the later stages of infection, PI3K is activated by the NS1 protein, and this prevents premature apoptosis and supports replication (Ehrhardt et al. 2007). Based on these findings, the regulation of PI3K in antiviral signaling can be proposed to play a role also in the early responses to IAV in infected primary human macrophages and to SeV in infected human epithelial cells, perhaps through crosstalk with other pathways, such as the mTOR signaling pathway.

A summary of the cell signaling pathways observed for SeV, IAV, and dsRNA and their proposed connections is presented in **Figure 13**.

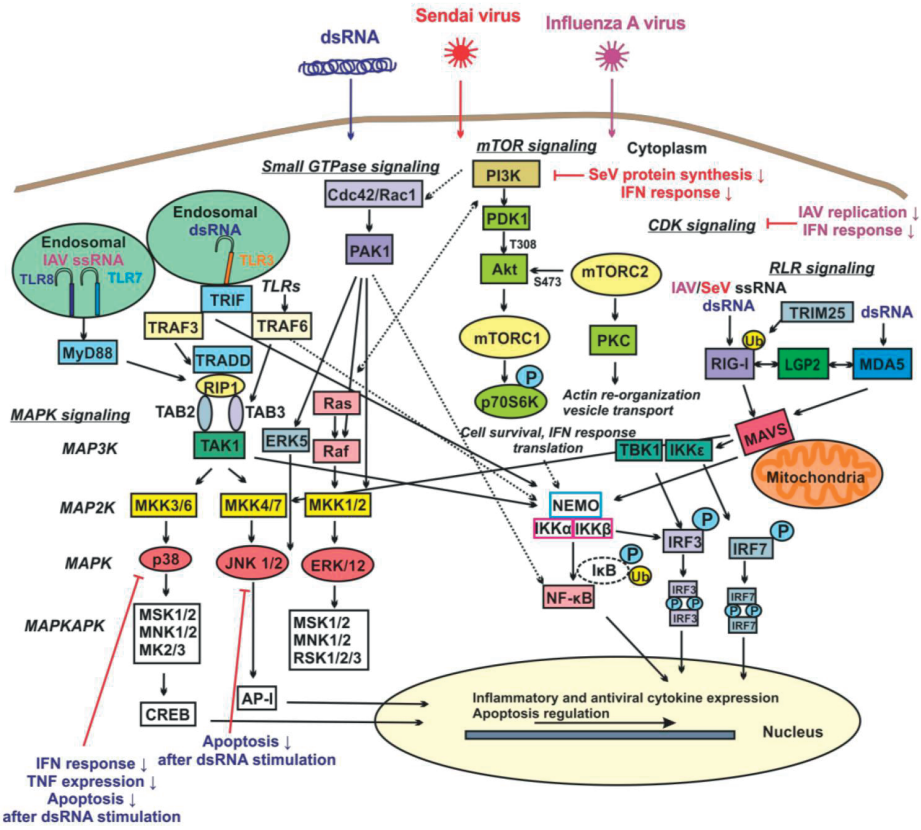

Figure 13. Selected signaling pathways identified to be regulated upon SeV and IAV infection as well as dsRNA stimulation (studies I-IV). The MAPK, small GTPase, mTOR, RLR and TLR signaling pathways have roles in the regulation of the innate immune response to viral infections. The TLR signaling can activate IRF3 (through TRAF3 (Oganesyan et al. 2006) and TRAF6 (Sato et al. 2003)) and TRADD/RIP1 (Meylan et al. 2004), as well as IRF7, NF-κB transcription factors, and MAPK signaling. By inhibiting the p38 signaling, the dsRNA-induced IFN response, TNF expression, and apoptosis were attenuated. Inhibiting the JNK activity decreased the dsRNA induced apoptosis. The small GTPase signaling affects MAPK signaling, but also mTOR signaling, mainly through the action of PAK1. Inhibiting mTOR signaling decreased the IFN response to SeV and the replication and translation of SeV. Similarly, IAV replication and the IFN response as well as cytokine expression were reduced after treating cells with inhibitors targeting specific CDKs. The main receptors for detection of cytoplasmic dsRNA and ssRNA are RIG-I and MDA5, which mediate signaling through the adaptor protein MAVS. MAVS, together with NEMO, TBK1, and IKKε, activate NF-κB, IRF3, and IRF7. These transcription factors initiate the expression of antiviral and pro-inflammatory cytokines. MAVS also affects MAPK signaling, in particular JNK signaling through MKK7.

3. TARGETING CYCLIN-DEPENDENT KINASES IMPAIRS IAV INFECTION (IV)

Study IV demonstrated that the phosphorylation profile of CDK substrates was altered after IAV infection and that specific CDK inhibitors (CDKIs) were able to suppress antiviral cytokine production and virus-induced cell death both *in vitro* and *in vivo*. Based on these findings, the role of CDKs in IAV replication and IAV-host response was studied in more detail. The results indicated that CDK activity is required for effective IAV replication and antiviral cytokine production in primary human macrophages. A variety of CDKIs that are currently under evaluation in clinical trials for their anticancer effects were tested, and it was found that two specific CDKIs, SNS-032 and flavopiridol, exerted antiviral effects at non-cytotoxic concentrations. SNS-032 inhibits CDK2, CDK7, and CDK9, while flavopiridol most effectively inhibits CDK1, CDK2, CDK4, and CDK6 (Chen et al. 2009). SNS-032 and flavopiridol antagonized the viral replication in primary human macrophages and rescued the infected cells from virus induced cell death (IV, Fig. 3B, Fig. 4A-B, Fig. 5B). SNS-032 decreased especially caspase-3/7 activity *in vitro* (IV, Fig. 5A). SNS-032 was also effective *in vivo*, rescuing infected mice (IV, Fig. 6). In addition to reducing cellular IAV-induced apoptosis, the inhibitors evoked strong immunomodulating effects. SNS-032 and flavopiridol decreased the secretion of CXCL10, CCL2, CCL3, CCL4, CCL5, IL-6, and IL-16 upon IAV infection (IV, Supporting Information Fig. S4A). SNS-032 and flavopiridol reduced the mRNA expression of *CXCL10* and *CXLX9* (IV, Fig. 4C), which have important roles in the clearance of viral infections, attracting activated NKs and T_H1 cells (Bonecchi et al. 1998, Inngjerdingen, Damaj & Maghazachi 2001). SNS-032 and flavopiridol decreased the mRNA expression of *IL-29*, but had only a minor effect on the mRNA levels of *IFN-β* after infection (IV, Fig. 4C). The TNF secretion was significantly decreased in flavopirdol treated, but not in SNS-032 treated, IAV infected cells (IV, Supporting Information Fig. S4B). The IL-18 secretion was decreased by both CDKIs, suggesting that the NLRP3 inflammasome assembly, activation, or caspase-1 processing of IL-18 was affected in the infected, but CDKI treated cells (IV, Fig. 5C). However, the antiviral effect was only observed if the CDKIs were added in the early stage of infection (IV, Supporting Information Fig. S2A). The immunomodulatory effects of SNS-032 were also observed on the transcription level in primary human macrophages infected with the wild type and a NS1 mutant ($WSN^{RK/AA}$) WSN virus (Söderholm et al. 2015). In summary, the functional studies with flavopiridol and SNS-032 revealed that CDK activity is needed for efficient IAV replication and host response and that SNS-032 is also able to rescue IAV infected mice.

Since CDKs are known to regulate the checkpoints in the cell cycle, a dysfunction of these kinases has been linked to various cancers and tumors. CDKIs were developed for cancer therapy, owing to their potential to restore the cell cycle control (Lapenna, Giordano 2009). CDKIs can also inhibit the phosphorylation of S5 (mainly phosphorylated by CDK7) and S2 (mainly phosphorylated by CDK9) within the CTD of RNA polymerase II and therefore act as global transcriptional repressors, affecting mainly short-lived proteins such as anti-apoptotic Mcl-1 and XIAP, known to be important for the survival of cancer cells (Cai et al. 2006). CDKIs affecting the RNA polymerase II have also been shown to accumulate and activate p53, inducing apoptosis in cancer cells (Cirstea et al. 2013, Lemke et al. 2014).

Herpes simplex virus and other DNA viruses depend on CDKs for their replication (Munakata et al. 2014). One clinical trial demonstrated that one particular CDKI, PHA-793887, reactivated latent herpes simplex virus infections, and that this activation was due to its ability to impair TLR signaling in DCs (Zoubir et al. 2011). It was shown in primary T cells and macrophages that CDK6-dependent phosphorylation of CDK2 plays a critical role in the phosphorylation and activation of sterile α motif and HD domain-containing protein-1 (SAMHD1) (Pauls et al. 2014a, Pauls et al. 2014b). SAMDH1 regulated the intracellular pool of dNTP, a metabolite on which the HIV-1 reverse transcriptase depends, and specific CDKIs inhibited HIV-1 infection through blocking the SAMHD1-dependent dNTP pool regulation. The inhibition or depletion of CDK2 inhibits the phosphorylation of S90 in CDK9, which is important for the association of CDK9 with PTEF-b. Inhibition of CDK9 by this mechanism downregulated HIV-1 transcription (Breuer et al. 2012). HMCV establishes transcriptosomes in the beginning of the infection, and these consist of cellular and viral proteins. Cellular RNA polymerase II is known to direct HMCV transcription, and the inhibition of CDK7 and CDK9, which phosphorylate the CTD of RNA polymerase II, led to impaired relocalization of particularly CDK9 to the viral transcriptosomes (Kapasi, Spector 2008). Moreover, the specific CDK7 inhibitor LDC4297 displayed antiviral activity towards HMCV in primary human fibroblasts (Hutterer et al. 2015). Hyperphosphorylated CTD of cellular RNA polymerase II associate with the IAV trimeric polymerase complex, and Zhang *et al.* described that cyclin T1/CDK9 is important for this association, functioning as an adapter between the RNA polymerase II and the viral polymerase complex (Zhang, Li & Ye 2010). When these investigators knocked down cyclin T1/CDK9, this inhibited the viral mRNA, cRNA, and vRNA synthesis, and blocked binding of the viral polymerase complex to RNA polymerse II. In addition to the direct link between specific CDKIs and the regulation of viral transcription, for instance flavopiridol modulated cellular immune responses, by suppressing AP-1 and NF-kB activities and p38 as well as JNK signaling pathways (Takada et al. 2008). CDK9 possesses immunomodulating activity in RSV infection; the infection enhanced the transcriptional elongation activity of CDK9, and CDK9 was implicated in the expression of ISGs (Tian et al. 2013). However, similarly to the present results, the expression of *IFN-β* was not influenced by inhibiting CDK9.

Primary human macrophages are not actively dividing cells. Therefore, it might be that the CDKIs are effective against the IAV, either by inhibiting the viral entry or its replication, or by modulating the immune response or cellular functions on which the virus cycle is dependent. The possible antiviral effects of SNS-032 and flavopiridol in primary human macrophages are shown in **Figure 14**.

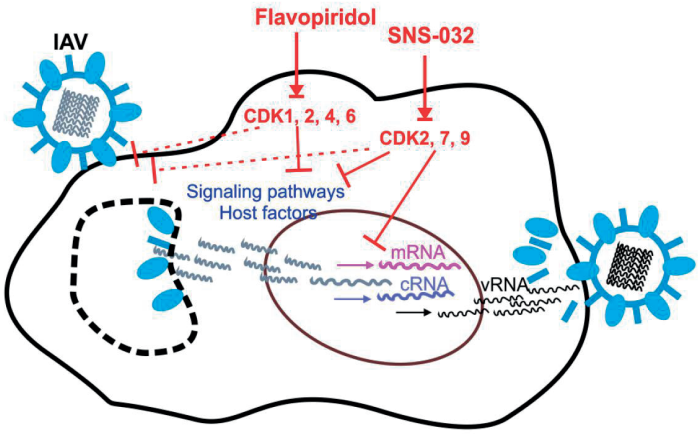

Figure 14. The potential targets of SNS-032 and flavopiridol during IAV infection in primary human macrophages. SNS-032 and flavopiridol inhibit the viral replication and decrease the expression and secretion of antiviral and pro-inflammatory cytokines in IAV infected primary human macrophages. The molecular mechanism behind the antiviral effects of SNS-032 and flavopiridol are not fully characterized. The effects might be a result of the CDKIs inhibiting either the entry or the replication of IAV. Inhibition of CDK2, CDK9 and CDK7 might lead to impaired function of the cellular RNA polymerase II, on which the IAV depends for its own replication purposes. The antiviral effects of the CDKIs might also be a result of immunomodulating effects by targeting host cellular signaling pathways or critical host factors on which the IAV depends.

IAVs depend on host factors for their life cycle. They manipulate cellular processes and cell signals for their own benefit. These manipulations can be observed in many ways, for instance as alterations in the gene expression and in the PTMs of the cellular proteins. IAVs are fast mutating viruses, and therefore they rapidly develop resistance to antiviral compounds targeting their own proteins. All of the current compounds on the market with recognized anti-influenza activity target IAV proteins. Currently, there is a keen interest in finding non-cytotoxic compounds targeting conserved virus-host interactions, since these are essential for virus replication and propagation. Inhibiting such interactions provides better opportunities to reduce the appearance of drug-resistant mutants and to minimize the morbidity and mortality caused by IAVs. Flavopiridol, and especially SNS-032, could serve as potential antiviral compounds for treating IAV infections.

V CONCLUSIONS AND FUTURE PERSPECTIVES

In this PhD project, MS-based phosphoproteomics, bioinformatics, and immunological studies were combined to characterize the virus induced changes in primary human macrophages, A549, and HaCaT cells. It was demonstrated that the global protein phosphorylation was altered in IAV and SeV infected as well as pI:C transfected cells, and that these alterations could, with the assistance of bioinformatics, be exploited to identify the cell signaling pathways and cell functions with roles in the innate immune response to viruses. Based on the findings from the phosphoproteomics data and bioinformatics analyses, the functional roles of specific host factors and cell signaling pathways were elucidated.

Detailed information about the nature of kinase substrates and their site-specific phosphorylation regulation is needed for achieving better understanding of their pathophysiological roles. The qualitative change in phosphorylation of a protein, more specifically the repertoire of phosphorylated serine, threonine, and tyrosine residues, can reveal whether the protein has been activated by a particular kinase and whether it participates in a specific signaling pathway. It was found that the mTOR signaling pathway regulates the SeV replication and SeV-induced IFN response in human lung epithelial cells. It was also demonstrated that CDK activity is essential for antiviral cytokine expression and IAV replication in infected primary human macrophages. The phosphorylation of proteins either directly affect the protein or the effect is transferred through the action of adaptor proteins such as the 14-3-3 proteins. The combination of phosphoproteomic and 14-3-3 interactome characterizations of dsRNA transfected keratinocytes revealed novel information on cell signaling pathways and host factors, identifying p38 and JNK signaling pathways as well as RAI and SIRT1 as novel regulators of the host response to viral dsRNA in human keratinocytes.

In conclusion, the present studies provide evidence that phosphoproteomics is a very powerful tool for characterizing the cellular antiviral signaling pathways and defence responses to viral infections. This approach is rather novel in virus-host interaction studies, as there is a limited amount of publications where MS-based phosphoproteomics has been applied. Large-scale phosphoproteomics studies provide insight into the regulation of the virus-host interactions and the cell signaling pathways involved in these interactions. In order to perform this type of research and in order to interpret the data effectively, seamless collaboration is required between scientists working in the fields of proteomics, bioinformatics and cell biology.

Neither the fact that the human genome has been mapped, nor the knowledge of the expression of these genes is enough to provide a complete appreciation of all biological processes and functions. In order to truly understand these phenomena more precisely, it is important to integrate information on the genome, transcriptome, metabolome, and proteome levels together with the PTMs. Proteomics is now prevalent, in particular because investigators are finally able to determine proteomes with accuracy; and thus soon the complete human proteome will also become a reality. The future will likely bring more

scientific projects comprising combinations of various 'omics' techniques and proteomic characterizations of many different PTMs. This will require more comprehensive databases and tailor-made computational analysis tools, which enable integration and processing of large amount of data acquired from the different 'omics' technologies. In the future, it is predicted that these strategies will provide valuable information also about virus-host interactions at the transcriptome, metabolome, proteome, and PTM levels, which can be utilized in the development of antiviral drugs and vaccines.

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